Kudo, N. [*1], Kumagai, K. [*2], Tomishige, N. [*2], Yamaji, T. [*2], Wakatsuki, S. [*1], Nishijima, M. , Hanada, K. [*2], and Kato, R. [*1] : **Structural basis for specific lipid recognition by CERT responsible for nonvesicular trafficking of ceramide**

Proc. Natl. Acad. Sci.USA., **105**, 488-493（2008）

In mammalian cells, ceramide is synthesized in the endoplasmic reticulum and transferred to the Golgi apparatus for conversion to sphingomyelin. Ceramide transport occurs in a nonvesicular manner and is mediated by CERT, a cytosolic 68-kDa protein with a C-terminal steroidogenic acute regulatory protein-related lipid transfer (START) domain. The CERT START domain efficiently transfers natural D-erythro-C_{16}-ceramide, but not lipids with longer (C_{20}) amide-acyl chains. The molecular mechanisms of ceramide specificity, both stereo-specific recognition and length limit, are not well understood. Here we report the crystal structures of the CERT START domain in its apo-form and in complex with ceramides having different acyl chain lengths. In these complex structures, one ceramide molecule is buried in a long amphiphilic cavity. At the far end of the cavity, the amide and hydroxyl groups of ceramide form a hydrogen bond network with specific amino acid residues that play key roles in stereo-specific ceramide recognition. At the head of the ceramide molecule, there is no extra space to accommodate additional bulky groups. The two aliphatic chains of ceramide are surrounded by the hydrophobic wall of the cavity, whose size and shape dictate the length limit for cognate ceramides. Furthermore, local high-crystallographic B-factors suggest that the α-3 and the Ω1 loop might work as a gate to incorporate the ceramide into the cavity. Thus, the structures demonstrate the structural basis for the mechanism by which CERT can distinguish ceramide from other lipid types yet still recognize multiple species of ceramides.

Keywords: crystal structure, lipid transport, ceramide

[*1] Structural Biology Research Center, Photon Factory, Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK)

[*2] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases

Aizaki, H. [*1], Morikawa, K. [*1], Fukasawa, M. [*2], Hara, H. [*1], Inoue, Y. [*1], H. Tani, H. [*3], Saito, K. [*2], Nishijima, M. , Hanada, K. [*2], Matuura, Y. [*3], M. M. Lai. [*4], Miyamura, T. [*1], Wakita, T. [*1], and Suzuki, T. [*1] : **Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection**

J. Virol., **82**, 5715-5724（2008）

In this study, we establish that cholesterol and sphingolipid associated with hepatitis C virus (HCV) particles are important for virion maturation and infectivity. In a recently developed culture system enabling study of the complete life cycle of HCV, mature virions were enriched with cholesterol as assessed by the molar ratio of cholesterol to phospholipid in virion and cell membranes. Depletion of cholesterol from the virus or hydrolysis of virion-associated sphingomyelin almost completely abolished HCV infectivity. Supplementation of cholesterol-depleted virus with exogenous cholesterol enhanced infectivity to a level equivalent to that of the untreated control. Cholesterol-depleted or sphingomyelin-hydrolyzed virus had markedly defective internalization, but no influence on cell attachment was observed. Significant portions of HCV structural proteins partitioned into cellular detergent-resistant, lipid-raft-like membranes. Combined with the observation that inhibitors of the sphingolipid biosynthetic pathway block virion production, but not RNA accumulation, in a JFH-1 isolate, our findings suggest that alteration of the lipid composition of HCV particles might be a useful approach in the design of anti-HCV therapy.

Keywords: hepatitis C virus, cholesterol, sphingolipid

[*1] Department of Virology Ⅱ, National Institute of Infectious Diseases

[*2] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases

[*3] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University

[*4] Department of Molecular Microbiology and Immunology, University of Southern California

Okemoto-Nakamura, Y. [*1], Yamakawa, Y. [*1], Hanada, K. [*1], Tanaka, K. [*2], Miura, M. [*1], Tanida, I. [*1], Nishijima, M. , and Hagiwara, K. [*1] : **Synthetic fibril peptide promotes clearance of scrapie prion protein by lysosomal degradation.**

Microbiol. Immunol., **52**, 357-365（2008）

Transmissible spongiform encephalopathies are infectious and neurodegenerative disorders that cause neural deposition of aggregates of the disease-associated form of PrP(Sc). PrP(Sc) reproduces by recruiting and converting the cellular PrP(C), and ScN2a cells support PrP(Sc) propagation. We found that incubation of ScN2a cells with a fibril peptide named P9, which comprises an intrinsic sequence of residues 167-184 of mouse PrP(C), significantly reduced the amount of PrP(Sc) in 24 hr. P9 did not affect the rates of synthesis and degradation of PrP(C). Interestingly, immunofluorescence analysis showed that the incubation of ScN2a cells with P9 induced colocalization of the accumulation of PrP with cathepsin D-positive compartments, whereas the accumulation of PrP in the cells without P9 colocalized mainly with lysosomal associated membrane proteins (LAMP)-1-positive compartments but rarely with cathepsin D-positive compartments in perinuclear regions. Lysosomal enzyme inhibitors attenuated the anti-PrP(Sc) activity; however, a proteasome inhibitor did not impair P9 activity. In addition, P9 neither promoted the ubiquitination of cellular proteins nor caused the accumulation of LC3-II, a biochemical marker of autophagy. These results indicate that P9 promotes PrP(Sc) redistribution from late endosomes to lysosomes, thereby attaining PrP(Sc) degradation.

Keywords: endsomal-lysosomal degradation pathway, fibril peptide, prion protein

[*1] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases
[*2] Department of Pathology, National Institute of Infectious Diseases

Okemoto, K. [*1], Hanada, K. [*1], Nishijima, M. , and Kawasaki, K. [*1*3] : **The preparation of a lipidic endotoxin affects its biological activities.**

Biol. Pharm.Bull., **31**, 1952-1954（2008）

Bacterial membrane constituents, such as Ornithine-containing lipid (OL) and the lipid A portion of lipopolysaccharide, trigger various immune responses through recognition by Toll-like receptor (TLR) 4. Usually, these lipids are dissolved in a small amount of aqueous or organic solvent before being added to the culture medium for examination of their biological activities. Macrophages stimulated with OL or lipid A sonically dissolved in saline released both interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha). In contrast, macrophages stimulated with OL or lipid A sonically dissolved in ethanol or dimethyl sulfoxide (DMSO) secreted much TNF-alpha, but very little IL-1beta. These results, taken together, indicate that how an endotoxin is prepared affects its biological activities. In addition, electromicroscopic analysis revealed that sonication of air-dried OL or lipid A in DMSO produced larger particles than those produced in saline, suggesting that the process of preparing lipidic TLR4-ligands affects their physical state including particle size, and that the physical state might be an important determinant of biological activity.

Keywords: endotoxin, lipopolysaccharide, Toll-like receptor 4

[*1] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases
[*2] National Institute of Health Sicenses
[*3] Faculty of Pharmaceutical Sciences, Doshisha Women's College

Takao Ashikaga[*1], Hitoshi Sakaguchi[*2], Kenji Okamoto[*3], Makoto Mizuno[*4], Jun Sato[*5], Takaaki Yamada[*6], Mayumi Yoshida[*7], Naoko Ota[*7], Seiji Hasegawa[*6], Tatsuji Kodama[*5], Yuko Okamoto[*4], Hirofumi Kuwahara[*3], Nanae Kosaka[*2], Sakiko Sono[*1], Yasuo Ohno : **Assessment of the human cell line activation test (h-CLAT) for skin sensitization; Results of the first Japanese inter-laboratory study**

AATEX, **13**, 27-35（2008）

The human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on the enhancement by sensitizers of CD86 and/or CD54 expression on THP-1 cells. The aim of this study is to confirm the transferability and reproducibility of the h-CLAT protocol. Seven Japanese laboratories participated in this h-CLAT ring study. First, two well-known sensitizers (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni) and one non-sensitizer (sodium lauryl sulfate (SLS)) were evaluated at each laboratory with the same protocol at the same application dose. All laboratories correctly evaluated the skin sensitization potential of these three chemicals. Next, four sensitizers and one non-sensitizer were tested as a second trial. There were two false-negatives (ethylene diamine and eugenol) in some laboratories. Finally, chemicals tested in the second trial were re-evaluated with doses individually determined by each laboratory as a third trial. The results were almost the same as the results obtained when all the laboratories tested the same application doses. These results suggest that for more precise evaluation of difficult samples (e.g., unstable or water-insoluble chemicals), modifications of the protocol

and prediction model are needed. However, the protocol was easily transferred to all laboratories and there were only a few false-negatives among 56 tests (8 chemicals and 7 laboratories).

Keywords: h-CLAT, skin sensitization test, validation, DCNB, SLS

[*1] Shiseido Corporation
[*2] Kao Corporation
[*3] Kanebo COSMETICS INC.
[*4] KOSE Corporation
[*5] LION Corporation
[*6] NIPPON MENARD COSMETIC CO., LTD.
[*7] POLA CORPORATION

Nanae Kosaka[*1], Kenji Okamoto[*2], Makoto Mizuno[*3], Takaaki Yamada[*4], Mayumi Yoshida[*5], Tatsuji Kodama[*6], Sakiko Sono[*7], Takao Ashikaga[*7], Jun Sato[*6], Naoko Ota[*5], Seiji Hasegawa[*4], Yuko Okamoto[*3], Hirofumi Kuwahara[*2], Hitoshi Sakaguchi[*1], Yasuo Ohno: **A study of the criteria for selection of THP-1 cells in the human cell line activation test (h-CLAT) : Results of the 2nd Japanese inter-laboratory study**
AATEX, **13**, 55-62（2008）

The Human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization method based on augmentation of CD86 and CD54 expression in THP-1 cells (human monocytic leukemia cell line). In our previous Japanese inter-laboratory study, we reported that the transferability and reproducibility of the h-CLAT is basically good. The aim of this study was to define the criteria for selecting appropriate THP-1 cells in the h-CLAT. In this study, new THP-1 cell lots were obtained from three cell banks: one in America, Europe and Japan. Using these three lots plus the cell lot we had previously used and obtained from ATCC we investigated the CD86/CD54 expression following exposure to two allergens (DNCB and Ni) and one non-allergen (SLS). Compared with the previous ATCC lot, two new lots showed similar results. Meanwhile, the third new lot showed distinctly different result in cell viability and CD86/CD54 augmentation induced by Ni compared to the other three lots. These results showed that the variability of cellular responses in the THP-1 cells depended on the cell source. In conducting the h-CLAT, it would be important to select appropriate THP cells to predict correctly the skin sensitization potential.

Keywords: h-CLAT, skin sensitization test, validation, DCNB, Ni, SLS

[*1] Kao Corp.,
[*2] Kanebo Cosmetics inc.,
[*3] Kose Corp.,
[*4] Nippon Menard Cosmetic Co., Ltd.,
[*5] Pola Chemical Industries, Inc.
[*6] Lion Corp.,
[*7] Shiseido Co. Ltd.

Sakiko Sono[*1], Takaaki Yamada[*2], Nanae Kosaka[*3], Kenji Okamoto[*4], Makoto Mizuno[*5], Jun Sato[*6], Mayumi Yoshida[*7], Naoko Ota[*7], Tatsuji Kodama[*6], Yuko Okamoto[*5], Hirofumi Kuwahara[*4], Hitoshi Sakaguchi[*3], Seiji Hasegawa[*2], Takao Ashikaga[*1], Yasuo Ohno. : A study on serum difference on test results in the human Cell Line Activation Test (h-CLAT): **Results of 3rd Japanese inter-laboratory study**
AATEX, **13**, 63-69（2008）

The Human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on enhancement of CD86 and/or CD54 expression on THP-1 cells. The aim of this study was to examine the effects of differences of serum source on the results of h-CLAT. Three different lots of serum, obtained from three sources, were compared with the serum used in the previous Japanese ring study. With each serum, cellular proliferation in subculture, cytotoxicity, and CD86/CD54 expression on THP-1 cells were measured following exposure to two known allergens (DNCB, and Ni) and one non-allergen (SLS). There was no clear difference of cellular proli8feration in subculture, cytotoxicity, or CD86/54 expression among cultures in the four sera. Although the source of serum does not appear to influence the result of h-CLAT, the validity of the test should nevertheless be confirmed when serum from a new source is introduced.

Keywords: h-CLAT, skin sensitization test, validation, serum, DCNB, Ni, SLS

[*1] Shiseido Co. Ltd.
[*2] Nippon Menard Cosmetic Co., Ltd.
[*3] Kao Corp.
[*4] Kanebo Cosmetics inc.
[*5] Kose Corp.
[*6] Lion Corp.
[*7] Pola Chemical Industries, Inc.

Makoto Mizuno[*1], Mayumi Yoshida[*2], Tatsuji Kodama[*3], Nanae Kosaka[*4], Kenji Okamoto[*5], Sakiko Sono[*6], Takaaki Yamada[*7], Seiji Hasegawa[*7], Takao Ashikaga[*6], Hirofumi Kuwahara[*5], Hitoshi Sakaguchi[*4], Jun Sato[*3], Naoko Ota[*2], Yuko Okamoto[*1], and Yasuo Ohno. : **Effect of pre-culture conditions on the human Cell Line Activation Test (h-CLAT) results: Results of the 4th Japanese inter-laboratory study**

AATEX, **13**, 70-82（2008）

The Human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on enhancement of CD86 and/or CD54 in THP-1 cells. Experimental conditions for h-CLAT were optimized in our previous study. This protocol defined that THP-1 cells are seeded between $0.1x10^6$ and $0.2x10^6$ cells/mL, and pre-cultured for 48h or 72h before treated with a test chemical. In this study we evaluated effects of pre-culture conditions on the h-CLAT results minutely. We cultivated the cells on nine pre-culture conditions before exposure to allergens (DNCB, Ni) or non-allergen (SLS), and then measured CD86 and CD54 expressions on these cells after the exposure. All laboratories almost correctly evaluated the skin sensitization potential of these three chemicals on any pre-culture conditions. However, only low CD86 and CD54 RFI values induced by DNCB tend to be obtained as the final cell concentration on pre-culture became higher. For maintaining the response of THP-1 cells to allergens and distinguishing allergens and non-allergens more clearly, THP-1 cells should be avoided being in over-growth conditions during pre-culture. Therefore, a supplementary experimental condition about pre-culture for h-CLAT that final cell concentration in pre-culture must not exceed $1.0x10^6$cells/mL was defined.

Key words: h-CLAT, skin sensitization test, validation, culture, DCNB, Ni, SLS

[*1] Kose Corp.
[*2] Pola Chemical Industries, Inc.
[*3] Lion Corp.
[*4] Kao Corp.
[*5] Kanebo Cosmetics inc.
[*6] Shiseido Co. Ltd.
[*7] Nippon Menard Cosmetic Co., Ltd.

Sanbuissho, A.[*1], Yoshida, M., Hisada, S.[*2], Sagami, F.[*3], Kudo, S.[*4], Kumazawa, T.[*5], Ube, M.[*6], Komatsu, S., Ohno, Y. : **Collaborative work on evaluation of ovarian toxicity by repeated-dose and fertility studies in female rats**

J Toxicol Sci. 34, SP1-22 (2009)

The National Institute of Health Sciences (NIHS) and 18 pharmaceutical companies of the Japan Pharmaceutical Manufacturers Association (JPMA) have conducted a validation study intended to evaluate whether a 2-week repeated general toxicity period with histopathological examination is sufficient to detect ovarian toxicity or not. The current repeated dose general toxicity study is considered to be insufficient in terms of evaluating female reproductive function due to a lack of evidence indicating that it is adequate. Evaluation of ovarian toxicity by comprehensive histopathological examination of the female reproductive organs based on the underlying morphology of a normal cycle of the reproductive tract including the ovary and additional immunohistochemical staining with proliferative cell nuclear antigen (PCNA) to identify small follicles may be a good tool to assess female reproductive function. In the collaborative study, 2- or 4-week repeated dose toxicity studies with ovarian histopathological examinations were conducted. A female fertility study was also conducted to compare the results with those of the ovarian histopathological findings. A total of 17 test substances were evaluated and categorized into hormone analogues, primordial follicle damaging agents, metabolite imbalance inducers, and endocrine imbalance inducers. Based on the results, ovarian toxicity could be detected by a careful histopatholgical examination. A 2-week dosing period may be sufficient for the evaluation of ovarian toxicity, except for cytotoxic compounds such as alkylating agents. The pathological findings of ovarian toxicity (decreases in follicles, increases in atretic follicles, increases in currently formed corpora lutea, etc) reflected the female fertility parameters (irregular estrous cycle, pre-implantation loss).

Key words: Collaborating study, ovary, toxicity

[*1] Daichi-Sankyo Co. Ltd.
[*2] Aska Pharmaceutical Co. Ltd.
[*3] Eisai Co. Ltd.
[*4] Sanwa-Kagaku Kenkyusho Co. Ltd.
[*5] Mitsubishi Tanabe Pharma Corporation
[*6] GlaxoSmithKline K.K.

Namekata, I.[*], Tsuneoka, Y.[*], Takahara, A.[*], Shimada, H.[*], Sugimoto, T.[*], Takeda, K.[*], Nagaharu, M.[*], Shigenobu, K.[*],

Kawanishi, T., Tanaka, H.* : **Involvement of the Na^+/Ca^{2+} Exchanger in the Automaticity of Guinea-Pig Pulmonary Vein Myocardium as Revealed by SEA0400**

J. Pharmacol. Sci., 110, 111-116 (2009)

We examined the involvement of the Na^+/Ca^{2+} exchanger in the automaticity of the pulmonary vein myocardium with a specific inhibitor, SEA0400. Action potentials were recorded from the myocardial layer of isolated guinea-pig pulmonary vein preparations, and Ca^{2+} transients were recorded from the cardiomyocytes. Spontaneous electrical activity was observed in 17.7% of the preparations, which was inhibited by either SEA0400 or ryanodine. In quiescent preparations, ouabain induced electrical activity and spontaneous Ca^{2+} transients, which were inhibited by SEA0400, as well as ryanodine. These results provide pharmacological evidence that the Na^+/Ca^{2+} exchanger underlies the automaticity of the pulmonary vein myocardium.

Keywords: Na^+-Ca^{2+} exchange, myocardium, automaticity

* 東邦大学

Tanaka, H.*, Namekata, I.*, Nouchi, H.*, Shigenobu, K.*, Kawanishi, T., Takahara, A.* : **New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: diversity in the excitation-contraction mechanisms of the heart**

J. Pharmacol. Sci., **109**, 327-333 (2009)

The waveform of the myocardial action potential (AP) triggering contraction differs among the species, developmental stage, and pathological state. The species difference in heart rate, which inversely correlates with body size, originates in the ion-channel mechanisms responsible for diastolic depolarization of the sinoatrial node. In some cases, such as the chronically AV-blocked dog and 11- to 13-day chick embryo, the repolarization reserve is decreased making the heart useful for drug evaluation. The degree of dependence of contraction on sarcoplasmic reticulum (SR) function increases during development. The large SR dependence and short AP of the adult mouse and rat support their rapid contraction under high heart rate. The function of the Na^+/Ca^{2+} exchanger is affected by AP waveform and ion concentrations; its major role is Ca^{2+} extrusion, but under pathological conditions such as ischemia-reperfusion, it allows Ca^{2+} influx and leads to myocardial injury, including loss of mitochondrial function. The role of mitochondria in ATP supply is less in the fetus where glycolysis plays a greater role. The pharmacological properties of the myocardium are affected by all of these factors and also by autonomic innervation and the hormonal status. Such comprehensive understanding is indispensable for the development of novel therapeutic strategies.

Keywords: cardiac muscles, excitation-contraction, action potential

* 東邦大学

四方田千佳子，保立仁美，伊豆津健一，川西徹，**皮膚適用製剤の溶出試験に関する研究（2）**

医薬品研究，**39**，436-441(2008)

第一報おいて，皮膚適用製剤の放出試験として，メンブランフィルター成型物を用いる方法を考案した．これらの方法を用いた試験結果を，ヘアレスマウスの皮膚を用いたフランツセルによる透過試験の結果と比較検討し，ある程度の相関を有することを明らかとした．さらに，考案した方法が軟膏や他の製剤にも応用可能であることを示した．

Keywords: 皮膚適用製剤，溶出試験，メンブランフィルター

柘植秀哉[*1]，中島辰巳[*1]，大内　正[*1]，青木光夫[*2]，大久保恒夫[*2]，四方田千佳子：**浸透圧測定法による機種間差による研究（第二報）**

医薬品研究，40，136-142 (2009)

製薬メーカー及び浸透圧測定用機器メーカーの協力を得て，市販製剤４品目を用いた浸透圧測定の共同実験を実施したところ，ブドウ糖が10%を超える濃度で（600mOs/kg）機種間差が認められた．機種間差の原因究明のために，特に凍結状態の異なる化合物及び高粘性溶液の測定を詳細に検討したところ，機種間差には，試料溶液の粘性が高いほど大きくなり，ガラス転移を有する非晶質物質で差が大きくなることが明らかとなった．

Keywords:浸透圧，機種間差，粘性

[*1] 社団法人東京医薬品工業協会局方委員会
[*2] 大阪医薬品協会技術研究委員会

Izutsu, K., Kadoya, S.*, Yomota, C., Kawanishi, T., Yonemochi, E.* and Terada, K.* : **Freeze-drying of proteins in glass solids formed by basic amino acids and dicarboxylic acids**

Chem. Pharm. Bull., **57**, 43-48 (2009)

The purpose of this study was to produce and characterize glass-state amorphous solids containing amino acids and organic acids that protect co-lyophilized proteins. Thermal analysis of frozen solutions containing a basic amino acid (e.g., L-arginine, L-lysine, L-histidine) and a hydroxy di- or tricarboxylic acid (e.g., citric acid, L-tartaric acid, DL-malic acid) showed glass transition of maximally freeze-concentrated solute at temperatures (T'_g) significantly higher than those of the individual solute solutions. Mixing of the amino acid with some dicarboxylic acids (e.g., oxalic acid) also suggested an upward shift of the transition temperature. Contrarily, combinations of the amino acid with monocarboxylic acids (e.g., acetic acid) had T'_gs between those of the individual solute solutions. Co-lyophilization of the basic amino acids and citric acid or L-tartaric acid resulted in amorphous solids that have glass transition temperatures (T_g) higher than the individual components. Mid- and near-infrared analysis indicated altered environment around the functional groups of the consisting molecules. Some of the glass-state excipient combinations protected an enzyme (lactate dehydrogenase, LDH) from inactivation during freeze-drying. The glass-state excipient combinations formed by hydrogen-bonding and electrostatic interaction network would be potent alternative to stabilize therapeutic proteins in freeze-dried formulations.

Keywords: freeze-drying, protein formulation, amorphous, stabilization

* Faculty of Pharmaceutical Sciences, Toho University

Izutsu, K., Hiyama, Y., Yomota, C. and Kawanishi, T. : **Near-Infrared analysis of hydrogen-bonding in glass- and rubber-state amorphous saccharide solids**

AAPS PharmSciTech, **10**, 524-529(2009)

Near-infrared (NIR) spectroscopic analysis of non-crystalline polyols and saccharides (e.g., glycerol, sorbitol, maltitol, glucose, sucrose, maltose) was performed at different temperatures (30-80 ℃) to elucidate the effect of glass transition on molecular interaction. Transmission NIR spectra (4000-12000 cm^{-1}) of the liquids and cooled-melt amorphous solids showed broad absorption bands that indicate random configuration of molecules. Heating of the samples decreased an intermolecular hydrogen-bonding OH vibration band intensity (6,200-6,500 cm^{-1}), with a concomitant increase in a free and intramolecular hydrogen-bonding OH group band (6,600-7100 cm^{-1}). Large reduction of the intermolecular hydrogen-bonding band intensity at temperatures above the glass transition (T_g) of the individual solids should explain the higher molecular mobility and lower viscosity in the rubber state. Mixing of the polyols with a high T_g saccharide (maltose) or an inorganic salt (sodium tetraborate) shifted both the glass transition and the inflection point of the hydrogen-bonding band intensity to higher temperatures. The implications of these results for pharmaceutical formulation design and process monitoring (PAT) are discussed.

Keywords: NIR, hydrogen-bond, glass transition, PAT

Shibata, H., Yoshioka, Y.[*1], Ohkawa, A.[*2], Abe, Y.[*2], Nomura, T.[*3], Mukai, Y.[*3], Nakagawa, S.[*3], Taniai, M.[*4], Ohta, T.[*4], Mayumi, T.[*5], Kamada, H.[*2], Tsunoda, S.[*2] and Tsutsumi, Y.[*3] : **The therapeutic effect of TNFR1-selective antagonistic mutant TNF-alpha in murine hepatitis models**

Cytokine, **44**, 229-33 (2008)

Tumor necrosis factor-alpha (TNF-alpha) is critically involved in a wide variety of inflammatory pathologies, such as hepatitis, via the TNF receptor-1 (TNFR1). To develop TNFR1-targeted anti-inflammatory drugs, we have already succeeded in creating a TNFR1-selective antagonistic mutant TNF-alpha (R1antTNF) and shown that R1antTNF efficiently inhibits TNF-alpha/TNFR1-mediated biological activity in vitro. In this study, we examined the therapeutic effect of R1antTNF in acute hepatitis using two independent experimental models, induced by carbon tetrachloride (CCl(4)) or concanavalin A (ConA). In a CCl(4)-induced model, treatment with R1antTNF significantly inhibited elevation in the serum level of ALT (alanine aminotransferase), a marker for liver damage. In a ConA-induced T-cell-mediated hepatitis model, R1antTNF also inhibited the production of serum immune activated markers such as IL-2 and IL-6. These R1antTNF-mediated therapeutic effects were as good as or better than those obtained using conventional anti-TNF-alpha antibody therapy. Our results suggest that R1antTNF may be a clinically useful TNF-alpha antagonist in hepatitis.

Keywords: Tumor necrosis factor-α, Liver failure, Inflammation, Therapy

[*1] 大阪大学MEIセンター
[*2] 医薬基盤研
[*3] 大阪大学大学院薬学研究科

*4 林原生物化学研究所京都
*5 神戸学院大学

Yoshida, H., Nishikawa, M.*, Yasuda, S.*, Mizuno, Y.* and Takakura, Y.* : **Cellular activation by plasmid DNA in various macrophages in primary culture**
J. Pharm. Sci., **97**, 4575-4585 (2008)

Macrophages are an important group of cells responsible for the inflammatory response to unmethylated CpG dinucleotide (CpG motif) in plasmid DNA (pDNA) via Toll-like receptor 9 (TLR9). This finding is primarily based on in vitro studies. Previous in vivo studies also have suggested that tissue macrophages are involved in inflammatory cytokine release in the circulation following intravenous administration of pDNA to mice. However, the relationship between the in vitro and in vivo studies has not been sufficiently clarified. To gain insight into which types of cells are responsible for the production of cytokines upon interaction with pDNA, peritoneal macrophages, splenic macrophages, hepatic nonparenchymal cells (NPCs) including Kupffer cells and mesangial cells were isolated from mice. All types of primary cultured cells, except for mesangial cells, express TLR9 at varying levels. Splenic macrophages and hepatic NPCs were activated to produce tumor necrosis factor-alpha (TNF-alpha) by naked pDNA, whereas peritoneal macrophages and mesangial cells were not. pDNA complexed with N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride/cholesterol liposome induced TNF-alpha in the splenic macrophages but not in the other cell types. These results indicate that splenic macrophages and hepatic NPCs are closely involved in TNF-alpha production in response to pDNA.
Keywords: plasmid DNA, CpG motif, TLR9

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* 京都大学大学院薬学研究科

Aso, Y., Yoshioka, S., Miyazaki, T., Kawanishi, T. : **Feasibility of ^{19}F-NMR for assessing the molecular mobility of flufenamic acid in solid dispersions**
Chem. Pharm. Bull., **57**, 61-64 (2009)

The purpose of the present study was to clarify the feasibility of ^{19}F-NMR for assessing the molecular mobility of flufenamic acid (FLF) in solid dispersions. Amorphous solid dispersions of FLF containing poly(vinylpyrrolidone) (PVP) or hydroxypropylmethylcellulose (HPMC) were prepared by melting and rapid cooling. Spin-lattice relaxation times (T_1 and $T_{1\rho}$) of FLF fluorine atoms in the solid dispersions were determined at various temperatures (-20 to 150°C). Correlation time (τ_c), which is a measure of rotational molecular mobility, was calculated from the observed T_1 or $T_{1\rho}$ value and that of the T_1 or $T_{1\rho}$ minimum, assuming that the relaxation mechanism of spin-lattice relaxation of FLF fluorine atoms does not change with temperature. The τ_c value for solid dispersions containing 20% PVP was 2-3 times longer than that for solid dispersions containing 20% HPMC at 50°C, indicating that the molecular mobility of FLF in solid dispersions containing 20% PVP was lower than that in solid dispersions containing 20% HPMC. The amount of amorphous FLF remaining in the solid dispersions stored at 60°C was successfully estimated by analyzing the solid echo signals of FLF fluorine atoms, and it was possible to follow the overall crystallization of amorphous FLF in the solid dispersions. The solid dispersion containing 20% PVP was more stable than that containing 20% HPMC. The difference in stability between solid dispersions containing PVP and HPMC is considered due to the difference in molecular mobility as determined by τ_c. The molecular mobility determined by ^{19}F-NMR seems to be a useful measure for assessing the stability of drugs containing fluorine atoms in amorphous solid dispersions.
Keywords: ^{19}F-NMR, molecular mobility, crystallization

Yoshioka, S. , Aso, Y., Osako, T., Kawanishi, T. : **Wide-Ranging Molecular Mobilities of Water in Active Pharmaceutical Ingredient (API) Hydrates as Determined by NMR Relaxation Times**
J. Pharm. Sci., **97**, 4258-4268 (2008)

In order to examine the possibility of determining the molecular mobility of hydration water in API hydrates by NMR relaxation measurement, spin-spin relaxation and spin-lattice relaxation were measured for the 11 API hydrates listed in the Japanese Pharmacopeia using pulsed ^{1}H-NMR. For hydration water that has relatively high mobility and shows Lorentzian decay, molecular mobility as determined by spin-spin relaxation time (T_2) was correlated with ease of evaporation under both non-isothermal and isothermal conditions, as determined by DSC and water vapor sorption isotherm analysis, respectively. Thus, T_2 may be considered a useful parameter which indicates the molecular mobility of hydration water. In contrast, for hydration water that has low mobility and shows Gaussian decay, T_2 was found not to correlate with ease of evaporation under non-isothermal conditions, which suggests that in this case, the

molecular mobility of hydration water was too low to be determined by T_2. A wide range of water mobilities was found among API hydrates, from low mobility that could not be evaluated by NMR relaxation time, such as that of the water molecules in pipemidic acid hydrate, to high mobility that could be evaluated by this method, such as that of the water molecules in ceftazidime hydrate.
Keywords: NMR relaxation time, dynamics, hydrate

Miyazaki T., Sivaprakasam K., Tantry J., Suryanarayanan R. : **Physical characterization of dibasic calcium phosphate dihydrate and anhydrate**
J. Pharm. Sci., **98**, 905-916 (2009)

The dehydration of different commercial brands of dibasic calcium phosphate dihydrate (DCPD; $CaHPO_4 \cdot 2H_2O$) was examined over a range of temperatures and water vapor pressures. To determine the main factors affecting the physical stability of DCPD, the baseline characterization of DCPD and dibasic calcium phosphate anhydrate (DCPA; $CaHPO_4$) was conducted by thermogravimetric analysis, differential scanning calorimetry and X-ray diffractometry. The surface area and the DCPA content (present as an impurity) depended on the commercial source of DCPD. The larger particles contained a higher concentration of DCPA and the anhydrate exhibited a concentration-dependent acceleratory effect on the dehydration of DCPD. Unlike DCPD, DCPA is physically stable and resisted hydration even when dispersed in water for over 7 months in the temperature range of 4-50 degrees C. In dosage forms containing DCPD, there is a potential for phase transformation to DCPA, while the reverse transition, that is, DCPA --> DCPD appears to be extremely unlikely. Thus, the risk of physical transformation can be minimized by using DCPA in formulations.
Keywords: calcium phosphate, dehydration, X-ray diffractometry

Sakamoto, T., Hiyama, Y. : **Rapid method for determining of nitazoxanide in tablets using reversed-phase ultra-performance liquid chromatography (UPLC) and high-performance liquid chromatography**
Pharmazie, **63**, 503-507 (2008)

A simple and rapid determination method for nitazoxanide (NTZ), an antiprotozoal agent, was developed using reverse-phase HPLC and Ultra Performance Liquid Chromatography™ (UPLC™). Only six minutes gradient condition for NTZ analysis using UPLC was achieved. The mobile phase consisted of a mixture of phosphate buffer (pH6.0) and acetonitrile. The repeatability (relative standard deviation (RSD), n=6) and the correlation coefficient from linearity (the range from 80% to 120% of amount) were 0.25 % and 0.9963 for UPLC and 0.15 % and 0.9988 for HPLC, respectively. The quantitative values of NTZ in tablets were 103.2% for HPLC and 98.7% for UPLC. The RSDs of quantitative values of sample solution were calculated to be 4.06 % to 4.64 % for HPLC and 0.15 % to 0.36 % for UPLC.
Keywords: Nitazoxanide, UPLC, HPLC, Quantitative analysis

Sakamoto, T., Fujimaki, Y.[*1], Hiyama, Y. : **NIR Spectroscopic Investigation of Two Fluoroquinolones, Levofloxacin and Ofloxacin, and Their Tablets for qualitative identification of commercial products on the market**
Pharmazie, **63**, 628-632 (2008)

A rapid and nondestructive identification method for ofloxacin (OFLX) and levofloxacin (LVFX) utilizing diffusion reflectance near-infrared (NIR) spectroscopy was developed. An obvious difference in spectral patterns between LVFX that is used for commercial tablets and LVFX HCl that can be purchased as a reagent at a low price was also observed. These quinolones are especially important for use as drugs against bio-terrorism because of their effectiveness against anthrax infection. Therefore, the possibility of a distribution of counterfeit drugs containing LVFX HCl on the market would be expected. NIR spectroscopic analysis would be applicable to on-site quality analysis that can be carried out easily and nondestructively.
Keywords : near-infrared spectroscopy, racemate, enantiomer, spectroscopic analysis, diffusion reflectance, counterfeit drugs

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[*1] Tokyo Metropolitan Industrial Technology Research Institute

Sakamoto, T., Matsubara, T.[*1], Sasakura, D.[*1], Takada, Y.[*2], Fujimaki, Y.[*3], Aida, K.[*2], Miura, T.[*1], Terahara T.[*2], Higo, N.[*2], Kawanishi, T., Hiyama, Y. : **Chemical mapping of tulobuterol in transdermal tapes using Microscopic Laser Raman Spectroscopy**
Pharmazie, **64**, 166-171 (2009)

Microscopic Laser Raman Spectroscopy and Mapping

(MLRSM) technique was used to investigate the distribution of tulobuterol (TBR) crystals in transdermal tapes. TBR is one of suitable compounds for the transdermal pharmaceuticals because it has high permeability into skin. In case of TBR transdermal tapes, some commercial products also contain TBR crystals in order to control a release rate from a matrix. Therefore, the presence of TBR crystals in the matrix is a critical factor for quality assurance of this type of TDDS tapes. The model tapes prepared here employed two kinds of matrices, i.e., rubber or acrylic, which are generally used for transdermal pharmaceuticals. TBR crystals in the matrix were observed by MLRSM. Accurate observation of the distribution of TBR in the tapes was achieved by creating a Raman chemical map based on detecting unique TBR peak in each pixel. Moreover, differences in the growth of TBR crystals in the two kinds of matrices were detected by microscopic observation. MLRSM also enabled the detection of TBR crystals in commercial products. The present findings suggest that Raman micro-spectroscopic analysis would be very useful for verifying and/or assessing the quality of transdermal pharmaceuticals in development, as well as for manufacturing process control.

Keywords : Raman spectroscopy, Raman mapping, TDDS, tulobuterol, transdermal tape, crystal

[*1] Bruker Optics K.K.
[*2] TDDS Laboratory, Hisamitsu Pharmaceutical Co. Inc.
[*3] Tokyo Metropolitan Industrial Technology Research Institute

Fujimaki, Y.[*1], Matsubara, T.[*2], Sakamoto, T., Sasakura, D.[*2], Miura, T.[*2], Takekawa M.[*3], Hiyama, Y. : **Study on distribution of ethenzamide and other ingredients on granule surfaces using Raman microspectroscopy and mapping**

Pharmazie, **64**, 316-322 (2009)

Distributions of API and medical additives in granules were analyzed using Raman microspectroscopy and mapping. In order to clearly detect ingredients present at low levels, the characteristic peak for each ingredient was used for identification. Two granulation processes, tumbling granulation and high-shear granulation were selected to examine the feasibility of Raman microspectroscopy for investigating granules. Ethenzamide, lactose monohydrate, cornstarch and methylcellulose were used to make model granules. Methylcellulose was distributed homogeneously from the early stage in both granulation methods. Cornstarch and lactose showed similar distribution properties in high-shear granulation. It was presumed from this observation that similar chemical structures with high-hydrophilic groups in the two compounds determined their similar distribution properties. These results suggest that Raman microspectroscopy using the unique absorption peak of each ingredient can detect each ingredient in the individual pixel size ($2 \times 2\mu m$). This analytical method can contribute to evaluation of granular conditions and granulation processes.

Keywords : Raman microspectroscopy, Raman mapping, granules, wet granulation, tambling granulation, high-shear granulation, granulation process

[*1] Bruker Optics K.K.
[*2] TDDS Laboratory, Hisamitsu Pharmaceutical Co. Inc.
[*3] Tokyo Metropolitan Industrial Technology Research Institute

Sakai-Kato, K., Kinouchi, T.[*1], Fujii, N.[*1], Imai, K.[*2], and Utsunomiya-Tate, N.[*2] : **Screening system for D-Asp-containing proteins using D-aspartyl endopeptidase and two-dimensional gel electrophoresis**

Amino Acids, **36**, 125-129 (2009)

D-Asp-containing proteins have been implicated in many aging-related diseases. To clarify the role of D-Asp-containing proteins in such diseases, we developed a screening system for these proteins using a D-aspartyl endopeptidase that specifically cleaves the proteins at the C-terminus. The digested proteins were detected by means of two-dimensional gel electrophoresis and identified using nano-liquid chromatography/tandem mass spectrometry. We were able to detect myelin basic protein, a known D-Asp-containing protein, in the brain tissues of mice; this indicates that our system is effective for screening D-Asp-containing proteins.

Keywords : D-amino acids, aging-related disease, proteomics

[*1] 京都大学原子炉実験所
[*2] 武蔵野大学薬学部

Akaishi, T.[*], Morimoto, T.[*], Shibao, M.[*], Watanabe, S.[*], Sakai-Kato, K., Utsunomiya-Tate, N.[*], Abe, K.[*] : **Structural requirements for the flavonoid fisetin in inhibiting fibril formation of amyloid beta protein**

Neurosci. Lett. **444**, 280-285 (2008)

Fisetin (3,3',4',7-tetrahydroxyflavone) has been found to be neuroprotective, induce neuronal differentiation, enhance memory, and inhibit the aggregation of the amyloid β protein (Aβ) that may cause the progressive neuronal loss in Alzheimer's disease. The diverse collection of biological activities of this compound may lead to a new type of therapeutic drug for Alzheimer's disease. As the first step to design even more effective drugs based upon the structure of fisetin, the present study investigated the structural requirements for the anti-amyloidogenic activity of fisetin by comparing the effects of several structurally related flavonoids on Aβ fibril formation in vitro. Aβ 1-42 (20μM) and the flavonoids were incubated for 0-48h at 37℃, and fibril formation was quantitatively determined by the thioflavin T fluorescence assay. Among ten flavonoids tested, fisetin, 3',4',7-trihydroxylflavone, 3,3',4'-trihydroxyflavone, luteolin, quercetin and myricetin inhibited Aβ fibril formation. On the other hand, 3,3',7-trihydroxyflavone, 5-deoxykaempferol, chrysin and kaempferol enhanced Aβ fibril formation. These results suggest that the 3',4'-dihydroxyl group, but not the 3- or 7-hydroxyl group, is essential for the inhibitory effect of fisetin on Aβ fibril formation.

Keywords: amyloid β protein, Alzheimer's disease, fisetin

* 武蔵野大学薬学部

Hatayama, M.[*1], Tomizawa, T.[*1], Sakai-Kato, K., Bouvagnet, P.[*2], Kose, S.[*1], Imamoto, N.[*1], Yokoyama, S.[*1,3], Utsunomiya-Tate, N.[*4], Mikoshiba, K.[*1], Kigawa, T.[*1,5], Aruga, J.[*1] : **Functional and structural basis of the nuclear localization signal in the ZIC3 zinc finger domain**

Hum. Mol. Genet. **17**, 3459-73 (2008)

Disruptions in ZIC3 cause heterotaxy, a congenital anomaly of the left-right axis. ZIC3 encodes a nuclear protein with a zinc finger (ZF) domain that contains five tandem C2H2 ZF motifs. Missense mutations in the first ZF motif (ZF1) result in defective nuclear localization, which may underlie the pathogenesis of heterotaxy. Here we revealed the structural and functional basis of the nuclear localization signal (NLS) of ZIC3 and investigated its relationship to the defect caused by ZF1 mutation. The ZIC3 NLS was located in the ZF2 and ZF3 regions, rather than ZF1. Several basic residues interspersed throughout these regions were responsible for the nuclear localization, but R320, K337 and R350 were particularly important. NMR structure analysis revealed that ZF1-4 had a similar structure to GLI ZF, and the basic side chains of the NLS clustered together in two regions on the protein surface, similar to classical bipartite NLSs. Among the residues for the ZF1 mutations, C253 and H286 were positioned for the metal chelation, whereas W255 was positioned in the hydrophobic core formed by ZF1 and ZF2. Tryptophan 255 was a highly conserved inter-finger connector and formed part of a structural motif (tandem CXW-C-H-H) that is shared with GLI, Glis and some fungal ZF proteins. Furthermore, we found that knockdown of Karyopherin alpha1/alpha6 impaired ZIC3 nuclear localization, and physical interactions between the NLS and the nuclear import adapter proteins were disturbed by mutations in the NLS but not by W255G. These results indicate that ZIC3 is imported into the cell nucleus by the Karyopherin (Importin) system and that the impaired nuclear localization by the ZF1 mutation is not due to a direct influence on the NLS.

Keywords: ZIC3, zinc finger domain, nuclear localization signal

[*1] 理化学研究所
[*2] INSERM, Equipe de Recherche Methodologique
[*3] 東京大学大学院理学系研究科
[*4] 武蔵野大学薬学部
[*5] 東京工業大学大学院総合理工学研究科

Sakai-Kato, K., Ishiguro, A.[*1], Mikoshiba, K.[*1], Aruga, J.[*1], Utsunomiya-Tate, N.[*2] : **CD spectra show the relational style between Zic-, Gli-, Glis-zinc finger protein and DNA**

Biochim. Biophys. Acta, **1784**, 1011-1019 (2008)

Zic family proteins have five C2H2-type zinc finger motifs. The Zic-zinc finger domains show high homology to the corresponding domains of the Gli and Glis families, which also contain five C2H2-type zinc finger motifs. The zinc finger motifs of the proteins of these three protein families form an α-helix conformation in solution. The addition of oligo DNA that included a Gli-binding sequence increased the α-helix content estimated by using circular dichroism spectroscopy. Comparison of the Zic-, Gli-, and Glis-zinc fingers indicated that the α-helix content after the addition of oligo DNA correlated well with the affinity of each zinc finger for the oligo DNA (correlation coefficient, 0.85). The importance of the zinc ion for protein folding was reflected in a reduction in the α-helix content upon

removal of the zinc ion. Owing to the compact globular structure, the α-helix structure of the proteins of these three protein families is extremely thermally stable. These results suggest that the α-helix structure is important for DNA binding and profoundly related to functional and structural diversity among the three families.

Key words: Zic, zinc finger motifs, circular dichroism

[*1] 理化学研究所
[*2] 武蔵野大学薬学部

Satoh, K.[*1], Iwata-Takakura, A.[*1], A. Yoshikawa, A.[*1], Gotanda, Y.[*1], T. Tanaka, T.[*2], T. Yamaguchi, T., Mizoguchi, H[*1] : **A new method of concentrating hepatitis B virus (HBV) DNA and HBV surface antigen: an application of the method to the detection of occult HBV infection**

Vox Sanguinis, **95**, 174-180 (2008)

Background The risk of post-transfusion hepatitis B virus (HBV) infection has been reduced after the implementation of HBV nucleic acid amplification technology (NAT). However, the problem of HBV DNA-positive and HBV surface antigen (HBsAg)-negative occult HBV infections remains to be solved. This is in part due to the HBV DNA load being too low to detect these occult HBV infections using mini-pool NAT. In Japan, the assay for the antibody against the HBV core antigen (anti-HBc) has not completely excluded occult HBV infection. To solve this problem, we have developed a new method of concentrating HBV DNA and HBsAg simultaneously to increase the sensitivity of detection tests. **Methods** Virus concentration is achieved by the enhancement of the agglutination of viruses using poly-L-lysine in the presence of a bivalent metal. Poly-L-lysine-coated magnetic beads are used to shorten the time of each step of the concentration procedure. Seventy-seven anti-HBc-positive and HBsAg-negative donations were examined. HBsAg and anti-HBc were tested by enzyme immunoassay (EIA) (AxSYM; Abbott) and haemagglutination inhibition test (Japanese Red Cross), respectively. **Results** HBV surface antigen and HBV DNA levels were concentrated up to four- to sevenfold. Using this method, 35 of the 77 anti-HBc-positive and HBsAg-negative donors were HBV DNA-positive by individual NAT and a further five donors became HBV DNA-positive by HBV concentration. Twenty-seven of 40 occult HBV infections became HBsAg-positive by HBsAg concentration. Conclusion Our new method of concentrating HBV and HBsAg increased the sensitivities of EIA and HBV NAT, and enabled us to detect 27 of 40 occult HBV infections by HBsAg EIA.

Keywords: anti-HBc, concentration of HBV DNA, concentration of HBsAg

[*1] Japanese Red Cross Saitama Blood Center
[*2] Tokyo Women's Medical University, International Research and Educational Institute for Integrated Medical Sciences (IREIIMS)

Kawasaki, N.[*1], Lin, CW[*2,3], Inoue, R.[*1], Kay-Hooi Khoo, KH.[*2,3], Kawasaki, N., Ma, BY.[*1], Shogo Oka, S.[*4], Ishiguro, M.[*5], Sawada, T.[*6], Ishida, H.[*6], Hashimoto, T.[*7], Kawasaki, T.[*1] : **Highly fucosylated N-glycan ligands for mannan-binding protein expressed specifically on CD26 (DPPVI) isolated from a human colorectal carcinoma cell line, SW1116**

Glycobiology, **19**, 430-450 (2009)

The serum mannan-binding protein (MBP) is a host defense C-type lectin specific for mannose, *N*-acetylglucosamine, and fucose residues, and exhibits growth inhibitory activity toward human colorectal carcinoma cells. The MBP-ligand oligosaccharides (MLO) isolated from a human colorectal carcinoma cell line, SW1116, are large, multiantennary *N*-glycans with highly fucosylated polylactosamine-type structures having Le^b–Le^a or tandem repeats of the Le^a structure at their nonreducing ends. In this study, we isolated the major MBP-ligand glycoproteins from SW1116 cell lysates with an MBP column and identified them as CD26/dipeptidyl peptidase IV (DPPIV) (110 kDa) and CD98 heavy chain (CD98hc)/4F2hc (82 kDa). Glycosidase digestion revealed that CD26 contained such complex-type *N*-glycans that appear to mediate the MBP binding. MALDI-MS of the *N*-glycans released from CD26 by PNGase F demonstrated conclusively that CD26 is the major MLO-carrying protein. More interestingly, a comparison of the *N*-glycans released from the MBP-binding and non-MBP-binding glycopeptides suggested that complex-type N-glycans carrying a minimum of 4 Le^a/Le^b epitopes arranged either as multimeric tandem repeats or terminal epitopes on multiantennary structures are critically important for the high affinity binding to MBP. Analysis of the *N*-glycan attachment sites demonstrated that the high affinity MLO was expressed preferentially at some *N*-glycosylation sites, but this site preference was not so stringent. Finally, hypothetical 3D models of tandem repeats of the Le^a epitope and the MBP-Lewis oligosaccharide complex were presented.

Keywords: CD26, Le[a] epitope, mannan-binding lectin

[*1] Research Center for Glycobiotechnology, Ritsumeikan University
[*2] Institute of Biological Chemistry
[*3] Graduate Institute of Biochemical Sciences, National Taiwan University
[*4] Human Health Sciences, Graduate School of Medicine, Kyoto University
[*5] Suntory Institute for Bioorganic Research
[*6] Faculty of Applied Biological Sciences, Gifu University
[*7] Faculty of Regional Studies, Gifu University

Itoh, S., Hachisuka, A., Kawasaki, N., Hashii, N., Teshima, R., Hayakawa, T., Kawanishi, T., Yamaguchi, T. : **Glycosylation analysis of IgLON family proteins in rat brain by liquid chromatography and multiple-stage mass spectrometry**
Biochemistry, **47**, 10132-10154 (2009)

IgLON family proteins, including limbic-associated membrane protein (LAMP), opioid-binding cell adhesion molecule (OBCAM), neurotrimin, and Kilon, are immunoglobulin (Ig) superfamily cell adhesion molecules. These molecules are composed of three Ig domains and a glycosylphosphatidylinositol (GPI) anchor and contain six or seven potential N-glycosylation sites. Although their glycosylations are supposed to be associated with the development of the central nervous system like other Ig superfamily proteins, they are still unknown because of difficulty in isolating individual proteins with a high degree of homology in performing carbohydrate analysis. In this study, we conducted simultaneous site-specific glycosylation analysis of rat brain IgLON proteins by liquid chromatography and multiple-stage mass spectrometry (LC-MS (n)). The rat brain GPI-linked proteins were enriched and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The four proteins were extracted from the gel, and subjected to LC-MS (n) after proteinase digestions. A set of glycopeptide MS data, including the mass spectrum, the mass spectrum in the selected ion monitoring mode, and the product ion spectra, was selected from all data based on carbohydrate-related ions in the MS/MS spectrum. The peptide portion and the carbohydrate structure were identified on the basis of peptide-related ion and carbohydrate-related ions, and the accurate mass. The site-specific glycosylations of four proteins were elucidated as follows. N-Glycans near the N-terminal were disialic acid-conjugated complex- and hybrid-type oligosaccharides. The first Ig domains were occupied by Man-5-9. Diverse oligosaccharides, including Lewis a/x-modified glycans, a brain-specific glycan known as BA-2, and Man-5, were found to be attached to the third Ig domain. Three common structures of glycans were found in the GPI moiety of LAMP, OBCAM, and neurotrimin.

Keywords: IgLON, liquid chromatography/multiple-stage mass spectrometry, GPI-anchor

Hashii, N., Kawasaki, N., Itoh, S., Nakajima, Y., Kawanishi, T., Yamaguchi, T. : **Alteration of N-glycosylation in the kidney in a mouse model of systemic lupus erythematosus: relative quantification of N-glycans using an isotope-tagging method**
Immunology, **126**, 336-345 (2009)

Changes in the glycan structures of some glycoproteins have been observed in autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis. A deficiency of alpha-mannosidase II, which is associated with branching in N-glycans, has been found to induce SLE-like glomerular nephritis in a mouse model. These findings suggest that the alteration of the glycosylation has some link with the development of SLE. An analysis of glycan alteration in the disordered tissues in SLE may lead to the development of improved diagnostic methods and may help to clarify the carbohydrate-related pathogenic mechanism of inflammation in SLE. In this study, a comprehensive and differential analysis of N-glycans in kidneys from SLE-model mice and control mice was performed by using the quantitative glycan profiling method that we have developed previously. In this method, a mixture of deuterium-labelled N-glycans from the kidneys of SLE-model mice and non-labelled N-glycans from kidneys of control mice was analysed by liquid chromatography/mass spectrometry. It was revealed that the low-molecular-mass glycans with simple structures, including agalactobiantennary and paucimannose-type oligosaccharides, markedly increased in the SLE-model mouse. On the other hand, fucosylated and galactosylated complex type glycans with high branching were decreased in the SLE-model mouse. These results suggest that the changes occurring in the N-glycan synthesis pathway may cause the aberrant glycosylations on not only specific glycoproteins but also on most of the glycoproteins in the SLE-model mouse. The changes in glycosylation might be involved in autoimmune pathogenesis in the model mouse kidney.

Keywords: isotope-tagging method, liquid chromatog-

raphy/multiple-stage mass spectrometry, systemic lupus erythematosus

Urayama, S.[*1]. Harada, Y.[*1], Nakagawa, Y.[*1], Ban, S.[*1,2], Akasaka, M.[*1], Kawasaki, N., Sawada, H.[*1] : **Ascidian sperm glycosylphosphatidylinositol-anchored CRISP-like protein as a binding partner for an allorecognizable sperm receptor on the vitelline coat**

J.Biol. Chem., **283**, 21725-21733 (2008)

Although ascidians are hermaphroditic, many species including *Halocynthia roretzi* are self-sterile. We previously reported that a vitelline coat polymorphic protein HrVC70, consisting of 12 EGF (epidermal growth factor)-like repeats, is a candidate allorecognition protein in H. roretzi, because the isolated HrVC70 shows higher affinity to nonself-sperm than to self-sperm. Here, we show that a sperm 35-kDa glycosylphosphatidylinositol-anchored CRISP (cysteine-rich secretory protein)-like protein HrUrabin in a low density detergent-insoluble membrane fraction is a physiological binding partner for HrVC70. We found that HrVC70 specifically interacts with HrUrabin, which had been separated by SDS-PAGE and transferred onto a nitrocellulose membrane. HrUrabin has an *N*-linked sugar chain, essential for binding to HrVC70. *HrUrabin* mRNA is expressed in the testis but not in the ovary, and the protein appears to be localized on the surface of sperm head and tail. Anti-HrUrabin antibody, which neutralizes the interaction between HrUrabin and HrVC70, potently inhibited fertilization and allorecognizable sperm-binding to HrVC70-agarose. However, no significant difference in the binding ability of HrUrabin to HrVC70 was observed in autologous and allogeneic combinations by Far Western analyses. These results indicate that sperm-egg binding in *H. roretzi* is mediated by the molecular interaction between HrUrabin on the sperm surface and HrVC70 on the vitelline coat, but that HrUrabin *per se* is unlikely to be a direct allorecognition protein.

Keywords: ascidian sperm, HrVC70, HrUrabin

[*1] Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University

[*2] Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University

Harazono, A., Kawasaki, N., Itoh, S., Hashii, N., Matsuishi-Nakajima, Y., Kawanishi, T., Yamaguchi, T. : **Simultaneous glycosylation analysis of human serum glycoproteins by high-performance liquid chromatography/tandem mass spectrometry**

J Chromatogr. B Analyt Technol Biomed Life Sci., **869**, 20-30 (2008)

Changes in the glycosylation of some serum proteins are associated with certain diseases. In this study, we performed simultaneous site-specific glycosylation analysis of abundant serum glycoproteins by LC/Qq-TOF MS of human serum tryptic digest, the albumin of which was depleted. The glycopeptide peaks on the chromatogram were basically assigned by database searching with modified peak-list text files of MS/MS spectra and then based on mass differences of glycan units from characterized glycopeptides. Glycopeptide of IgG, haptoglobin and ceruloplasmin were confirmed by means of a comparison of their retention times and m/z values with those obtained by LC/MS of commercially available glycoproteins. Mass spectrometric carbohydrate heterogeneity in the assigned glycopeptides was analyzed by an additional LC/MS. We successfully demonstrated site-specific glycosylation of 23 sites in abundant serum glycoproteins.

Keywords: glycosylation analysis, human serum, LC/MS

Sano, K.[*], Asahi, M.[*], Yanagibashi, M.[*], Hashii, N., Itoh, S., Kawasaki, N., Ogawa, H.[*] : **Glycosylation and ligand-binding activities of rat plasma fibronectin during liver regeneration after partial hepatectomy**

Carbohydr. Res., **343**, 2329-2335 (2008)

Fibronectin (FN) is a multifunctional glycoprotein present in the extracellular matrix (ECM) and plasma. We previously reported that the glycosylation and ligand-binding of vitronectin (VN) change markedly after partial hepatectomy (PH). Here we show the changes of FN during liver regeneration. The yields of purified sham-operated (SH-) and PH-FN were higher than that of non-operated (NO)-FN, while binding activities of FNs to ECM ligands were changed only slightly by hepatectomy. The carbohydrate concentration of PH-FN decreased to 66% of that of NO- and SH-FN. By using LC/MS(n), eight kinds of complex-type N-glycan structures were found to be present in all FNs, and bi- and trisialobiantennary glycans were the major structures. Fucosylation was markedly increased, while O-acetylation of sialic acid was found to be decreased in PH-FN. The alterations in glycosylation and biological activities of FN after PH are different from those of VN, suggesting that these glycoproteins play different biological functions

in tissue remodeling.
Keywords: fibronectin, vitronectin, glycosylation

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* Graduate School of Humanities and Sciences, Ochanomizu University

Kawasaki, N., Itoh, S., Hashii, N., Harazono, A., Takakura, D., Yamaguchi, T. : **Mass spectrometry for analysis of carbohydrate heterogeneity in characterization and evaluation of glycoprotein**
Trends. Glycosci. Glycotechnol., **20**, 97-116 (2008)

Analysis of the carbohydrate heterogeneity of glycoprotein-based substances is crucial for establishing the nomenclature and deˆnition of biological substances, ensuring consistency in the quality of these products, comparatively assessing the products obtained after the implementation of changes in the manufacturing process, and developing biosimilar or follow-on biological products. Liquid chromatography/mass spectrometry is recognized as one of the most useful techniques for analyzing the carbohydrate heterogeneity of glycoprotein substances. Here, we demonstrate the utility of LC/MS for analyzing the carbohydrate heterogeneity by using some representative glycoproteins such as tissue-plasminogen activator, a monoclonal antibody, the folliclestimulating hormone, and human chorionic gonadotropin. Further, we demonstrate that MS-based glycoprotein analysis has potential applications in glycomics.
Keywords: LC/MS, glycoprotein, heterogeneity

Suzuki, T., Tamehiro, N., Sato, Y., Kobayashi, T., Ishii-Watabe, A., Shinozaki, Y., Nishimaki-Mogami, T., Hashimoto, T.[*1], Asakawa, Y.[*1], Inoue, K.[*2], Ohno, Y., Yamaguchi, T., Kawanishi, T. : **The novel compounds that activate farnesoid X receptor: the diversity of their effects on gene expression**
J. Pharmacol. Sci., **107**, 285-294 (2008)

Farnesoid X receptor (FXR) controls the expression of critical genes in bile acid and cholesterol homeostasis. To study FXR and to develop a regulator of cholesterol, some non-steroidal and steroidal ligands have been found in addition to endogenous ligands for FXR. In this study, we discovered five bile acid derivatives (methyl cholate, methyl deoxycholate, 5beta-cholanic acid, 5beta-cholanic acid-7alpha,12alpha-diol, and NIHS700) and two natural products (marchantin A and marchantin E) that activated FXR in the reporter assay. These compounds activated FXR to a high level comparable to the most potent endogenous bile acid, chenodeoxycholic acid, although it was not predicted from their structures; five of them were similar to the lower potency bile acids, and two were structurally much different from bile acids. The elevation levels of reporter gene expression by some of the screened compounds were varied in Cos-7, HepG2, HuH-7, and Caco-2 cells. These compounds also controlled the expression of genes regulated by FXR, and some of the compounds regulated these genes in a cell-type-specific and/or gene-selective fashion. Therefore, molecular design of the compounds can cause selective modulation of the expression of FXR target genes.
Keywords: farnesoid X receptor, reporter assay, ginkgolic acid

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[*1] Faculty of Pharmaceutical Sciences, Tokushima Bunri University
[*2] Graduate School of Pharmaceutical Sciences, Kyushu University

Shibata, H.[*], Nakano, T.[*], Parvez, MA.[*], Furukawa, Y.[*], Tomoishi, A.[*], Niimi, S., Arakai, N.[*], and Higuti, T.[*] : **Triple combinations of lower and longer alkyl gallates and oxacillin improve antibiotic synergy against methicillin-resistant Staphylociccus aureus.**
Antimicrob, Agents, Chemother., **53**, 2218-2220 (2009)

Using liposome systems, we found that gallates with short alkyl chains were located in the external medium and those with longer alkyl chains were located in the surface region of lipid bilayer. Combinations of these gallates remarkably reduced oxacillin MICs against methicillin-resistant Staphylococcus aureus to below the antibiotic breakpoint (< or = 2 microg/ml).
Keywords: gallates, oxacillin, MRSA

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* Institute of Health Biosciences, The University of Tokushima Graduate School

Parvez, MA.[*1], Shibata, H.[*1], Nakano, T.[*1], Niimi, S., Fujii, N.[*2], Arakaki, N.[*1] and Higuti, T.[*1] : **No relationship exists between PBP 2a amounts expressed in different MRSA strains obtained clinically and their beta-lactam MIC values**
J. Med. Inves., **55**, 246-253 (2008)

After establishing a linear relationship between the amount of penicillin-binding protein (PBP) 2a and membrane proteins of methicillin-resistant Staphylococcus aureus (MRSA)

COL by dot-blot analysis using an antibody against PBP 2a, we determined the PBP 2a quantities in membrane fractions prepared from 14 different MRSA cells. Methicillin-sensitive S. aureus ATCC 6538P was used as a quality control strain. The amounts of PBP 2a diverged among the strains, and no relationship to beta-lactam MIC values were observed in the corresponding strains.

Keywords: MRSA, PBP, β-lactan, MIC values

[*1] Institute of Health Biosciences, The University of Tokushima Graduate School

[*2] Faculty of Human Life Sciences, Tokushima Bunri Univesity

Harashima, M.[*], Harada K.[*], Ito, Y.[*], Hyuga, M., Seki, T.[*], Ariga, T.[*], Yamaguchi, T. and Niimi, S. : **Annexin A3 expression Increases in Hepatocytes and is regulated by hepatocyte growth factor in rat liver regeneration**

J. Biochem., **143**, 537-545 (2008)

Annexin (Anx) A3 increases and plays important roles in the signalling cascade in hepatocyte growth in cultured hepatocytes. However, no information is available on its expression and role in rat liver regeneration. In the present study, AnxA3 expression was investigated to determine whether it also plays a role in the signalling cascade in rat liver regeneration. AnxA3 protein and mRNA level both increase in liver after administration of carbon tetrachloride (CCl4) or 70% partial hepatectomy. AnxA3 protein level increases in isolated parenchymal hepatocytes, but not in non-parenchymal liver cells, in these rat liver regeneration models. AnxA3 mRNA increases in hepatocytes after CCl4 administration. Anti-hepatocyte growth factor antibody suppresses this increase in AnxA3 mRNA level. These results demonstrate that AnxA3 expression increases in hepatocytes through a hepatocyte growth factor-mediated pathway in rat liver regeneration models, suggesting that AnxA3 plays an important role in the signalling cascade in rat liver regeneration.

Keywords: annexin A3, carbon tetrachloride, hepatocyte growth factor, parenchymal hepatocytes, partial hepatectomy

[*] Nihon University College of Bioresource Sciences

橋井則貴，川崎ナナ，髙倉大輔，伊藤さつき，川原信夫，正田卓司，杉本直樹，葩島由二，品川麻衣[*1]，榛葉信久[*1]，宮田一義[*2]，塚本秀樹[*3]，千秋和久[*3]，長谷川泰介[*4]，河合健蔵[*5]，余田　光[*5]，木下充弘[*6]，掛樋一晃[*6]，合田幸広，奥田晴宏，棚元憲一，山口照英：**ヘパリン純度試験に関する研究（第1報）^{1}H-NMRによるヘパリンナトリウム純度試験に関する研究**

医薬品研究, **39**, 651-659 (2008)

^{1}H-NMR による 過硫酸化コンドロイチン硫酸及びデルマタン硫酸分析法を確立するとともに，日本薬局方各条ヘパリンナトリウム純度試験としての適用可能性を検証した.

Keywords: ヘパリンナトリウム，過硫酸化コンドロイチン硫酸，^{1}H-NMR

[*1] 味の素（株）ライフサイエンス研究所
[*2]（株）大塚製薬工場
[*3] テルモ（株）研究開発センター
[*4] 日本臓器製薬（株）生物活性科学研究所
[*5] 扶桑薬品工業（株）研究開発センター
[*6] 近畿大学薬学部

橋井則貴，川崎ナナ，髙倉大輔，伊藤さつき，福原潔，品川麻衣[*1]，榛葉信久[*1]，有村雅敏[*2]，辰巳昌史[*2]，奥田晴宏，山口照英：**ヘパリン純度試験に関する研究（第2報）^{1}H-NMRによるヘパリンカルシウム純度試験に関する研究**

医薬品研究, **39**, 660-712 (2008)

^{1}H-NMR による 過硫酸化コンドロイチン硫酸及びデルマタン硫酸分析法を確立するとともに，局外規ヘパリンカルシウム純度試験としての適用可能性を検証した.

Keywords: ヘパリンカルシウム，過硫酸化コンドロイチン硫酸，^{1}H-NMR

[*1] 味の素（株）ライフサイエンス研究所
[*2] 沢井製薬（株）

掛樋一晃[*1], 栂　直孝[*1]，木下充弘[*1], 橋井則貴, 川崎ナナ, 寺尾敏光[*2]，河合健蔵[*3], 余田　光[*3],山口照英：**ヘパリンナトリウム純度試験に関する研究（第3報）キャピラリー電気泳動法によるヘパリンナトリウム不純物の分析**

医薬品研究, **39**, 713-720 (2008)

キャピラリー電気泳動法による過硫酸化コンドロイチン硫酸及びデルマタン硫酸分析法を確立するとともに，日本薬局方各条ヘパリンナトリウム純度試験としての適用可能性を検証した.

Keywords: ヘパリンナトリウム，過硫酸化コンドロイチン硫酸，キャピラリー電気泳動

[*1] 近畿大学薬学部
[*2]（株）大塚製薬工場
[*3] 扶桑薬品工業（株）研究開発センター

川崎ナナ，橋井則貴，杉本直樹，髙倉大輔，秦　艶，細山沙織[*1]，戸井田敏彦[*1]，山口照英：**ヘパリン純度試験に関する研究（第4報）合成過硫化コンドロイチン硫酸の日局標準品としての適用性の評価**

医薬品研究, **39**, 721-729 (2008)

合成した過硫酸化コンドロイチン硫酸 (OSCS) のOSCS標準品としての適用可能性を明らかにすることを目的として，有害事象を引き起こしたヘパリンナトリウムから精製したOSCS及び合成OSCSの品質特性を比較した．

Keywords: ヘパリンナトリウム，合成過硫酸化コンドロイチン硫酸，日局標準品

[*1] 千葉大学大学院薬学研究科

原園　景，川崎ナナ，伊藤さつき，小林　哲，石川リカ[*1]，高井俊紀[*1]，古賀明子[*2]，岡本寿美子[*2]，山口秀人[*3]，濱詰康樹[*4]，佐藤貴之[*4]，窪田雅之[*5]，掛樋一晃[*6]，木下充弘[*6]，島　圭介[*7]，山田真希[*7]，山口照英：**質量分析法を用いたペプチド及びタンパク質性医薬品の確認試験法に関する研究**

医薬品研究, **39**, 627-646 (2008)

Since mass spectrometry (MS) and tandem mass spectrometry (MS/MS) make it possible to measure an accurate mass of peptides and proteins and provide structural information, they have been used for not only analysis of primary structure and post-translational modification but also identification tests of peptide and protein products. However, assay methods for identification tests have not been fully standardized due to different ionization methods, many types of analyzers and various analytical conditions. In this paper, mass spectrometry for identification tests of peptide and protein products has been standardized and validated in a collaborative study using several types of mass spectrometers equipped with ESI or MALDI sources. The results of molecular mass measurement from the collaborative study suggest the following acceptance criteria: i) 0.3 Da (monoisotopic mass) for peptides with masses <1,000 Da, ii) 300 ppm (monoisotopic mass) and 500 ppm (average mass) for peptides with masses 1,000~6,000 by ESI-MS and MALDI-MS, and iii) 500 ppm and 1,600 ppm (average mass) for proteins with masses 6,000~22,000 Da by ESI-MS and MALDI-MS, respectively. Although peptides with masses 1,000~4,000 Da yielded 5~10 of b- and y- series fragments by CID-MS/MS and PSD, the detected ions were not identical among laboratories. Further study is necessary for setting of optimization and standardization of MS/MS conditions.

Keywords: Mass spectrometry, Peptide/Protein, Identification test

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[*1] キリンファーマ（株）
[*2] 中外製薬（株）
[*3] アステラス製薬（株）
[*4] 大日本住友製薬（株）
[*5] サーモフッシャーサイエンティフィック（株）
[*6] 近畿大学薬学部
[*7] 島津製作所（株）

後藤洋子[*]，新見伸吾：**ラクトース修飾フィブロイン基材上における初代培養ラット肝細胞のスフェロイド形成と維持**

高分子論文集 (Kobunshi Ronbunshu), **65**, 312-316 (2008)

We examined the formation and maintenance of spherical multicellular aggregates (spheroids) of rat primary hepatocytes on dishes coated with lactose-silk fibroin conjugates bearing galactose residues (Lac-CY-SF) as substrate materials. Rat hepatocytes that had attached onto the conjugate-coated dishes were subsequently cultured in a medium supplemented with epidermal growth factor (EGF) and insulin. After the rat hepatocytes extended flat on the conjugate-coated dishes in 2 days of culture, these hepatocytes formed spheroids about 100 to 300μm in diameter at days 4. After 6 days of culture the spheroids were maintained without any obvious change in size on the conjugate-coated dishes. However, the detachment of the spheroids from the conjugate-coated dishes began from day 8, and the spheroids clearly shrank at day 10. These results suggested that hepatocyte spheroid formation was induced on the Lac-CY-SF conjugate-coated dishes in the presence of EGF and insulin, but the conjugate-coated dishes were not capable of keeping the spheroids for more than 5 days.

Keywords: lactose-silk fibroin conjugates, rat hepatocytes, epidermal growth factor, insulin, spheroids

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[*] National Institute of Agrobiological Sciences

Watanabe, K.[*], Hyuga, S.[*], Hyuga, M., Sekiguchi, A.[*], Endo, M.[*], Tsuda, T.[*], Oikawa, T.[*], Yamaguchi, T., and Hanawa, T.[*] : **Unkeito, a traditional Kampo for-**

mula, exhibts a selective estrogen receptor modulator-like activity

J. Trad. Med., **26**, 18-24 (2009)

Unkeito is a Kampo formula used to treat several menstrual disorders and menopausal symptoms. Our aim was to determine the effects of unkeito on trabecular bone mineral density (BMD) and uterus in the ovariectomized (OVX) mouse to predict the effects of unkeito on women in the post-reproductive period Design: Ten-week-old Balb/c mice were ovariectomized to induce both osteoporosis and loss of ovarian function. Two weeks after surgery, the mice were divided into control and experimental groups. The control mice were given unlimited access to tap water. The experimental mice were given an estrogen solution (6.25 or 12.5 mg/day), unkeito suspension (60, 120, 240, or 480 mg/day), or a combination of 12.5 mg/day estrogen and unkeito (240 or 480 mg/day) orally for two weeks. The trabecular BMD, uterine weight, and endometrial thickness were then measured. Results: Trabecular BMD, uterine weight, and endometrial thickness in OVX mice were markedly decreased as compared with the sham-operated mice. The trabecular BMD in OVX mice that received unkeito was significantly increased in comparison with that of OVX mice that did not receive unkeito. No significant differences in uterine weight or endometrial thickness were noted between the unkeito groups and the OVX groups. Moreover, the increment in both uterine weight and endometrial thickness in OVX mice induced by estradiol was reduced by oral administration of unkeito. Conclusion: OVX mice showed recovery from trabecular BMD loss without both uterine weight gain and increase in endometrial thickness, after treatment with unkeito for two weeks. Thus, we found for the first time that unkeito exhibits a selective estrogen receptor modulator-like activity.

Keywords: Kampo, estrogen, selective estrogen receptor modulator

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* 北里研究所東洋医学総合研究所

Goda, Y., Kawahara, N., Kiuchi, F.[*1], Hirakura, K.[*2], Kikuchi, Y.[*2], Nishimura, H.[*2], Takao, M. [*2], Marumoto, M.[*2], Kitazaki, H.[*2] : **A guanidine derivative from seeds of *Plantago asiatica***

J. Nat. Med., **63**, 58-60 (2009)

A new guanidine derivative named plantagoguanidinic acid was isolated from the seeds of *Plantago asiatica*. The structure was elucidated by two-dimensional (2D) nuclear magnetic resonance (NMR) spectral and other spectral methods.

Keywords: *Plantago asiatica*, Plantaginaceae, guanidine derivative

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[*1] Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation

[*2] Tsumura & Co.

Makino, T.[*1], Hishida, A.[*2], Goda, Y., Mizukami, H.[*1] : **Comparison of major flavonoid contents in *Scutellaria baicalensis*, *S. lateriflora* and their commercial products**

J. Nat. Med., **62**(3), 294-299 (2008)

According to the notification for definition of pharmaceuticals from the Director-General of the Pharmaceutical and Food Safety Bureau, Ministry of Health Labour and Welfare of Japan, the roots of *Scutellaria baicalensis* (Chinese skullcap) and *S. lateriflora* (skullcap) are classified as "the raw materials exclusively used as pharmaceuticals", but their aerial parts are classified as "non-pharmaceuticals" so, in principle, there are no health claims for these materials and no descriptions of drug-like dosages or administration directions. Dried root of *S. baicalensis* is also registered in Japanese Pharmacopoeia XV as scutellaria root. Scutellaria root is considered to have the adverse drug reactions of interstitial pneumonia and drug-induced hepatopathy in kampo medicines (Japanese traditional herbal formulations), and baicalin, its major constituent, is considered to be the cause of the adverse reaction. This study was conducted to evaluate the validity of this borderline between pharmaceuticals and non-pharmaceuticals by analyzing the amounts of four flavonoids, including baicalin, in the roots, stems, and leaves of *S. baicalensis* and *S. lateriflora*, and in the commercial products herbal tea and dietary supplements prepared from *S. lateriflora*. These flavonoids were found in the root of *S. baicalensis*; its aerial parts, however, did not contain them. On the other hand, the amounts of those flavonoids in the aerial parts of *S. lateriflora* were larger than in the root. Herbal tea and dietary supplements of *S. lateriflora* obtained commercially also contained those flavonoids, and the dietary supplements contained amounts of them comparable with that in kampo medicine. These results suggest that classification that the aerial parts of *S. lateriflora* as non-pharmaceuticals in Japan needs reconsideration.

Keywords: *Scutellaria baicalensis, Scutellaria lateriflora*, flavonoid composition

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[*1] Graduate School of Pharmaceutical Sciences, Nagoya City University

[*2] Tsukuba Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation

Amakura, Y.[*1], Yoshimura, M.[*1], Mouri, C.[*2], Mikage, M.[*2], Kawahara, N., Goda, Y., Yoshida, T.[*1] : **Convenient TLC-based identification test for the crude drug "Pogstemoni Herba"**

Yakugaku Zasshi, **128**(12), 1833-1837 (2008)

TLC and HPLC were used to identify possible chemical markers for evaluating the quality of the crude drug "Pogostemoni herba" (aerial part of *Pogostemon cablin*), which is a component of Kampo medicines. In addition to the reported patchouli alcohol and 2-hydroxy-6-methyl-3-(4-methylpentanoyl)-4-pyrone, three phenylethanoids were isolated from this plant material for the first time: acteoside, isoacteoside, and crenatoside. The usefulness of these compounds as indicators of the crude commercial drug under various TLC conditions was examined, and patchouli alcohol was found to give a definite spot with a reproducible Rf value. Therefore, we propose TLC of the methanol (MeOH) extract using patchouli alcohol as a marker as a convenient method for identifying the crude drug Pogostemoni herba.

Keywords: *Pogostemon cablin*, Pogostemoni herba, patchouli alcohol

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[*1] 松山大学薬学部

[*2] 金沢大学大学院自然科学研究科

Terabayashi, S.[*1], Sakai, E.[*2], Yamaji, H.[*3], Kondo, K.[*3], Kawahara, N., Goda, Y. : **Authentication and standardization of botanical origin and morphology of Coix fruit in the Japanese Pharmacopoeia**

Journal of Japanese Botany, **84**(2), 77-84 (2009)

Definition (botanical origin) and description (morphology, etc.) of Coix fruit in the Japanese Pharmacopoeia were presented for authentication and standardization of crude drug. The crude drug, Coix fruit is defined as a fruit enveloped with an involucre of *Coix lacryma-jobi* Linné var. *mayuen* Stapf. The smell, taste, and external morphological and anatomical features of Coix fruit were described based mainly on market samples. The term of organ enveloping fruit is also discussed.

Keywords: Coix fruit, *Coix lacryma-jobi* Linné var. mayuen Stapf, morphology

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[*1] 横浜薬科大学

[*2] 岐阜薬科大学

[*3] (株) ツムラ

Hasegawa, T.[*], Saijo, M.[*], Ishii, T.[*], Nagata, T.[*], Haishima, Y., Kawahara, N., Goda, Y. : **Structural elucidation of a tadalafil analogue found in a dietary supplement**

J. Food Hyg. Soc. Japan, **49**(4), 311-315 (2008)

A tadalafil analogue was detected in a dietary supplement marketed for tonic effect, along with hydroxyhomosildenafil and aminotadalafil. The tadalafil analogue was isolated by preparative thin layer chromatography (TLC) and its structure was elucidated using high-performance liquid chromatography (HPLC), liquid chromatography electrospray ionization-mass spectrometry (LC-ESI-MS), Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) and nuclear magnetic resonance (NMR) spectroscopy. The compound was determined to be methyl-1-(1,3-benzodioxol-5-yl)-2-(chloroacetyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylate. This is the first report of detection of this compound in a dietary supplement.

Keywords: tadalafil analogue, tonic effect, dietary supplement

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[*] 千葉県衛生研究所

Kawahara, N., Ido, Y.[*1], Nakajima, I.[*1], Kawasaki, T.[*1], Sakai, E.[*2], Goda, Y. : **Comparative study on testing methods and specification values for crude drugs in Pharmacopoeias among four western pacific regional countries (Japan, China, Korea and Vietnam) (IV) Comparative study on TLC identification for crude drugs considering harmonization and clean analysis**

Shoyakugaku Zasshi, **62**(2), 72-78 (2008)

Recently, from the viewpoint of prevention of environmental pollution and the health protection of researcher, clean analysis removing harmful chemical reagents such as benzene and chloroform is recommended worldwide. At the fourth Western Pacific Regional Forum for the Harmonization of Herbal Medicines (FHH) Standing

Committee in Tokyo 2006, we proposed the collaborative study of the developing solvent for TLC identification in Pharmacopoeia, considering clean analysis. The proposed collaboration study is as follows: each member state tests the TLC analysis using non-toxic solvent systems which are described in other members' Pharmacopoeia with regard to the designgted crude drugs in the comparative table. In this paper, we show the results of the task work. From our comparative study, it is suggested that for almost all TLC identification of crude drugs using chloroform (or benzene) as a developing solvent, non-toxic solvent systems will be able to replace the toxic solvents.

Keywords: FHH, TLC identification, clean analysis

[*1]（株）ウチダ和漢薬
[*2] 岐阜薬科大学

Hirasawa, Y.[*1], Tanaka, T.[*1], Kobayashi, J.[*2], Kawahara, N., Goda, Y., Morita, H.[*1] : **Malycorins A-C, new Lycopodium alkaloids from *Lycopodium phlegmaria***

Chem. Pharm. Bull., **56**(10), 1473-1476 (2008)

A novel C_{19}N-type *Lycopodium* alkaloid, malycorin A (1) consisting of a serratinane skeleton with 2-propanol unit has been isolated from the club moss *Lycopodium phlegmaria*, together with two new C_{16}N-type alkaloids, malycorins B (2) and C (3), and the structures and relative stereochemistry were elucidated on the basis of spectroscopic data.

Keywords: *Lycopodium* alkaloid, malycorin, *Lycopodium phlegmaria*

[*1] 星薬科大学
[*2] 北海道大学大学院薬学研究科

Hirasawa, Y.[*1], Kato, E.[*1], Kobayashi, J.[*2], Kawahara, N., Goda, Y., Shiro, M.[*3], Morita, H.[*1] : **Lycoparins A-C, new alkaloids from *Lycopodium casuarinoides* inhibiting acetylcholinesterase**

Bioorg. Med. Chem., **16**, 6167-6171 (2008)

Three new *Lycopodium* alkaloids, lycoparins A-C (1-3), have been isolated from the club moss *Lycopodium casuarinoides*. Structures and stereochemistry of 1-3 were elucidated on the basis of 2D NMR correlations. Lycoparins C (3) exhibited an inhibitory activity against acetylcholinesterase, while lycoparins A (1) and B (2) did not show activity.

Keywords: alkaloid, lycoparin, *Lycopodium casuarinoides*

[*1] 星薬科大学
[*2] 北海道大学大学院薬学研究科
[*3]（株）リガク

Hasegawa, T.[*], Takahashi, K.[*], Saijo, M.[*], Ishii, T.[*], Nagata, T.[*], Kurihara, M., Haishima, Y., Goda, Y., Kawahara, N. : **Isolation and structural elucidation of cyclopentynafil and *N*-octylnortadalafil found in a dietary supplement**

Chem. Pharm. Bull., **57**(2), 185-189 (2009)

A new sildenafil analogue, cyclopentynafil (1) and a new tadalafil analogue, *N*-octylnortadalafil (2) were isolated from a dietary supplement illegally marketed for erectile dysfunction. The structures of the sildenafil and tadalafil analogues were elucidated by using HPLC-photodiode array (PDA), LC-MS, high-resolution MS, NMR and circular dichroism (CD). These compounds were determined to be 5-[2-ethoxy-5-(4-cyclopentylpiperazin-1-ylsulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one and (6*R*,12a*R*)-2-octyl-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydropyrazino[1',2:1,6]pyrido[3,4-*b*]indole-1,4-dione, respectively. Recently, a large number of phosphodiesterase-5 (PDE-5) inhibitors, including their analogues, have been isolated from dietary supplements, while cyclopentynafil and *N*-octylnortadalafil are the first compounds reported to be new sildenafil and tadalafil analogues, respectively. Quantitative HPLC analysis showed that the contents of 1 and 2 in the product were about 130 mg/tablet (301μg/mg) and about 27 mg/tablet (64.1μg/mg), respectively.

Keywords: cyclopentynafil, *N*-octylnortadalafil, phosphodiesterase-5 inhibitor

[*] 千葉県衛生研究所

Hirasawa, Y.[*1], Miyama, S.[*1], Kawahara, N., Goda, Y., Rahman, A.[*2], Ekasari, W.[*2], Widyawaruyanti, A.[*2], Indrayanto, G.[*2], Zaini, N. C.[*2], Morita, H.[*1] : **Indole Alkaloids from the Leaves of *Alstonia scholaris***

Heterocycles, **79**(1), 1107-1112 (2009)

A new indole alkaloid, akuammidine-*N*-oxide (1) was isolated from the leaves of *Alstonia scholaris* (Apocynaceae) together with akuammidine (2), and the structure was elucidated by NMR spectral analysis and chemical correlation. Akuammidine (2) showed a moderate antiplasmodial activity.

Keywords: indole alkaloid, akuammidine-*N*-oxide, *Alstonia*

scholaris

[*1] 星薬科大学
[*2] Airlangga University

Kawahara, N., Anjiki, N.[*1, 2], Hosoe, J., Kim, I-H., Ikezaki, H.[*2], Mikage, M.[*1], Goda, Y. : **Studies on relationship between taste and content of sulfur dioxide in crude drugs obtained from the Japanese market**

Pharmaceutical Regulatory Science, **40**(3), 129-135 (2009)

Sulfur dioxides and sulfites are registered in "The Japan's Specifications and Standards for Food Additives", and are mainly used as bleach and anti-oxidants. The Food Sanitation Law prohibits their use with sesame, legumes and vegetables. In China, sulfur fumigation is performed for the purpose of bleaching, drying, insecticide and antibacterial process, in the preparation of some crude drugs. Recently, it has been reported that large quantities of sulfur dioxides may be present in sulfur fumigated crude drugs. In the course of our survey of impurity in herbal materials, we analyzed the content of sulfur dioxides for 31 kinds of crude drugs purchased from the Japanese market. Furthermore, with the aim of development a new simple method for the measurement of sulfur dioxides, we investigated correlation between the color value obtained by spectrophotometry and the sulfur dioxides content in 19 kinds of crude drugs. A good correlation between the color index L^* value and the sulfur dioxide content, and the good inverse correlation between the color index C^* value and the sulfur dioxide content were observed in 4 powdered crude drugs. However, other crude drugs did not show any correlations between color and sulfur dioxide content. Seeking other new methodology for the measurement of sulfur dioxide, we examined the correlation between taste intensity and sulfur dioxide content in 5 kinds of crude drugs with the use of a taste-sensing system. High levels of sulfur dioxide (more than 80 mg/kg) influenced the taste intensity of umami, and astringency and anionic bitterness of 4 crude drugs (Platycodon Root, Fritillaria Bulb, Ginger and Forsythia Fruit). Therefore, measurement of the taste intensity may be suitable as a screening procedure for sulfur dioxide content in these crude drugs.

Keywords: sulfur dioxide, taste-sensing system, crude drugs

[*1] 金沢大学大学院自然科学研究科
[*2] (株) インテリジェントセンサーテクノロジー

Tokumoto, H., Shimomura, H., Katsuki, S.[*], Goda, Y. : **Morphological discrimination of *Curcuma longa* L. and *Curcuma aromatica* Salisb**

Shoyakugaku Zasshi, **62**(2), 54-65 (2008)

The first step for ensuring the quality of natural products is to use the right parts from the right origin. By using a microscope we studied morphological differences between the rhizomes of *Curcuma longa* L. and *C. aromatica* Salisb., both of which are used as health food ingredients. We found a sharp contrast between the shapes of starch grains of *C. longa* and those of *C. aromatica*, namely long triangle to long ovate in *C. longa* and ovate to wide ovate in *C. aromatica*, regardless of the degree of processing. There is a clear difference in the ratio of the major diameter to the minor one between these two species. In addition, we also found that the color of the contents of the secreting cells differed between the species: reddish-brown in *C. longa*, colorless or pale yellow in *C. aromatica*. We then applied the morphological discrimination method to samples of health foods, the origins of which had been determined by DNA analyses. The result was identical with that determined by molecular biology techniques. This study shows that microscopic identification is a useful method for the authentication of the origin of health food products.

Keywords: *Curcuma longa*, *Curcuma aromatica*, anatomy

[*] 医薬基盤研究所薬用植物資源研究センター

Ohtsuki, T., Miyagawa, T.[*1], Koyano, T.[*2], Kowithayakorn, T.[*3], Kawahara, N., Goda, Y., Ishibashi, M.[*1] : **Acylated triterpenoid saponins from *Schima noronhae* and their cell growth inhibitory activity**

J. Nat. Prod., **71**(5), 918–921 (2008)

Two new acylated triterpenoid saponins were isolated from the branches of *Schima noronhae* by bioassay-guided purification. Their chemical structures were established on the basis of spectroscopic analysis and chemical means as 3-*O*-α-l-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloyl-A1-barrigenol (1) and 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucuronopyranosyl 22-*O*-angeloylerythrodiol (2). Compounds 1 and 2 showed cell growth inhibitory activity against both HeLa and DLD1 cells at a concentration of less than 10 μM.

Keywords: *Schima noronhae*, triterpenoid saponin, cell growth inhibitory activity

[*1] 千葉大学大学院薬学研究院
[*2] テムコ
[*3] コンケン大学農学部

Abe, Y., Tera, M.[*1], Sasaki, N. [*1], Okamura, M. [*2], Umemoto, N. [*3], Momose, M. [*3], Kawahara, N., Kamakura, H., Goda, Y., Nagasawa, K. [*1], Ozeki, Y. [*1] : **Detection of 1-*O*-malylglucose: Pelargonidin 3-*O*-glucose-6" -*O*-malyltransferase activity in carnation (*Dianthus caryophyllus*)**

Biochem. Biophys. Res. Commun., **373**, 473–477 (2008)

Carnations have anthocyanins acylated with malate. Although anthocyanin acyltransferases have been reported in several plant species, anthocyanin malyltransferase (AMalT) activity in carnation has not been identified. Here, an acyl donor substance of AMalT, 1-*O*-β-D-malylglucose, was extracted and partially purified from the petals of carnation. This was synthesized chemically to analyze AMalT activity in a crude extract from carnation. Changes in the AMalT activity showed close correlation to the accumulation of pelargonidin 3-malylglucoside (Pel 3-malGlc) during the development of red petals of carnation, but neither AMalT activity nor Pel 3-malGlc accumulation was detectable in roots, stems and leaves.

Keywords: anthocyanin, carnation, malyltransferase

[*1] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology
[*2] Plant Research Center, Kirin Agribio Co. Ltd.
[*3] Central Laboratories for Frontier Technology, Kirin Holdings Co. Ltd.

Abe, Y., Sawada, A.[*], Momose, T.[*], Sasaki, N.[*], Kawahara, N., Kamakura, H., Goda, Y., Ozeki, Y.[*] : **Structure of an anthocyanin–anthocyanin dimer molecule in anthocyanin-producing cells of a carrot suspension culture**

Tetrahedron Lett., **49**, 7330-7333 (2008)

A novel anthocyanin, an anthocyanin–anthocyanin dimer, was isolated from the cells of an anthocyanin-producing carrot cell-line culture, and its structure was elucidated using spectroscopic methods. It consists of two molecules of the anthocyanin, cyanidin 3-[xylosyl-(sinapoyl-glucosyl)-galactoside], with a CH–CH_3 linkage at the 8–8 position. This is the first report of the identification and isolation of an anthocyanin–anthocyanin dimer with a CH–CH_3 linkage from intact plant cells.

Keywords: anthocyanin, dimer, carrot

[*] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology

Abe, Y., Shoji, T.[*1], Kawahara, N., Kamakura, H., Kanda, T.[*1], Goda, Y., Ozeki, Y.[*2] : **Structural characterization of a procyanidin tetramer and pentamer from the apple by low-temperature NMR analysis**

Tetrahedron Lett., **49**, 6413-6418 (2008)

The structures of a procyanidin tetramer and pentamer from unripe apple (*Malus pumila*) were elucidated by low-temperature NMR analysis at −34 ℃. These structures were [epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (1)] and [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (2)].

Keywords: apple, NMR, procyanidin

[*1] Asahi Breweries, Ltd.
[*2] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology

Sasaki, N.[*1], Abe, Y., Goda, Y., Adachi, T.[*2], Kasahara, K.[*3], Ozeki, Y.[*1] : **Detection of DOPA 4,5-Dioxygenase (DOD) Activity Using Recombinant Protein Prepared from *Escherichia coli* Cells Harboring cDNA Encoding *DOD* from *Mirabilis jalapa***

Plant Cell Physiol., **50**, 1012-1016 (2009)

Betalains are synthesized in flowers, fruits and other tissues of the plant order Caryophyllales. Betalamic acid is the chromophore of betalain pigments synthesized by a ring-cleaving enzyme reaction on L-dihydroxyphenylalanine (DOPA). Although reverse genetic evidence has proven that DOPA 4,5-dioxygenase (DOD) is a key enzyme of betalain biosynthesis, all attempts to detect recombinant plant DOD activity in vitro have failed. Here, we report on the formation of betalamic acid from DOPA under suitable assay conditions using recombinant MjDOD produced by *Escherichia coli*. This is the first report showing biochemical evidence for DOD activity in vitro.

Keywords: Betalain, Betalamic acid, DOPA dioxygenase

[*1] Department of Biotechnology and Life Science, Tokyo

University of Agriculture and Technology
[*2] Institute for Plant Biotechnology Research & Development, Ltd.
[*3] Yokohama, Kanagawa

Shintani, A.[*1], Ohtsuki, T., Yamamoto, Y.[*2], Hakamatsuka, T., Kawahara, N., Goda, Y., Ishibashi, M.[*1] : **Fuligoic acid, a new yellow pigment with a chlorinated polyene-pyrone acid structure isolated from the myxomycete *Fuligo septica* f. *flava***

Tetrahedron Lett., **50**, 3189-3190 (2009)

Fuligoic acid, a new yellow pigment with a chlorinated polyene–pyrone acid structure, was isolatedfrom field-collected fruit bodies of the myxomycete *Fuligo septica* f. *flava*, and its structure was elucidatedby spectral data, including its absolute configuration based on CD data.

Keywords: myxomycetes, *Fuligo septica* f. *flava*, polyene–pyrone

[*1] Chiba University
[*2] Yamamoto Laboratory

Maruyama, T., Kamakura, H., Miyai, M., Komatsu, K.[*1], Kawasaki, T.[*2], Fujita, M.[*2], Shimada, H.[*3], Yamamoto, Y.[*3], Shibata, T.[*4], Goda, Y. : **Authentication of the traditional medicinal plant, *Eleutherococcus senticosus* by DNA and chemical analyses**

Planta Medica, **74**, 787-789 (2008)

Shigoka (SGK), the rhizome of *Eleutherococcus senticosus*, is a traditional medicine used as a tonic in northeast Asia and far eastern Russia. We analyzed the nuclear ribosomal DNA, internal transcribed spacer (ITS) sequence of the medicine available on the Japanese and Chinese markets, and found that at least 3 species were used as the source plant of the commercial SGKs and that only 70% of all samples was made from the correct species. Furthermore, we performed the quantitative determination of 3 marker compounds, eleutheroside B (EB), syringaresinol diglucoside (Syr) and isofraxidin (Iso) by ultraperformance liquid chromatography (UPLC)/mass spectrometry (MS). We found that EB and Iso are specific to the correct source plant of SGK. Of them, EB is thought to be the best marker compound for quality assurance of the SGK from the viewpoint of its pharmacological activity.

Keywords: *Eleutherococcus senticosus*, ITS sequence, UPLC/MS

[*1] 富山大学和漢医薬学総合研究所
[*2]（株）ウチダ和漢薬
[*3]（株）栃本天海堂
[*4] 医薬基盤研究所薬用植物資源研究センター

Wakana, D., Hosoe, T.[*1], Itabashi, T.[*1], Okada, H[*1], Fukushima, K.[*2], Kawai, K.[*1] : **Structures of new triterpene glycosides, Malbrancheosides A-D, from *Malbranchea filamentosa***

Heterocycles, **75**(5), 1109-1122 (2008)

In the course of searching for new biologically active metabolites in *Malbranchea filamentosa*, four new triterpene glycosides, malbrancheosides A (1) - D (4), were isolated. The structures of 1- 4 were confirmed by the chemical and spectroscopic investigation. Malbrancheosides A (1) - D (4) are the first example of triterpenoidal glycosides having $_{D}$-glucosamine derivatives from the fungal sources.

Keywords: *Malbranchea filamentosa*, triterpene glycoside, Malbrancheoside

[*1] 星薬科大学
[*2] 千葉大学真菌医学研究センター

Moriyama, H.[*1, 3], Hosoe, T.[*1], Wakana, D., Itabashi, T.[*1], Kawai, K.[*1], Iizuka, T.[*2], Hoshi, K.[*3], Fukushima, K.[*4], Francis Chun Lau[*5] : **Assay-guided informatory screening method for antiplatelet effect of adenosine isolated from *Malbranchea filamentosa* IFM 41300: inhibitory behaviors of adenosine in different solvents**

Journal of Health Science, **55**(1), 103-108 (2009)

A particle-counting aggregometer employing laser-light scattering was used in systematic manners to screen and to detect inhibitor(s) of platelet aggregation from the extract of *Malbranchea filamentosa* (*M. filamentosa*) IFM41300. The inhibitor was determined as adenosine on the basis of the ^{13}C- and ^{1}H-NMR spectral data. Although adenosine was previously reported as an inhibitor of platelet aggregation, we isolated the compound from the fungus for the first time. Because the method was able to provide us with information on the developmental formation of platelet aggregates in different sizes with incubation time, we further elaborated the inhibitory behaviors of adenosine, as an example, at varied concentrations in different solvents such as dimethyl sulfoxide (DMSO) and saline. We found that DMSO could facilitate to dissolve less water soluble materials from herbs and fungi by using the present assay

method.

Keywords: *Malbranchea filamentosa*, adenosine, platelet aggregation

*1 星薬科大学
*2 横浜薬科大学
*3 昭和薬科大学
*4 千葉大学真菌医学研究センター
*5 InterHealth Research Center

Itabashi, T.*1, Hosoe, T.*1, Wakana, D., Fukushima, K.*2, Takizawa, K.*2, Yaguchi, T.*2, Okada, K.*3, Takaki, Galba Maria de campos*3, Kawai, K.*1 : **A new indoloditerpene derivatives, penijanthine A, isolated from *Penicillium janthinellum***

J. Nat. Med., **63**, 96-99 (2009)

In a screen searching for new bioactive agents, a new indoloditerpene, penijanthine A (1), was isolated from *Penicillium janthinellum* IFM 55557. The structure of 1 was established on the basis of spectroscopic and chemical investigation, as well as detailed comparison with the spectroscopic and physico-chemical data of paxilline (2), which was isolated along with 1.

Keywords: Indoloditerpene, Penijanthine A, *Penicillium janthinellum*

*1 星薬科大学
*2 千葉大学真菌医学研究センター
*3 Universidade Católica de Pernambuco

Wakana, D., Hosoe, T.*1, Wachi, H.*1, Itabashi, T.*1, Fukushima, K.*2, Yaguchi, T.*2, Kawai, K.*1 : **The cytotoxic and antifungal activities of two new sesqueterpenes, malfilanol A and B, derived from *Malbranchea filamentosa***

J. Antibiotics, **62**(5), 217-219 (2009)

Fungi of the *Malbranchea* belong to the family *Onygenaceae* and are taxonomically close to human and animal pathogenic fungi. These facts prompted us to investigate the chemical constitutes of *Malbranchea* fungi. We recently reported the isolation and structure characterization of 4-benzyl-3-phenyl-5*H*-furan-2-one as a vasodilator, malfilamentosides A and B as furanone glycosides and malbrancheosides A-D as triterpene glycosides, from the fungus *Malbranchea filamentosa* IFM41300. Further purification of extracts of rice cultivated by the above fungus allowed us to isolate two new sesqueterpenes, designated malfilanol A (1) and B (2). Characterization of their structures, cytotoxic activities and antifungal activities are described in this paper.

Keywords: *Malbranchea filamentosa*, sesqueterpene, antifungal activity

*1 星薬科大学
*2 千葉大学真菌医学研究センター

Makita, N.*, Suzuki, M.*, Asami, S.*, Takahata, R.*, Kohzaki, D.*, Kobayashi, S.*, Hakamatsuka, T., Hozumi, N.* : **Two of four alternatively spliced isoforms of RUNX2 control osteocalcin gene expression in human osteoblast cells**

Gene, **413**, 8-17 (2008)

Runx2は骨芽細胞の分化と骨形成を調節する転写因子であるが，細胞の種類によってスプライシング位置の異なる3つのisoformが発現しており，そのうち2つは核内に局在化せず，遺伝子結合活性を持たないことを明らかにした．また，Runx2はいくつかのisoformの組み合わせにより標的遺伝子の発現を増強のみならず抑制もできることを明らかにした．

Keywords: osteoblast, Runx2, transcription factor

* Research Institute for Biological Sciences, Tokyo University of Science

Iwashita, K., Nagashima, H.* : **Rubratoxin B causes lipid accumulation in fatty mouse liver**

Mycotoxins, **58**, 83-87 (2008)

The potent hepatotoxic mycotoxin rubratoxin B causes fatty liver. To elucidate the lipid accumulation mechanism, we investigated the type of lipid droplets accumulated and the activities of the lipogenic enzymes glucose-6-phosphate dehydrogenase (G6PD) and fatty acid synthase in mouse livers treated for 24 h with rubratoxin B. Oil Red O staining revealed numerous microvesicular lipid droplets in the liver cells of rubratoxin B-treated mice. In addition, treatment with rubratoxin B notably induced the activity of G6PD, a crucial member of the pentose phosphate cycle, which produces and supplies NADPH for fatty acid synthesis. Unexpectedly, rubratoxin B decreased the activity of fatty acid synthase, which facilitates fatty acid chain elongation. However, because fatty acid synthase is not a key enzyme in fatty acid synthesis, its activity level in rubratoxin B-treated mice may be sufficient for lipid accumulation.

Keywords: rubratoxin B, fatty liver, lipid accumulation

* National Food Research Institute

Iwashita, K., Nagashima, H.* : **Rubratoxin B induces interleukin-6 secretion in mouse white adipose tissues and 3T3-L1 adipocytes**

Toxicology Letters, **182**, 79–83 (2008)

Rubratoxin B is a mycotoxin that causes hepatic fatty changes. We examined whether white adipose tissue (WAT) contributes to rubratoxin B toxicity through effects on interleukin (IL)-6. Rubratoxin B was intraperitoneally injected into mice at 1.5 mg/kg. Urinary albumin and macrophage inflammatory protein (MIP)-2 secretion were increased 24 h after treatment with rubratoxin B. Rubratoxin B was previously reported to induce IL-6 secretion, although the secreting tissue was unknown. Here, rubratoxin B prominently augmented *IL-6* transcription in epididymal WAT and to a lesser extent in perirenal WAT and liver. Rubratoxin B may thus exert its toxicity partly through IL-6 secretion from WATs. In contrast, *MIP-2* gene expression increased only in liver. To examine the specific involvement of adipocytes, we used mouse 3T3-L1 cells, an *in vitro* differentiation model of adipocytes. Expression of *IL-6* and *MIP-2* mRNA in 3T3-L1 adipocytes after 24 h of rubratoxin B treatment increased dose-dependently. Rubratoxin B also increased IL-6 and MIP-2 secretion from 3T3-L1 adipocytes. The increase in IL-6 secretion was markedly higher than the increase in *IL-6* gene transcription, indicating that rubratoxin B-induced secretion of IL-6 from 3T3-L1 adipocytes is chiefly controlled post-transcriptionally. Rubratoxin B is thus the first mycotoxin known to exert its toxicity through effects on WATs.

Keywords: Rubratoxin B, Adipose tissue, 3T3-L1 cell

* National Food Research Institute

Seshime, Y., Juvvadi, PR.*1, Tokuoka, M.*2, Koyama, Y,*2, Kitamoto, K.*1, Ebizuka, Y.*1, Fujii, I.*3 : **Functional expression of the *Aspergillus flavus* PKS-NRPS hybrid CpaA involved in the biosynthesis of cyclopiazonic acid**

Bioorg. Med. Chem. Lett., **19**(12), 3288-3292 (2009)

α-Cyclopiazonic acid (CPA) is an indole tetramic acid mycotoxin. Based on our identification of the polyketide synthase-nonribosomal peptide synthase (PKS-NRPS) hybrid gene *cpaA* involved in cyclopiazonic acid biosynthesis in *Aspergillus* fungi, we carried out heterologous expression of *Aspergillus flavus cpaA* under α-amylase promoter in *Aspergillus oryzae* and identified its sole product to be the CPA biosynthetic intermediate *cyclo*-acetoacetyl-L-tryptophan (cAATrp). This result rationalized that the PKS-NRPS hybrid enzyme CpaA catalyzes condensation of the diketide acetoacetyl-ACP formed by the PKS module and L-Trp activated by the NRPS module. This CpaA expression system provides us an ideal platform for PKS-NRPS functional analysis, such as adenylation domain selectivity and product releasing mechanism.

Keywords: cyclopiazonic acid, *Aspergillus flavus*, biosynthesis

*1 東京大学大学院
*2 (財) 野田産業科学研究所
*3 岩手医科大学

Mitsuguchi, H.*1, Seshime, Y., Fujii, I.*2, Shibuya, M.*1, Ebizuka, Y.*1, Kushiro, T.*1 : **Biosynthesis of Steroidal Antibiotic Fusidanes: Functional Analysis of Oxidosqualene Cyclase and Subsequent Tailoring Enzymes from *Aspergillus fumigatus***

Journal of the American Chemical Society, **131**(18), 6402-6411 (2009)

Three putative oxidosqualene cyclase (OSC) genes exist in the genome of the fungus *Aspergillus fumigatus* that produces a steroidal antibiotic, helvolic acid. One of these genes, *Afu4g14770*, designated *AfuOSC3*, is clustered with genes of cytochrome P450 monooxygenases (P450s), a short-chain dehydrogenase/reductase (SDR), and acyltransferases, which presumably function in triterpene tailoring steps, suggesting that this gene cluster codes for helvolic acid biosynthesis. *AfuOSC3* was PCR amplified from A. *fumigatus* IFO8866 genomic DNA and expressed in yeast. The yeast transformant accumulated protosta-17(20)*Z*,24-dien-3*β*-ol, an established precursor for helvolic acid. Its structural isomer, (20*R*)-protosta-13(17),24-dien-3*β*-ol, was also isolated from the transformed yeast. To further identify the function of triterpene tailoring enzymes, four P450 genes (*CYP5081A1-D1*) and a SDR gene (*AfuSDR1*) in the cluster were each coexpressed with *AfuOSC3* in yeast. As a result, coexpression of AfuSDR1 gave a 3-keto derivative of protostadienol. On the other hand, coexpression with CYP5081A1 gave protosta-17(20)*Z*,24-diene-3*β*,29-diol and protosta-17(20)*Z*,24-dien-3*β*-ol-29-oic acid. These metabolites are in well accord

with the oxidative modification involved in helvolic acid biosynthesis. AfuSDR1 and CYP5081A1 presumably function together to catalyze demethylation of C-29 methyl group. These results provided a firm ground for identification of the present gene cluster to be involved in helvolic acid biosynthesis.

Keywords: helvolic acid, *Aspergillus fumigatus*, biosynthesis

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[*1] 東京大学大学院
[*2] 岩手医科大学

Tokuoka, M.[*1], Seshime, Y., Fujii, I.[*2], Kitamoto, K.[*3], Takahashi, T.[*1], Koyama, Y.[*1] : **Identification of a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae***

Fungal Genetics and Biology, **45**(12), 1608-1615 (2008)

Cyclopiazonic acid (CPA) is a mycotoxin produced by several strains of *Penicillium* and *Aspergillus* species. *Aspergillus oryzae* strains used in fermented foods do not produce CPA; however, several wild-type *A. oryzae* strains produce CPA. Here, we identified a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene involved in CPA production by comparing the telomere-adjacent region of a CPA-producing strain (*A. oryzae* NBRC 4177) with that of a nonproducing strain (*A. oryzae* RIB40). NBRC 4177 has an additional 17-18-kb sequence beyond the region corresponding to the telomere repeat in RIB40 and this additional regions contains 3'region of the PKS-NRPS gene, while RIB40 has only the 5'region of the PKS-NRPS gene. Gene disruption of the PKS-NRPS gene in NBRC 4177 resulted in elimination of CPA production. Thus, the PKS-NRPS gene is required for CPA biosynthesis, and the truncation of this gene is presumed as one of the determinants of CPA nonproductivity in *A. oryzae* RIB40.

Keywords: cyclopiazonic acid, *Aspergillus oryzae*, polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS)

[*1] (財) 野田産業科学研究所
[*2] 岩手医科大学
[*3] 東京大学大学院

Chung, MH., Suzuki, S.[*1], Nishihara, T.[*2], and Hattori, M.[*1] : **Estrogenic effects of a Kampo formula, *Tokishakuyakusan*, in parous ovariectomized rats**

Biol. Pharm. Bull., **31**(6), 1145-1149 (2008)

Female hormone-dependent cancers and other diseases pose a serious health threat for women, and low-risk medicines against such cancers have not yet been discovered. The present study examines the effects of the traditional Chinese herbal mixture, *Tokishakuyakusan* (TS) and 17β-estradiol on the uterus of parous ovariectomized rats. Uterine atrophy that causes a reduction in uterine tissue and the uterine cavity area, was induced by ovariectomy, and slightly recovered by the daily oral administration of TS for two weeks (1,000 mg/kg body weight). TS restored the decreased plasma estradiol concentration due to ovariectomy. However the yeast two-hybrid assay showed that TS did not bind estrogen receptors α and β and immunohistochemical staining revealed that 17β-estradiol stimulated the protein expression of estrogen receptor α, progesterone receptor, c-fos and c-jun in the uterus, whereas TS did not. These results suggest that TS might be useful for treating menopausal syndromes among women, as well as for patients when hormone replacement therapy (HRT) with estrogen is contraindicated.

Keywords: *Tokishakuyakusan*, Kampo formula, parous ovariectomized rat

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[*1] Division of Metabolic Engineering, Institute of Natural Medicine, University of Toyama
[*2] Faculty of Pharmaceutical Sciences, Hyogo University of Health Sciences

Min, J. Z.[*], Shimizu, Y.[*], Toyo'oka, T.[*], Inagaki, S.[*], Kikura-Hanajiri, R., Goda, Y. : **Simultaneous determination of 11 designated hallucinogenic phenethylamines by ultra-fast liquid chromatography with fluorescence detection**

J. Chromatogr. B, **873**(2), 187-194 (2008)

To avoid the spreading of illegal drugs, a designated drug regulation system was introduced along with revision of the Pharmaceutical Affairs Law in Japan in 2006, and 32 substances including phenethylamine-type drugs were listed in April 2007. In this study, a new simultaneous determination method, based on ultra-fast liquid chromatography coupled with fluorescence detection (UFLC-FL), was developed for the 11 designated phenethylamine drugs. The phenethylamines were labeled with 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) at 60 degrees C for 2h in 0.1M borax (pH 9.3).

The resulting 11 fluorophores were completely separated by reversed-phase chromatography using an ACQUITY UPLC BEH C(18) column (2.1 mm x 100 mm 1.7 microm) and fluorometrically detected at 550 nm (excitation at 450 nm). The calibration curves obtained from the peak areas versus the injection amounts of the phenethylamines showed a good linearity. The limits of detection (signal-to-noise ratio of 3: S/N=3) on the chromatogram were in the range from 10 fmol (PMMA) to 2.5pmol (MMDA-2). Good accuracy (%) and precision (CV) by intra-day assay and inter-day assay were also obtained using the present procedure. The method was applied to the qualitative and quantitative analyses of phenethylamine in real products obtained from the Japanese market. As the results, BDB (0.24 mg/mg), MMDA-2 (0.98 mg/mL) and 2C-I (0.016 mg/mg) were identified from the different products (powder, liquid and mushroom like). Because the procedure is simple, selective and sensitive, the present method seems to be useful for the qualitative and quantitative analyses of the designated phenethylamines in various samples including biological specimens.

Keywords: Designated hallucinogenic phenethylamines, Fluorescence labeling, 4-(*N*,*N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

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* University of Shizuoka

Kawamura, M., Kikura-Hanajiri, R., Goda, Y. : **Survey of current trends in the abuse of psychotropic plants using LC-MS**

Jpn. J. Food Chemistry, **15**, 73-78 (2008)

In recent years, various products of non-controlled psychotropic plants have become popular in place of chemical psychotropic substances, which are now subject to stricter controls, and they are causing concern in Japan. To survey current trends in the abuse of psychotropic plants, 127 kinds of plant products (which were distributed from April 2004 to May 2007 in Japan) were analyzed using LC-ESI-MS. As a results of the analyses, 6 typical psychotropic plant components, *N*,*N*-dimethyltryptamine (DMT) (1.2% contents in the sample), mescaline (0.1-1.7%), salvinorin A (0.1-4.5%), lysergamide (LSA) (0.005-0.4%), harmine (0.04-3.1%), and harmaline (0.008-4.5%) were determined in the 51 products. Although one-third of the products were mixtures of several plant materials or they did not list their ingredients, the raw materials of some products could be estimated by the determination of the typical plant's components. On the other hand, the compounds in some products were not consistent with the ingredients listed on the products. The products which contained raw materials exclusively used as pharmaceuticals were also found to be distributed. Moreover, the contents of the active compounds in some products were sufficient to produce hallucinogenic effects in humans and there is concern about the damage to the health of their users. The proposed analytical method could be useful in the investigation of these plant products in the market.

Keywords: psychotropic plant, LC-MS, hallucinogenic constituent

Kikura-Hanajiri, R., Kawamura, M., Uchiyama, N., Ogata, J., Kamakura, H., Saisho, K., Goda, Y. : **Analytical data of designated substances (Shitei-Yakubutsu) controlled by the Pharmaceutical Affairs Law in Japan, part I: GC-MS and LC-MS**

Yakugaku Zasshi, **128**(6), 971-979 (2008)

In the last 10 years, many analogs of narcotic substances have been widely distributed in Japan as easily available psychotropic substances and have become a serious problem. They have been sold as video cleaners, incense and reagents with various forms via the Internet or in video shops. They are not controlled under the Narcotics and Psychotropics Control Law because their pharmacological effects have not yet been proved, scientifically. For countermeasures against these substances, the Ministry of Health, Labor and Welfare amended the Pharmaceutical Affairs Law in 2006 and the non-controlled psychotropic substances (31 compounds (11 tryptamines, 11 phenethylamines, 6 alkyl nitrites, 2 piperazines and salvinorin A) and 1 plant (*Salvia divinorum*)) have been controlled by the amended Law as "Designated Substances (Shitei-Yakubutsu)" since April in 2007. Moreover, 5 compounds (4 phenethylamines and 1 piperazine) was added to this category from January 2008. In this study, we developed simultaneous analytical methods for these designated substances using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) and showed their data of the retention times and the spectra of ultraviolet (UV), electron ionization (EI) GC-MS and electrospray ionization (ESI) LC-MS.

Keywords: psychotropic substances, Designated Substances, Shitei-Yakubutsu

Uchiyama, N., Kikura-Hanajiri, R., Kawahara, N.,

Haishima, Y., Goda, Y. : **Identification of a cannabinoid analog as a new type of designer drug in a herbal product**

Chem. Pharm. Bull., **57**(4), 439-441 (2009)

A new type of designer drug, a cannabinoid analog (1), was found in a herbal product distributed on the illegal drug market in Japan in expectation of its narcotic effect. The structure of 1 was identified by LC-MS, GC-MS, high-resolution MS, and NMR analyses. Compound 1 showed a molecular weight of 332, and accurate mass measurement exhibited its elemental composition to be $C_{22}H_{36}O_2$. Together, the mass and NMR spectrometric data revealed that 1 was (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol, which was first synthesized in 1979 by a group at Pfizer Inc. and reported as a potent cannabinoid analog possessing cannabinoid receptor binding activity and analgesic activity in the 1990s. This is the first report to identify a cannabinoid analog in an illegal drug.

Keywords: cannabinoid analog, designer drug, (1*RS*,3*SR*)-3-[4-(1,1-dimethyloctyl)-2-hydroxyphenyl]cyclohexan-1-ol

Uchiyama, N., Kikura-Hanajiri, R., Kawahara, N., Goda, Y. : **Analysis of designer drugs detected in the products purchased in fiscal year 2006**

Yakugaku Zasshi, **128**(10), 1499-1505 (2008)

Many psychotropic substances are easily available in Japan via the Internet, thus the spread of drug abuse is becoming more serious problem. To avoid drug abuse, 32 substances have been controlled in Japan since April in 2007 by the Pharmaceutical Affairs Law as designated substances (Shitei-Yakubutsu, classified as 11 tryptamines, 11 phenethylamines, 2 piperazines, 6 alkyl nitrites, 1 diterpene and 1 plant). Although the distributions of these drugs have been decreased through this regulation, new designer drugs are still being found. In this study, we detected 7 designer drugs in 15 products, which purchased just before the amendment of the law, by NMR, GC-MS and LC-MS analyses. Three methylone derivertives (1-(3,4-methylenedioxyphenyl-2-(pyrrolidin-1-yl)-1-pentanone: MDPV, 2-methylamino-1-(3,4-methylenedioxyphenyl)butan-1-one: bk-MBDB, 2-ethylamino-1-(3,4-methylenedioxyphenyl)propan-1-one): bk-MDEA, a MDMA derivative (*N*-hydroxy-1-(3,4-methylenedioxyphenyl)-2-aminopropane: *N*-OH-MDMA), a methamphetamine derivative (*N*-methyl-1-(4-fluorophenyl) propan-2-amine: *N*-Me-4-FMP), a tryptamine derivative (5-methoxy-*N*-ethyl-*N*-isopropyltryptamine: 5-MeO-EIPT) and indan-2-amine were detected. 5-MeO-EIPT was newly identified in this study.

Keywords: psychotropic substances, NMR, designer drug

Uchiyama, N., Saisho, K., Kikura-Hanajiri, R., Haishima, Y., Goda, Y. : **Determination of a new type of phosphodiesterase-5 inhibitor, Thioquinapiperifil, in a dietary supplement promoted for sexual enhancement**

Chem. Pharm. Bull., **56**(9), 1331-1334 (2008)

A new type of phosphodiesterase-5 (PDE-5) inhibitor, thioquinapiperifil (1), was found in dietary supplements. LC-MS analysis indicated that the supplements contain two major compounds. One was identified as thiodenafil (synonym: thiosildenafil) by direct comparison with the authentic compound. The other showed a molecular weight of 448, and accurate mass measurement showed its elemental composition to be $C_{24}H_{28}N_6O_1S_1$. Together, the mass and NMR spectrometric data revealed that the compound is an imidazoquinazoline derivative: 3-ethyl-1,3-dihydro-8-[[[2-[4-(hydroxymethyl)-1-piperidinyl]phenyl] methyl]amino]-2*H*-imidazo[4,5-g]quinazoline-2-thione. This compound had been synthesized as a PDE-5 inhibitor, formerly reported as KF31327 by Kyowa Hakko Kogyo Co., Ltd. Considering this compound's general properties, it has been renamed thioquinapiperifil with the agreement of Kyowa Hakko Kogyo Co., Ltd. The detection of imidazoquinazoline-type compounds in dietary supplements has not been reported. Quantitative analysis showed that the contents of 1 and thiodenafil in the products were about 13 ~ 15 mg/tablet (43 ~ 48 μg/mg) and about 0.4 mg/tablet (1 μg/mg), respectively.

Keywords: thioquinapiperifil, phosphodiesterase-5 inhibitor, erectile dysfunction

Uchiyama, N., Kawamura, M., Kamakura, H., Kikura-Hanajiri, R., Goda, Y. : **Analytical Data of Designated Substances (Shitei-Yakubutsu) Controlled by the Pharmaceutical Affairs Law in Japan Part II: Color test and TLC**

Yakugaku Zasshi, **128**(6), 981-987 (2008)

Many psychotropic substances are easily available in Japan via the Internet. To avoid the spread of drug abuse, some drugs have been controlled in Japan since 2007 by the Pharmaceutical Affairs Law as designated substances (Shitei-Yakubutsu). 29 designated substances (classified as tryptamine, phenethylamine and piperazine types) were analyzed using color tests and TLC. The

color tests were examined with the Marquis reagent, the Ehrlich reagent, the Simon's reagent, the Liebermann-Burehard's reagent and the Mandelin reagent. The color of β-carbonyl-methylenedioxyphenetylamines produced by the Marquis reagent was yellow, and 4-halo-2,5-dimethoxy phenethylamines reacted with the Marquis reagent to give deep yellow green and/or a deep green color. Although all designated substances of the tryptamine type reacted with the Ehrlich reagent to give a brown colors, only 1-(2,4,6-trimethoxyphenyl)propan-2-amine (TMA-6) in phenethylamines showed a red color on treatment with the reagent. However, 3,4,5-trimethoxy isomer and 2,4,5-trimethoxy isomer of TMA-6 were not colored with the reagent. Thus, TMA-6 could be distinguished from isomers by using the Ehrlich reagent. We also analyzed the designated substances with TLC developed with two different solvent conditions. All substances were detected by $UV_{254\ nm}$ and an iodoplatinate reagent. These results suggested that color tests and TLC are available for preliminary identification of designated substances followed by GC-MS and LC-MS analyses.

Keywords: color test, TLC, Designated Substances

Ogata, J., Kikura-Hanajiri, R., Yoshimatsu, K.[*], Kiuchi, F.[*], Goda, Y. : **Detection method for the ability of hemp (*Cannabis sativa* L.) seed germination by the use of 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC)**

Yakugaku Zasshi, **128**(11), 1707-1711 (2008)

Cannabis plants show a high Δ^9-tetrahydrocannabinol content and are used as a psychoactive drug. Therefore the cultivation of hemp and its possession are prohibited by law in Japan. Meanwhile, Cannabis seeds have been used as a component of *Shichimi-togarashi* (a Japanese spice), bird feed, or a crude drug (*mashinin*). To exclude the possibility of the germination, it is officially noticed that hemp seeds must be killed. However, the number of violators has increased in recent years. To judge the ability of seed germination, a germination test is performed. However, the test requires several days and thus has not been used for on-site inspection. In this study, we developed a rapid detection method to determine the ability of *Cannabis* seed to germinate using 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC). The principle of the assay is as follows. The endogenous respiratory enzymes in hemp seeds convert added colorless TTC into red 1,3,5-triphenylformazan. Consequently, a living embryo is stained red, while red does not appear in the dead seeds. The reaction was active over a pH range of 8.0-9.0, and the optimum activity was found from 40 to 50℃. Under the optimum conditions, we were able to determine the ability of seeds to germinate based on the presence of color within 20 min. Since this method is rapid and simple, it is applicable to on-site inspections. In addition, it could be used as an alternative technique to the germination test, because erroneous decisions is cannot occur under the assay principle.

Keywords: *Cannabis sativa* L., tetrazolium salt, germination test

[*] 医薬基盤研究所薬用植物資源研究センター

Kikuchi, H., Ohtsuki, T., Koyano, T.[*1], Kowithayakorn, T.[*2], Sakai, T.[*3], Ishibashi, M.[*4] : **Death receptor 5 targeting activity-guided isolation of isoflavones from *Millettia brandisiana* and *Ardisia colorata* and evaluation of ability to induce TRAIL-mediated apoptosis**

Bioorg. Med. Chem., **17**, 1181-1186 (2009)

Death receptor 5 (DR5) is an apoptosis-inducing membrane receptor for TNF-related apoptosis-inducing ligand (TRAIL). On screening for compounds that enhance DR5 expression using a luciferase assay with DLD-1/*SacI*, we previously identified 4'-demethyltoxicarol isoflavone (1) isolated from the leaves of *Millettia brandisiana*. In this study, we revealed that 1 sensitized TRAIL-resistant human gastric adenocarcinoma (AGS) cells to TRAIL-induced apoptosis by up-regulating the expression of DR5. 1 induced DR5 expression at both the mRNA and protein level. A human recombinant DR5/Fc chimera remarkably inhibited 1-induced apoptosis. These results suggest that the enhancement of DR5 expression by 1 was critical to the cell death. Furthermore, a MeOH extract of the bark of *Ardisia colorata* markedly enhanced DR5 activity in this screening system. Bioassay-guided fractionation of *A. colorata* led to the isolation and identification of a new isoflavone, coloratanin A (3), together with ten known compounds. The chemical structure of the new compound was elucidated on the basis of a spectroscopic analysis.

Keywords: TRAIL, DR5, isoflavone

[*1] テムコ
[*2] コンケン大学農学部
[*3] 京都府立医科大学
[*4] 千葉大学大学院薬学研究院

Ohtsuki, T., Hiraka, T.[*1], Kikuchi, H., Koyano, T. [*2], Kowithayakorn, T. [*3], Sakai, T. [*4], Ishibashi, M. [*1] : **Flavonoids from *Eupatorium odoratum* with death receptor 5 promoter enhancing activity**

Heterocycles, **77**, 1379-1388 (2009)

Sixteen flavonoids including two new ones (1 and 2) were isolated from the leaves of *Eupatorium odoratum* (Compositae) through bioassay-guided isolation. The chemical structures of 1 and 2 were established on the basis of spectroscopic analysis. Compounds 2, 7, 9, and 14 led to more than 2-fold increase in death receptor 5 (DR5) promoter activity at 17.5 or 35 μM.

Keywords: TRAIL, DR5, flavonoid

[*1] 千葉大学大学院薬学研究院

[*2] テムコ

[*3] コンケン大学農学部

[*4] 京都府立医科大学

Tanabe, S., Sato, Y., Suzuki, T., Suzuki, K., Nagao, T. and Yamaguchi, T. : **Gene Expression Profiling of Human Mesenchymal Stem Cells for Identification of Novel Markers in Early- and Late-Stage Cell Culture**

J. Biochem., **144**, 399-408 (2008)

Human mesenchymal stem cells (hMSCs) are multipotent cells that differentiate into several cell types, and are expected to be a useful tool for cellular therapy. Although the hMSCs differentiate into osteogenic cells during early to middle stages, this differentiation capacity decreases during the late stages of cell culture. To test a hypothesis that there are biomarkers indicating the differentiation potential of hMSCs, we performed microarray analyses and profiled the gene expression in six batches of hMSCs (passages 4-28). At least four genes [necdin homolog (mouse) (*NDN*), EPH receptor A5 (*EPHA5*), nephroblastoma overexpressed gene (*NOV*) and runt-related transcription factor 2 (*RUNX2*)] were identified correlating with the passage numbers in all six batches. The results showed that the osteogenic differentiation capacity of hMSCs is down-regulated in the late stages of cell culture. It seemed that adipogenic differentiation capacity was also down-regulated in late stage of the culture. The cells in late stage are oligopotent and the genes identified in this study have the potential to act as quality-control markers of the osteogenic differentiation capacity of hMSCs.

Keywords: cellular therapy, gene expression, stem cell

Fujishita, K.[*1], Ozawa, T.[*1], Shibata, K.[*1], Tanabe, S., Sato, Y., Hisamoto, M.[*2], Okuda, T.[*2], Koizumi, S.[*1] : **Grape Seed Extract Acting on Astrocytes Reveals Neuronal Protection Against Oxidative Stress via Interleukin-6-mediated Mechanisms**

Cell. Mol. Neurobiol., in press

Grape polyphenols are known to protect neurons against oxidative stress. We used grape seed extract (GSE) from "Koshu" grapes (*Vitis vinifera*) containing a variety of polyphenols, and performed transcriptome analysis to determine the effects of GSE on primary cultures of astrocytes in the hippocampus. GSE upregulated various mRNAs for cytokines, among which interleukin-6 (IL-6) showed the biggest increase after treatment with GSE. The GSE-evoked increase in IL-6 mRNAs was confirmed by quantitative RT-PCR. We also detected IL-6 proteins by ELISA in the supernatant of GSE-treated astrocytes. We made an oxidative stress-induced neuronal cell death model in vitro using a neuron rich culture of the hippocampus. Treatment of the neurons with H_2O_2 caused neuronal cell death in a time- and concentration-dependent manner. Exogenously applied IL-6 protected against the H_2O_2-induced neuronal cell death, which was mimicked by endogenous IL-6 produced by GSE-treated astrocytes. Taken together, GSE acting on astrocytes increased IL-6 production, which functions as a neuroprotective paracrine, could protect neuronal cells from death by oxidative stress.

Keywords: grape seed extract, astrocytes, IL-6

[*1] 山梨大学大学院医学工学総合研究部

[*2] 山梨大学ワイン科学研究センター

Kiyonaka, S.[*1], Kato, K.[*1], Nishida, M.[*2], Mio, K.[*3], Numaga, T.[*1], Sawaguchi, Y.[*1], Yoshida, T.[*1], Wakamori, M.[*1], Mori, E.[*1], Numata, T.[*1], Ishii, M.[*4], Takemoto, H.[*1], Ojida, A.[*1], Watanabe, K.[*2], Uemura, A.[*2], Kurose, H.[*2], Morii, T.[*5], Kobayashi, T.[*6], Sato, Y., Sato, C.[*3], Hamachi, I.[*1] and Mori, Y.[*1] : **Selective and direct inhibition of TRPC3 channels underlies biological activities of a pyrazole compound**

Proc. Natl. Acad. Sci. USA., **106**, 5400-5405 (2009)

Canonical transient receptor potential (TRPC) channels control influxes of Ca^{2+} and other cations that induce diverse cellular processes upon stimulation of plasma membrane receptors coupled to phospholipase C (PLC). Invention of subtype-specific inhibitors for TRPCs is crucial for distinction of respective TRPC channels that

play particular physiological roles in native systems. Here, we identify a pyrazole compound (Pyr3), which selectively inhibits TRPC3 channels. Structure-function relationship studies of pyrazole compounds showed that the trichloroacrylic amide group is important for the TRPC3 selectivity of Pyr3. Electrophysiological and photoaffinity labeling experiments reveal a direct action of Pyr3 on the TRPC3 protein. In DT40 B lymphocytes, Pyr3 potently eliminated the Ca^{2+} influx-dependent PLC translocation to the plasma membrane and late oscillatory phase of B cell receptor-induced Ca^{2+} response. Moreover, Pyr3 attenuated activation of nuclear factor of activated T cells, a Ca^{2+}-dependent transcription factor, and hypertrophic growth in rat neonatal cardiomyocytes, and in vivo pressure overload-induced cardiac hypertrophy in mice. Thus, the TRPC3-selective inhibitor Pyr3 is a powerful tool to study in vivo function of TRPC3, suggesting a pharmaceutical potential of Pyr3 in treatments of TRPC3-related diseases such as cardiac hypertrophy.

Keywords: B lymphocytes, cardiac remodeling, TRPC3 channel

[*1] 京都大学工学部
[*2] 九州大学薬学部
[*3] 産業技術総合研究所脳神経情報研究部門
[*4] 昭和大学薬学部
[*5] 京都大学エネルギー理工学研究所
[*6] 田辺三菱製薬

Watanabe, T.[*1], Tanaka, G.[*2], Hamada, S.[*3], Namiki, C.[*4], Suzuki, T., Nakajima, M.[*5], Furihata, C.[*1] : **Dose-dependent alterations in gene expression in mouse liver induced by diethylnitrosamine and ethylnitrosourea and determined by quantitative real-time PCR**

Mutat. Res., **673**, 9-20 (2009)

We examined the dose-dependency of gene expression changes for 51 genes in mouse liver treated with two *N*-nitroso genotoxic hepatocarcinogens, diethylnitrosamine (DEN) and ethylnitrosourea (ENU) by quantitative real-time PCR (qPCR). The most characteristic result was a similar dose-dependency of gene expression changes with DEN and ENU. Twenty-one genes exhibited a distinct dose-dependent increase in expression at 4h for both carcinogens [*Bax, Btg2, Ccng1, Cdkn1a, Cyp4a10, Cyp21a1, Fos, Gadd45b, Gdf15, Hmox1, Hspb1, Isg20l1, Jun, Mbd1, Mdm2, Myc, Net1, Plk2, Ppp1r3c, Rcan1* and *Tubb2c*], although the increase in gene expression due to ENU was generally weaker than that due to DEN. Only *Gdf15* showed a dose-dependent increase in expression at 28 days for both carcinogens. The differences between DEN and ENU were in the expression of additional genes (7 for DEN and 8 for ENU). The present results show a distinct dose-dependency of gene expression changes induced by DEN and ENU. These changes were associated with cancer, cell cycle arrest, DNA replication, recombination, repair and cell death and were seen not only at 4h but also, for some, at 28 days after administration.

Keywords: gene expression, RT-PCR, genotoxicity

[*1] 青山学院大学理工学部
[*2] 大鵬薬品工業
[*3] 三菱安科研
[*4] エスエス製薬
[*5] 安評センター

Zhang, Y.[*1], Chen, Y.[*1], Bangaru, S.D.[*1], He, L.[*1], Abele, K.[*1], Tanabe, S., Kozasa, T.[*2] and Yang, J.[*1] : **Origin of the Voltage Dependence of G-Protein Regulation of P/Q-type Ca^{2+} Channels**

J. Neurosci., **28**, 14176-14188 (2008)

G-protein (Gβγ)-mediated voltage-dependent inhibition of N- and P/Q-type Ca^{2+} channels contributes to presynaptic inhibition and short-term synaptic plasticity. The voltage dependence derives from the dissociation of Gβγ from the inhibited channels, but the underlying molecular and biophysical mechanisms remain largely unclear. We investigated the role in this process of Ca^{2+} channel β subunit ($Ca_v\beta$) and a rigid α-helical structure between the α-interacting domain (AID), the primary $Ca_v\beta$ docking site on the channel α_1 subunit, and the pore-lining IS6 segment. Gβγ inhibition of P/Q-type channels was reconstituted in giant inside-out membrane patches from *Xenopus* oocyte. β-less channels were still inhibited by Gβγ, but without any voltage dependence, indicating that $Ca_v\beta$ is indispensable for voltage-dependent Gβγ inhibition. A truncated $Ca_v\beta$ containing only the AID-binding guanylate kinase (GK) domain could fully confer voltage dependence to Gβγ inhibition. Furthermore, voltage-dependent Gβγ inhibition was abolished when the rigid α-helix was disrupted by insertion of multiple glycines. These results suggest that depolarization-triggered movement of IS6, coupled to the subsequent conformational change of the Gβγ-binding pocket through a rigid α-helix induced partly by the $Ca_v\beta$ GK domain, causes the dissociation of Gβγ and is fundamental to voltage-dependent Gβγ inhibition.

Keywords: Gβγ, voltage-dependent modulation, β subunit

*1 Department of Biological Sciences, Columbia University
*2 Department of Pharmacology, University of Illinois College of Medicine

Nishida, M.[*1], Sato, Y., Uemura, A.[*1], Narita, Y.[*1], Tozaki-Saitoh, H.[*1], Nakaya, M.[*1], Ide, T.[*2], Suzuki, K., Inoue, K.[*1], Nagao, T. and Kurose, H.[*1] : **$P2Y_6$ receptor-G$\alpha_{12/13}$ signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis**

EMBO J., **27**, 3104-3115 (2008)

Cardiac fibrosis, characterized by excessive deposition of extracellular matrix proteins, is one of the causes of heart failure, and it contributes to the impairment of cardiac function. Fibrosis of various tissues, including the heart, is believed to be regulated by the signalling pathway of angiotensin II (Ang II) and transforming growth factor (TGF)-β. Transgenic expression of inhibitory polypeptides of the heterotrimeric G12 family G protein ($G\alpha_{12/13}$) in cardiomyocytes suppressed pressure overload-induced fibrosis without affecting hypertrophy. The expression of fibrogenic genes (TGF-β, connective tissue growth factor, and periostin) and Ang-converting enzyme (ACE) was suppressed by the functional inhibition of $G\alpha_{12/13}$. The expression of these fibrogenic genes through $G\alpha_{12/13}$ by mechanical stretch was initiated by ATP and UDP released from cardiac myocytes through pannexin hemichannels. Inhibition of G-protein-coupled P2Y6 receptors suppressed the expression of ACE, fibrogenic genes, and cardiac fibrosis. These results indicate that activation of $G\alpha_{12/13}$ in cardiomyocytes by the extracellular nucleotides-stimulated $P2Y_6$ receptor triggers fibrosis in pressure overload-induced cardiac fibrosis, which works as an upstream mediator of the signalling pathway between Ang II and TGF-β.

Keywords: cardiac fibrosis, cardiac hypertrophy, P2Y6 receptor

*1 九州大学薬学部
*2 九州大学医学部

伊佐間和郎，鹿庭正昭，土屋利江：**アクセサリー類を除く金属製品に含有する鉛及びカドミウムの分析調査**

中毒研究, **21**, 393-395 (2008)

鉛及びカドミウムを含有する製品範囲の特定とその含有量の測定を行うことを目的として，アクセサリー類等を除く金属製品に含有する鉛及びカドミウム量に関する試買調査を実施した．アクセサリー類を除く金属製品（212製品）及びそれらの容易に分離可能な金属部品のうち，乳幼児が飲み込むおそれがあるものを検体（312検体）として，エネルギー分散型蛍光X線（XRF）分析により鉛及びカドミウムの含有量を測定した．鉛含有量が米国消費者製品安全委員会の金属製子供用アクセサリーの暫定指針（Pb 0.06%）を超える製品の割合は，文具及び事務用具（33/59），家具等付属品（20/50）及び裁縫用小物用具（18/87）に多かった．また，カドミウム含有量が欧州連合のRoHS指令（Cd 0.01%）を超える製品の割合は，文具及び事務用具（7/59），裁縫用小物用具（7/87）及び家具等付属品（4/50）に多かった．金属製アクセサリー類に比べて含有量は少ないものの，鉛やカドミウムなどの有害金属を含有する金属製品は広く家庭内に存在することが確認された．

Keywords: lead, cadmium, metal product

中島晴信*，沢辺善之*，伊佐間和郎，土屋利江：**高分子材料中のオクチル酸スズ（2-エチルヘキサン酸スズ）の分析**

大阪府立公衆衛生研究所研究報告, **46**, 97-102 (2008)

Tin octylate (tin 2-ethylhexanoate) is a compound widely used as the polymerization catalyst applicable to biodegradable polylactide plastics. To figure on the quantity of a residual tin octylate in medical materials, a quantitative determination method was established for octylic acid (2-ethylhexanoic acid) that is a decomposition product of tin octylate under an acid condition with hydrochloric acid. Octylic acid was extracted from the medical materials with a mixture of acetone and n-hexane (3:7) containing a small amount of hydrochloric acid by shaking overnight at 37℃. The extract was trimethylsilylated(TMS), and the TMS derivative was analyzed by GC/MS. A capillary column of DB-5 ms (0.25 mmφ × 25 m with a film thickness of 0.25 μm) was used for GC, and the TMS derivative was determined using an ion of m/z 201 in the MS. Of four samples studied, TMS derivative of octylic acid was detected in two samples. Mean quantitative value converted into tin octylate was 134.4 μg/g (n=3, CV=14.3%) and 6.5 μg/g (n=3, CV=28.7%), respectively, which were detected at the same concentration level in the repeated analyses.

Keywords: tin 2-ethylhexanoate, 2-ethylhexanoic acid, polylactide plastic

* 大阪阪府立公衆衛生研究所

Nakamura, H.[*1], Kawakami, T., Niino, T.[*2], Takahashi,

Y.[*1] and Onodera, S.[*1] : **Chemical fate and changes in the mutagenic activity of the antibiotics nitrofurazone and furazolidone during aqueous chlorination**

J. Toxicol. Sci., **33**, 621-629 (2008)

Reactions of nitrofuran antibiotics (nitrofurazone (NFZ) and frazolidone (FZD)) with hypochlorite in aqueous solution were investigated under the conditions that simulate wastewater disinfection. The chlorination byproducts were determined by high performance liquid chromatography. At the levels of 5 μM, NFZ reacted rapidly with free chlorine in neutral pH (7.0), while the FZD-hypochlorite reaction was reasonably slow under the same pH. Nevertheless, the strong mutagenic parents disappeared completely after the hypochlorite reactions, and the chlorination byproducts were observed to exert a weak mutagenic effect on Salmonella typhimurium TA100 without S9-mix. The extent of the reactions depended on the chlorine dose, solution pH and compound structures.

Keywords: nitrofurans, water chlorination, mutagenicity

[*1] 東京理科大学薬学部
[*2] 三菱化学安全科学研究所

Kawakami, T., Nishi, I.[*1], Kishi, T.[*1] and Onodera, S.[*1] : **Formation of polybrominated and polychlorinated ethylphenoxyethylphenols (PXEPEPs) during aqueous chlorination of 4-ethylphenol solutions in the presence of bromide ions**

J. Environ. Sci. Health. Part A. **44,** 641-647 (2009)

This investigation was undertaken to determine the effect of the bromide concentration on the formation of polyhalogenated ethylphenoxyethylphenols (PXEPEPs), including predioxins, during the chlorination of 4-ethylphenol in solution. An aqueous solution of 4-ethylphenol was treated with hypochlorite in the presence of various concentrations of bromide ions. The changes in the compositions of the halogenated products in hexane extracts of the chlorinated solution were analyzed by gas chromatograph (GC) and a flame ionization detector (FID) and mass spectrometry (MS). 4-Ethylphenol was shown to from several halogenated compounds, including PXEPEPs, as by-products of chlorination. The number of substituted chlorine or bromine atoms ranged from 0 to 4. The formation of bromine-substituted PXEPEPs was observed in the presence of 0.1 equivalents of bromide ions per mole of 4-ethylphenol. The number of substituted bromine atoms increased with the amount of co-existing bromide ions. In the presence of more than one equivalent of bromide ions per mole of 4-ethylphenol, the number of bromine atoms substituted in the PXEPEPs increased, whereas the number of chlorine atoms substituted in the PXEPEPs decreased. GC-MS total ion chromatograms confirmed the formation of polybrominated and polychlorinated predioxins during the aqueous chlorination of 4-ethylphenol in the presence of bromide ions. However, at ten equivalents of bromide ions per mole of 4-ethylphenol, no predioxins were observed in the hexane extract obtained from the aqueous 4-ethylphenol solution after being treated with chlorine. The formation of PXEPEPs during the chlorination of 4-ethylphenol in the presence of bromide ions was also influenced by the reaction pH.

Keywords: 4-ethylphenol, chlorination, polybrominated and polychlorinated predioxin

[*1] 東京理科大学薬学部

Kishi, T.[*1], Suzuki, S.[*1], Takagi, M.[*1], Kawakami, T. and Onodera, S.[*1] : **Influence of experimental conditions on the formation of PCDD/Fs during the thermal reactions of 2,4,6-trichlorophenol**

Chemosphere, **76**, 205-211 (2009)

In order to obtain information on thermochemical reactions of chlorophenols, which are well known as dioxin precursors, occurring during the combustion of municipal solid wastes, the combustion of 2,4,6-trichlorophenol (2,4,6-T3CP) in an air stream was investigated over a temperature range of 500–800 ℃ for a residence time of 1–20 s using a quartz flow reactor. Gas chromatographic/mass spectrometric (GC/MS) analysis of the gaseous products and residues showed that 2,4,6-T3CP began to decompose at 500℃ and produced several compounds, with 1,3,6,8- and 1,3,7,9-tetrachlorodibenzo-p-dioxins (T4CDD) as the major products. In addition, more than fifty organic products were observed in our experimental conditions. The yields of polychlorinated benzenes, phenols, dibenzofurans, and dibenzo-p-dioxins formed during the combustion of 2,4,6-T3CP were plotted as a function of temperature and residence time. Furthermore, it was found that the yields and the compositions of these gaseous products were strongly dependent on the residence time in the flow reactor.

Keywords: PCDD/Fs, 2,4,6-Trichlorophenol, combustion condition

*1 東京理科大学薬学部

Nakajima, M.[*1], Kawakami, T., Niino, T.[*2], Takahashi, Y.[*1] and Onodera, S.[*1] : **Aquatic fate of sunscreen agents octyl-4-methoxycinnamate and octyl-4-dimethylaminobenzoate in model swimming pools and the mutagenic assays of their chlorination byproducts**

J. Health. Sci. **53**, 363-372 (2009)

Reactions of sunscreen agents, octyl dimethyl-p-aminobenzoate (ODPABA) and octyl-p-metoxycinnamate (OMC), with hypochlorite in aqueous solution were investigated under the conditions that simulate swimming pool disinfection sites. Chlorination byproducts were determined by GC/MS. At a concentration of 9μM, ODPABA reacted rapidly with free chlorine in the buffered solution at pH 7.0, OMC reacted with hypochlorite reasonably slowly under the same condition. ODPABA and OMC produced chlorine-substituted compounds as intermediates, which were decomposed to cleavaged products of ester-bond during the aqueous chlorination process. The chlorination intermediates of OMC exhibited weak mutagenic on Salmonella typhimurium TA100 strain without the S9-mix. The extent of the reactions depended on the chlorine dose, solution pH, and compound structures.

Keywords: sunscreen agents, water chlorination, mutagenicity

*1 東京理科大学薬学部
*2 三菱化学安全科学研究所

Machida, K.[*], Suemizu, H. [*], Kawai, K. [*], Ishikawa, T., Sawada, R., Ohnishi , Y.[*], Tsuchiya, T., : **Higher susceptibility of NOG mice to xenotransplanted tumors**

J. Toxicol. Sci, **34(1)**, 123-127 (2009)

The purpose of tumorigenicity testing, as applied not only to cell substrates used for viral vaccine manufacture but also stem cells used for cell-based therapy, is to discriminate between cells that have the capacity to form tumors and cells that do not. Therefore, tumorigenicity testing is essential in assessing the safety of these biological materials. Recently developed NOD/Shi-scid IL2Rg(null) (NOG) mice have been shown to be superior to NOD/Shi-scid (SCID) mice for xenotransplantation of both normal and cancerous cells. To select a suitable mouse strain as a xenogenic host for tumorigenicity testing, we compared the susceptibility of NOG (T, B, and NK cell-defective), SCID (T and B cell-defective), and the traditionally used nude (T cell-defective) mice to tumor formation from xenotransplanted HeLa S3 cells. When 10(4) HeLa S3 cells were subcutaneously inoculated into the flanks of these mice, the tumor incidence on day 22 was 10/10 (100%) in NOG, 2/10 (20%) in SCID, and 0/10 (0%) in nude mice. The subcutaneous tumors formed reproducibly and semiquantitatively in a dose-dependent manner. Unexpectedly, half of the NOG mice (5/10) that had been inoculated with a mere 10^1 HeLa S3 cells formed progressively growing subcutaneous tumors on day 78. We confirmed that the engrafted tumors originated from inoculated HeLa S3 cells by immunohistochemical staining with anti-HLA antibodies. These data suggest that NOG mice may be the best choice as a suitable strain for testing tumorigenicity.

Keywords: HeLaS3, NOD/Shi-*scid* IL2Rg^{null} (NOG), Tumorigenicity testing

* （財）実験動物中央研究所

Sawada, R., Matsuoka, A., Matsuda, Y., Tsuchiya, T. : **Change in characteristics of human mesenchymal stem cells during the in vitro culture — c-myc gene expression and chromosome aberrations at the c-myc locus —**

YAKUGAKU ZASSHI, **128**(12), 1851-1856 (2008)

We investigated mRNA expression of c-myc and chromosome aberrations at the c-myc locus in the same passage number of human mesenchymal stem cells (hMSCs). To understand the sensitivity of mRNA expression and the induction of chromosome aberrations, we first tested them in hMSC and cancer cell lines (HeLa S3, HOS, and OUMS-27). The c-myc mRNA expressions in HeLa S3 and OUMS-27 were significantly higher than those in hMSC, but then those in HOS were not. On the other hand, c-myc aberrant cells detected by fluorescence in situ hybridization in HeLa S3, HOS, and OUMS-27 were significantly higher than that in hMSC. Both analyses were performed in hMSCs derived from five donors for the culture period of 50 days. In hMSCs from one donor, the frequency of c-myc aberrant cells significantly increased at 20 and 50 days respectively, and each mRNA expressions had a tendency to increase, but there is no significant change among 3, 20 and 50 days. In hMSCs from the others, both endpoints did not change for 50 days. For safe use

of somatic stem cells in the regenerative medicine, the investigation of characteristic change of them during the in vitro culture is important. In the present study, we showed the mRNA expressions and chromosome aberrations of hMSCs in in vitro culture as the first step for establishing of safety evaluation of tissue engineered medical devices using normal hMSCs.

Keywords: human mesenchymal stem cells (hMSCs), c-myc gene expression, copy number of the c-myc locus

Tam P. S. Y.[*1], Sawada, R., Cui, Y.[*1], Matsumoto, A.[*2], Fujiwara, Y[*1]. : **The metabolism and distribution of docosapentaenoic acid (n-6) in the liver and testis of growing rats**

Biosci. Biotechnol. Biochem., **72**(10), 2548-2554 (2008)

To investigate the metabolism and distribution of docosapentaenoic acid (22:5n-6, DPA) in the liver and testis of growing rats, 22:5n-6 was administered to their dams. Newborn rats with a low hepatic arachidonic acid (20:4n-6, AA) level were generated by administrating a diet rich in docosahexaenoic acid (22:6n-3, DHA) but n-6 fatty acid (FA) free to pregnant dams. After parturition, 22:5n-6 or linoleic acid (18:2n-6, LA) was administered with a high level of 22:6n-3 to the dams until weaning. At weaning, the hepatic 20:4n-6 level was significantly highest in the DPA-DHA but not LA-DHA diet-fed animals. The hepatic delta-6 desaturase (D6D) mRNA abundance was significantly lower in both the LA-DHA and DPA-DHA diet-fed animals, connoted with the 20:4n-6 content recovered by 22:5n-6 that did not involve D6D and supporting the occurrence of retroconversion in the liver of the growing rats. The low D6D level in the 3-week-old testis was not in proportion to the elevated 22:5n-6 level, implying that early testicular 22:5n-6 accumulation might require supply from the circulation system.

Keywords: docosapentaenoic acid, docosahexaenoic acid, arachidonic acid

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[*1] お茶の水女子大学
[*2] 城西大学

Yamada, T., Sawada, R., Tsuchiya, T. : **The effect of sulfated hyaluronan on the morphological transformation and activity of cultured human astrocytes**

Biomaterials, **29**, 3503-3513 (2008)

We demonstrated the effect of synthesized sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan, and basic fibroblast growth factor 2 (FGF-2) on normal human astrocytes (NHA) activity and its morphological transformation in vitro study. Astrocyte is a kind of glial cell and stellated astrocyte (activating astrocyte) supports axons network, neurons survival and synaptic plasticity. Treatment of SHya hardly affected NHA proliferation. However combination treatment of SHya and FGF-2 increased NHA proliferation. Treatment of SHya promoted transformation of normal astrocyte into a stella morphology (stellation) and combination treatment of SHya and FGF-2 promoted stellation than that of SHya only. Treatment of SHya increased glial fibrillary acidic protein (GFAP), nestin mRNA and GFAP protein expression in the stellated NHA. The cell-cell adhesion of NHA increased by treatment of SHya. Treatment of SHya increased heparin-binding trophic factors FGF-2, midkine, and some other trophic factors mRNA level in the NHA. These results suggested that the treatment of SHya promoted NHA activity due to enhancing neurotrophins production and the morphological transformation of NHA and the effect of SHya on astrocytes partly involved FGF-2 activity. These findings indicate that SHya may be involved in the astrocyte activity and support neurons survivals.

Keywords: Hyaluronan, Sulfated groups, Astrocyte

Yamada, T., Jung, DY, Sawada, R., Tsuchiya, T. : **Intracerebral microinjection of stannous 2-ethylhexanoate affects dopamine turnover in cerebral cortex and locomotor activity in rats**

J Biomed Mater Res B : Appl Biomater., **87B(2)**, 381-386 (2008)

Stannous 2-ethylhexanoate [$Sn(Oct)_2$] is used as a catalyst for production of poly-L-lactic acid and copolymers that are implanted in cranial surgery, but reports on its effects on the central nervous system are few. We examined the effects of $Sn(Oct)_2$ on cell viability in vitro and on neurotransmission and behavior in the rat. Treatment of normal human astrocytes with 10 mg/mL $Sn(Oct)_2$ reduced mitochondrial activity to 16% of the control. Injection of $Sn(Oct)_2$ at 6.28 mg/kg BW (2 mg/kg BW Sn) into right lateral ventricle of the rat brain tended to increase the ambulation distance after 30 days when compared with the control group. The turnover of dopamine neurotransmission was increased in the cerebral cortex. These results suggest that $Sn(Oct)_2$ is cytotoxic to astrocytes in vitro. Injection of $Sn(Oct)_2$ into the brain had no or very weak immediate neurotoxicity, but long-term exposure to $Sn(Oct)_2$ increased

dopamine neurotransmission turnover.

Keywords: stannous 2-ethylhexanoate, open field test, dopamine

Ayada, T.[*1], Taniguchi, K.[*1], Okamoto, F.[*1], Kato, R., Komune, S.[*1], Takaesu, G.[*1] and Yoshimura, A.[*1,3] : **Sprouty4 negatively regulates protein kinase C activation by inhibiting phosphatidylinositol 4,5-biphosphate hydrolysis**

Oncogene **28**, 1076-1088(2009)

Sproutys have been shown to negatively regulate growth factor-induced extracellular signal-regulated kinase (ERK) activation, and suggested to be an anti-oncogene. However, molecular mechanism of the suppression has not yet been clarified completely. Sprouty4 inhibits vascular endothelial growth factor (VEGF)-A-induced ERK activation, but not VEGF-C-induced ERK activation. It has been shown that VEGF-A-mediated ERK activation is strongly dependent on protein kinase C (PKC), whereas that by VEGF-C is dependent on Ras. This suggests that Sprouty4 inhibits the PKC pathway more specifically than the Ras pathway. In this study, we confirmed that Sprouty4 suppressed various signals downstream of PKC, such as phosphorylation of MARCKS and protein kinase D (PKD), as well as PKC-dependent nuclear factor (NF)-B activation. Furthermore, Sprouty4 suppressed upstream signals of PKC, such as Ca^{2+} mobilization, phosphatidylinositol 4,5-biphosphate (PIP_2) breakdown and inositol 1,4,5-triphosphate (IP_3) production in response to VEGF-A. Those effects were dependent on the C-terminal cysteine-rich region, but not on the N-terminal region of Sprouty4, which is critical for the suppression of fibroblast growth factor (FGF)-mediated ERK activation. Sprouty4 overexpression or deletion of the *Sprouty4* gene did not affect phospholipase C (PLC) -1 activation, which is an enzyme that catalyzes PIP_2 hydrolysis. Moreover, Sprouty4 inhibited not only VEGF-A-mediated PIP_2 hydrolysis but also inhibited the lysophosphatidic acid (LPA)-induced PIP_2 breakdown that is catalyzed by PLC/ activated by G-protein coupled receptor (GPCR). Taken together, Sprouty4 has broader suppression activity for various stimuli than previously thought; it may function as an inhibitor for various types of PLC-dependent signaling as well as for ERK activation.

Keywords: negative regulation, ERK activation, PKC

[*1] 九州大学
[*2] 慶應大学

Taniguchi, K.[*1,2], Sasaki, K.[*3], Watari, K.[*1], Yasukawa, H.[*3], Imaizumi, T.[*3], Ayada, T.[*1], Okamoto, F.[*1], Ishizaki, T.[*2], Kato, R, Kohno, R.[*1], Kimura, H.[*4], Sato, Y.[*4], Ono M.[*1], Yonemitsu Y.[*1,5], Yoshimura A.[*2,6]. : **Suppression of Sproutys has a therapeutic effect for a mouse model of ischemia by enhancing angiogenesis**

PLoS ONE **4**, e5467(2009)

Sprouty proteins (Sproutys) inhibit receptor tyrosine kinase signaling and control various aspects of branching morphogenesis. In this study, we examined the physiological function of Sproutys in angiogenesis, using gene targeting and short-hairpin RNA (shRNA) knockdown strategies. Sprouty2 and Sprouty4 double knockout (KO) (DKO) mice were embryonic-lethal around E12.5 due to cardiovascular defects. The number of peripheral blood vessels, but not that of lymphatic vessels, was increased in Sprouty4 KO mice compared with wild-type (WT) mice. Sprouty4 KO mice were more resistant to hind limb ischemia and soft tissue ischemia than WT mice were, because Sprouty4 deficiency causes accelerated neovascularization. Moreover, suppression of Sprouty2 and Sprouty4 expression in vivo by shRNA targeting accelerated angiogenesis and has a therapeutic effect in a mouse model of hind limb ischemia. These data suggest that Sproutys are physiologically important negative regulators of angiogenesis in vivo and novel therapeutic targets for treating peripheral ischemic diseases.

Keywords: angiogenesis, ischemia, therapeutic effect

[*1] 九州大学
[*2] 慶應大学
[*3] 久留米大学
[*4] 東北大学
[*5] 千葉大学
[*6] CREST

Miyamoto, K.[*1], Miyamoto, T.[*1], Kato, R, Yoshimura, A[*1], Motoyama, N[*2], Suda, T[*1]. : **FoxO3a regulates hematopoietic homeostasis through a negative feedback pathway in conditions of stress or aging**

Blood, **112**, 4485-4493 (2008)

Stress or aging of tissue-specific stem cells is considered central to the decline of tissue homeostasis in the elderly, although little is known of molecular mechanisms underlying hematopoietic stem cell (HSC) aging and stress resistance. Here, we report that mice lacking the transcription factor forkhead box O3a (FoxO3a) develop

neutrophilia associated with inhibition of the up-regulation of negative regulator of cell proliferation, Sprouty-related Ena/VASP homology 1 domain-containing proteins 2 (Spred2) and AKT and ERK activation, in HSCs during hematopoietic recovery following myelosuppressive stress conditions. Compared with aged wild-type mice, more severe neutrophilia was also observed in aged Foxo3a-deficient mice. AKT and ERK activation and inhibition of Spred2 were detected in HSCs from aged FoxO3a-deficient mice. Spred2-deficient mice also developed neutrophilia during hematopoietic recovery following myelosuppressive stress, indicating that FoxO3a plays a pivotal role in maintenance, integrity, and stress resistance of HSCs through negative feedback pathways for proliferation. This will provide new insight into the hematopoietic homeostasis in conditions of aging and stress.

Keywords: FoxO3, hematopoietic homeostasis, negative feedback

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[*1] 慶應大学
[*2] 国立長寿医療センター

Hexig, B., Nakaoka, R., Tsuchiya, T. : **Safety evaluation of surgical materials by cytotoxicity testing**
J. Artif. Organs, **11**, 204-211 (2008)

The cytotoxicity of three kinds of commercially available absorbable hemostats [oxidized cellulose (Surgicel, gauze and cotton types), microfibrillar collagen (Avitene), and cotton-type collagen (Integran)] and one adhesion barrier [sodium hyaluronate and carboxymethyl-cellulose membrane (Seprafilm)] were comparatively assessed by a colony assay using V79 cells and a minimum essential medium (MEM) elution assay in combination with a neutral red assay using L929 cells. Strong cytotoxicity was detected for Surgicel by both the MEM elution assay and the colony assay. For Avitene, both methods revealed weak cytotoxicity. For Seprafilm, no cytotoxicity was detected by the MEM elution assay, while a moderate degree of cytotoxicity was observed in the colony assay. For Integran cytotoxicity was not detected by either the MEM elution or the colony assay. The results of the different methods showed some inconsistency in terms of the degree of cytotoxicity of the materials. It is proposed that the combination of two or more sensitive cytotoxicity testing methods for the evaluation of biomaterials is necessary to avoid false-negative results for biomaterials at the preclinical stage. Furthermore, investigation of the correlation between the cytotoxicity and the extraction period of the surgical materials is helpful for predicting the effect of prolonged in vivo use of biomaterials on surrounding cells, tissues, and organs.

Keywords: Safety evaluation, Surgical materials, Colony assay

田中和人[*1], 上村明正[*1], 片山傳生[*1], 木下定[*2], 迫田秀行, 藏本孝一[*3]：**超高分子量ポリエチレンの疲労き裂伸展特性に及ぼす環境劣化とVitamin-E添加の影響**
Journal of Society of Materials Science, Japan, **57**, 875-881 (2008)

Ultra High Molecular Weight Polyethylene (UHMWPE) has been used for the bearing materials of artificial knee joints owing to its superior mechanical properties and chemical resistance. In vivo, however, because of wear and fatigue of UHMWPE components, delamination fracture occurred. Although γ-irradiation and following aging was reported to accelerate the delamination fracture, the effects on the fatigue crack growth behavior have not been revealed yet. Moreover, the addition of Vitamin-E (α-Tocopherol) was reported to prevent the delamination wear, but the prevention mechanism has not been clarified yet. In this study, in order to understand the influence of γ-irradiation and accelerated aging, and the addition of Vitamin-E on the fatigue crack growth properties of UHMWPE, tensile tests and fatigue crack growth tests of UHMWPE were carried out. After the γ-irradiation and accelerated aging, the specimen surface was oxidized and its crystallinity was increased. However the addition of Vitamin-E reduced the oxidization of the specimen and the increase of its crystallinity. For the tensile tests, the yield stress was increased and the tensile strength was decreased by γ-irradiation and accelerated aging. For the fatigue crack growth tests, the addition of Vitamin-E reduced the decrease of ΔJ_{th} by γ-irradiation and accelerated aging. Although the fibrillation and the brittle fracture were observed on the fracture surface of γ-aged specimen, they were not observed on that of Vitamin-E added γ-aged specimen.

Keywords: Ultra High Molecular Weight Polyethylene, Knee joint, Fatigue Crack growth

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[*1] 同志社大学
[*2] 京都大学
[*3] ナカシマプロペラ株式会社

迫田秀行，鄭徳泳，佐藤道夫，土屋利江，脇谷滋之[*1]，天正恵治[*2]：**人工関節の不具合要因分析**

日本臨床バイオメカニクス学会誌, **29**, 361-365（2008）

Joint arthroplasty is an effective procedure for recovering the quality of life of patients with osteoarthritis or rheumatoid arthritis. There is still a strong need to improve the durability of joint prostheses and reduce the failure rate. Long-term clinical study is a very important tool for evaluating implants as in-vitro testing can not fully simulate the biological and biomechanical environment in vivo. Therefore, analysis of failed and retrieved implants and identification of factors related to the failure are very important.

To fully understand the factors promoting implant failure, clinical information is necessary, since the failure can be attributed not only to the implant itself but also to host conditions. However, obtaining clinical information and analyzing it is costly and time-consuming. This study investigated efficient means to identify the factors of implant failure and collect useful information from retrieved implants for the development of future implants.

Eleven retrieved knee implants were obtained without clinical information. After visual inspection, UHMWPE components of the implants were analyzed by FTIR.

Eight cases were considered to have failed due to oxidative degradation of UHMWPE components. This mechanism is well known and is not considered to happen to currently available products as their manufacturing processes have been improved to prevent oxidation of UHMWPE. Three cases were considered to have failed due to factors other than oxidative degradation of UHMWPE components and clinical information was considered necessary to identify the factors related to failure in these implants.

It was possible to extract cases that had failed due to factors other than oxidative degradation of UHMWPE components based on visual inspection and FTIR analysis. It was considered that detailed analysis of these selected cases using clinical information will provide useful information for the development of future implants.

Keywords: UHMWPE, implant failure, oxidation

[*1] 大阪市立大学
[*2] 信州大学

迫田秀行，鄭　徳泳，佐藤道夫，土屋利江，脇谷滋之[*1]，天正恵治[*2]：**微小試験片を用いた人工関節用UHMWPEの疲労特性評価**

日本臨床バイオメカニクス学会誌, **29**, 367-372（2008）

Highly crosslinked polyethylene (HXLPE) was developed to reduce wear on the ultra-high molecular weight polyethylene (UHMWPE) component of joint prostheses. The manufacturing process of HXLPE includes radiation crosslinking and thermal treatment to eliminate free radicals. Since these processes are known to degrade the fatigue property of UHMWPE, it might become one of the main factors limiting the durability of implants.

The fatigue property of UHMWPE has mainly been evaluated by fatigue crack propagation test using compact tension specimens. However, this cannot be applied to retrieved implants or final products due to required specimen size. Therefore, there is not sufficient data to relate the fatigue property of UHMWPE to clinical outcome. This study investigated a new test method to evaluate the fatigue property of UHMWPE of retrieved implants or final products.

Rectangular specimens were machined from virgin UHMWPE. An initial crack was created at the centre of the specimens and cyclic tensile load was applied at 1Hz by a conventional fatigue test machine. Nominal stress and the number of cycles until fracture were used for analysis. Specimens were also prepared from UHMWPE components of retrieved implants in the same manner.

The results of virgin material were plotted on a straight line showing a typical S-N curve. There were no apparent influences of specimen size, initial crack length or magnitude of applied load.. Specimens from retrieved implants showed inferior fatigue property.

Fatigue property of retrieved implants could be evaluated by the new test method developed in this study. Oxidized UHMWPE components showed degraded fatigue property. Further study of failed implants using this test method is expected to demonstrate the relation between the fatigue property of UHMWPE and failure of the implants, contributing to future improvements.

Keywords: UHMWPE, fatigue, retrieved implants

[*1] 大阪市立大学
[*2] 信州大学

迫田秀行，中岡竜介，松岡厚子，土屋利江：**三次元スキャフォールドを用いた細胞培養系の評価方法の検討**

国立医薬品食品衛生研究所報告，**126**，76-81（2008）

In tissue engineering and related studies, in vitro evaluations are often carried out by a three-dimensional cell culture, where cells are inoculated in three-dimensional

scaffolds. Cell number is one of the most fundamental parameter in cell cultures and especially important in three-dimensional cell cultures because cell behavior is sometimes dependent on the cell density. However, there are many studies where cell number is not specified, probably due to the difficulty of evaluating cell number in the three-dimensional cell culture.

In this study, we examined if existing methods to evaluate cell number established for conventional two-dimensional cell cultures could be applied to the three-dimensional cell cultures using collagen composite scaffolds and human articular chondrocytes as an example of the three-dimensional cell culture. The cells were inoculated on the conventional cell culture plate or the scaffolds and the cell number was estimated by different methods and the results were compared with each other. Firstly, DNA quantification method was shown to be able to estimate cell numbers in either two-dimensional or three-dimensional culture. Secondary, the results of non-destructive cell number estimation method using alamarBlue reagent were found to be consistent with those of DNA quantification method in either two-dimensional or three-dimensional culture. However, the results of the other non-destructive cell number estimation method using TetraColor ONE reagent were not consistent with those of other methods in the three-dimensional culture.

It was concluded that when applying existing evaluating methods established for the two-dimensional cell cultures to a three-dimensional cell culture, it is important to validate them for the three-dimensional cell culture.

Keywords: three-dimensional cell culture, scaffold, cell number

石川　格，澤田留美，加藤幸夫[*1,*2]，辻紘一郎[*1]，邵　金昌[*2]，山田貴史，加藤玲子，土屋利江：**新無血清培地STK2のヒト間葉系幹細胞増殖における有用性について**

薬学雑誌, **129**, 381-384 (2009)

To apply human mesenchymal stem cells (hMSC) to regenerative medicines, it is necessary to multiply hMSC in vitro in a short period. In addition, it is desirable that the medium which is used for the hMSC multiplication is not supplemented with the serum, because the addition of the serum has risks of infection. In this study, we cultured hMSC with three kinds of medium used for multiplying hMSC (DMEM, MSCGM, STK2) and compared hMSC proliferation in each medium. As a result, it was confirmed that hMSC proliferation was significantly higher in STK2 medium which is a novel serum-free medium developed for hMSC multiplication. Moreover, we compared the hMSC proliferation in these media under the environment that assumed bone reproduction. When we cultured hMSC in each medium with hydroxyapatite (HAp), the proliferative inhibition by HAp depended on the additive amount, and the degree of the proliferative inhibition was different among the media but the lowest inhibitory effect was observed in STK2 medium.

Keywords: Mesenchymal stem cell, Serum-free medium, Cell proliferation, Hydroxyapatite, Stem cell culture

[*1] 広島大学大学院医歯薬学総合研究科
[*2] JSTイノベーションプラザ広島

植松美幸，中野喜隆[*1]，松川紘大[*1]，宇都宮隆平[*1]，中村亮一[*2]，村垣善浩[*2]，伊関　洋[*2]，青見茂之[*2]，梅津光生[*1]：**大血管手術の安全性を高める画像支援ナビゲーションシステム**

日本バーチャルリアリティ学会論文誌, **14**（1）, 39-48 (2009)

筆者らはマルチスライスCT画像を用いたナビゲーションシステムを構築した．これは，正確に手術野のオリエンテーションをつけることを支援するシステムであり，胸腹部大動脈瘤手術でアダムキュービッツ動脈を選択的に温存する際に効果的に用いることができる．今回，30名の胸腹部大動脈瘤の患者に対して行った結果について報告する．まず，手術前にマルチスライスCTを撮影し，アダムキュービッツ動脈に血流を送る肋間動脈の位置を同定する．手術中のナビゲーションでは，手術野でのポインター位置をセンサーにて計測し，これと連動して解剖学的構造を3次元画像により表示する．これにより，目標とする肋間動脈を実空間上で特定でき，目標血管と臓器につながる主要な動脈が再建された．手術成績に関しては，院内での死亡は１人で対麻痺はゼロであった，この新たなナビゲーションシステムは正確なオリエンテーションの実現に有効であった．そして，筆者らのナビゲーションの臨床応用では．胸腹部大動脈瘤手術を安全かつ効率的に進められることが示された．

Keywords: surgical navigation system, aortic vascular surgery, three-dimensional imaging

[*1] 早稲田大学
[*2] 東京女子医科大学

Matsuoka, A., Haishima, Y., Hasegawa, C., Matsuda,

Y., and Tsuchiya, T. : **Organic-solvent extraction of model biomaterials for use in the in vitro chromosome aberration test**

J. Biomed.Mater.Res., **86A**, 13-22 (2008)

We prepared polyurethane (PU) containing 0.4% or 4% 4,4'-methylenedianiline (MDA) as model materials to investigate the effectiveness of sample preparation by organic-solvent extraction for the in vitro chromosome aberration (CA) test. MDA itself (0.4 mg/mL) was positive only in the presence of an exogenous metabolizing system (S9 mix). The culture medium extract of PU containing 4% MDA (PU/4% MDA) was negative with and without S9 mix. Methanol and acetone extracts, on the other hand, induced structural CAs without S9 mix, which we did not expect because MDA requires S9 mix for activity. On chemical analysis, however, we found that the ratio of MDA extracted by the organic solvents to that extracted by the culture medium of PU/4% MDA was about 15:1. Interestingly, oligomers consisting of poly(tetramethyleneglycol) derivatives (OTMG) were also extracted by the organic solvents. The data suggest that the induction of structural CAs in the absence of S9 mix may have been partly due to synergism of MDA and OTMG. CA tests of MDA and PTMG-1000 in combination confirmed that to be the case. Thus, organic-solvent extraction may be more effective than medium extraction in evaluating the biological safety of biomaterials.

Keywords: organic-solvent extraction, polyurethane

Matsuoka, A., Kodama, Y., Fukuhara, K., Honda, S.[*1], Hayashi, M.[*1], Sai, K., Hasebe, M.[*2], Fujiwara ,Y.[*2] : **A pilot study of the antioxidative activity of resveratrol and its analogue in a 6-month feeding test in young adult mice**

Food Chem. Toxicol., **46,** 1125-1130 (2008)

Resveratrol, a polyphenolic phytoalexin, has free-radical scavenging activity and we found that it induces chromosomal aberrations, micronuclei, and sister chromatid exchanges in vitro. We synthesized its analogue 4-hydroxy-*trans*-stilbene (4-OH) and found that it has the same in vitro clastogenic activities as resveratrol, suggesting that the 4'hydroxy group of resveratrol is responsible for the effect. We fed resveratrol and 4-OH to young adult ICR mice at 0, 0.2, 2, or 20ppm in their standard powder diet for 6 months and investigated the antioxidative effects. Half of each group was given 3000 ppm potassium bromate ($KBrO_3$) in water for the last week to cause oxidative damge. Body weight gain tended to increase in males at 0.2 ppm resveratrol or 4-OH, and in females at 2 ppm 4-OH. Micronucleus (MN) analysis in bone marrow erythrocytes showed that the $KBrO_3$ tendency to induce MN was not prevented by the dietary resveratrol or 4-OH, which themselves did not induce MN under the present conditions. In this pilot study, resveratrol and 4-OH showed no obvious effect, either beneficial or advese, at doses that are feasible in daily life for humans.

Keywords: 4-hydroxy-*trans*-stilbene, in vivo antioxidative activity

[*1] 富山県衛生研究所
[*2] お茶の水女子大学

Uchino, T., Takezawa[*], T., Ikarashi, Y. : **Reconstruction of three-dimensional human skin model composed of dendritic cells, keratinocytes, and fibroblasts utilizing a handy scaffold of collagen vitrigel membrane**

Toxicol. In Vitro , **23**, 333-337 (2009)

We previously we attempted to make a three-dimensional human skin model consisting of three different cells, dendritic cells, keratinocytes and fibroblasts (KDF-Skin) to evaluate immunoreactions in human skin; however, this model had various problems; for example, 1) the incubation period for the construction of this model is long (about three weeks); 2) to construct the collagen gel, high amounts of fibroblasts are needed; and 3) the horny layer of keratinocytes in this skin model is thinner than that of keratinocytes in real human skin. In order to overcome these problems, a new three-dimensional human skin model utilizing a handy scaffold of collagen vitrigel membrane (VG-KDF-Skin) was constructed. As a result, after 14-days incubation, the epidermis layer of normal human keratinocytes was thicker than the keratinocyte layer of KDF-Skin. The incubation period for VG-KDF-Skin construction was 7 days shorter than that of KDF-Skin, and the number of fibroblasts needed to seed VG-KDF-Skin was 4-times fewer than that of KDF-Skin. After the application of sensitizers such as DNCB, VG-KDF-Skin induced the expression of CD86 and cytokine release. These results suggest that the new three–dimensional human skin model consisting of dendritic cells, keratinocytes, fibroblasts and collagen vitrigel membrane was more useful for alternative animal testing than the KDF-Skin model.

Keywords: three-dimensional human skin model, collagen

vitrigel membrane, skin sensitization

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*National Institute of Agrobiological Sciences

久保田領志，鈴木俊也*，田原麻衣子，清水久美子，西村哲治：**水環境中のPPCPsのモニタリングと浄水工程を想定した処理性評価**

水環境学会誌，**31**，643-649（2008）

Pharmaceuticals and personal care products (PPCPs) and their metabolites continually flow into aquatic environments and are detected widely at significant concentrations. However, a comprehensive survey has not been conducted on PPCPs in aquatic environments in Japan. Moreover, the information about removal techniques for PPCPs, such as chlorination and ozonization, is limited. In this study, we investigated the occurrence of fifteen PPCPs in water samples (sewage water and river water) collected in urban areas of Japan and evaluated the PPCPs removal efficiencies of chlorination and powdered activated carbon treatment. PPCPs (except erythromycin, paroxetine, and fluvoxamine) were detected in almost all sewage water and river water samples. The highest value was observed in bezafibrate and detected at $\mu g \cdot l^{-1}$ level. The concentrations of other PPCPs were one order of magnitude lower than that of bezafibrate. The removal efficiency of chlorination varied with the type of PPCPs, and the percentages of relative residuals of fibrates, fluvoxamine, carbamazepine, and ethenzamide were more than 50% at four hours after treatment. On the other hand, although removal rate was relatively low compared with that of chlorination, almost all PPCPs were satisfactory removed by powdered activated carbon treatment. These results suggest that a combination of these treatments could be effective for the removal of PPCPs.

Keywords: pharmaceuticals and personal care products (PPCPs), removal efficiency, human impact

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* 東京都健康安全研究センター

Ihara, T.*, Saito, T.*, Sugimoto, N. : **Expansion of organic reference materials for the analysis of hazardous substances in foods and environments. -Realization of an efficient metrological traceability using the quantitative NMR method-**

Synthesiology, **2**, 12-22 (2009)

Reference materials are indispensable for accurate analysis of hazardous substances in foods and environments. For organic substances, however, the dissemination of reference materials is hopelessly unable to catch up with today's rapidly proliferating analytical needs. To solve this problem, analytical techniques were improved to develop a method in which a single primary reference material could provide accurate quantitative measurements for a wide variety of organic compounds. In this approach, we turned our attention to the ^{1}H NMR method. We improved the method to perform precise comparisons of signal quantities from protons at different chemical shifts, enabling calibration at an acceptable level of uncertainty for a variety of organic reference materials by using a primary reference material for protons. This result opens the prospect of highly efficient metrological traceability, reducing the required number of national reference materials to a minimal level.

Keywords: metrological traceability, nuclear magnetic resonance spectroscopy, primary method of measurement

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*（独）産業技術総合研究所

青柳光敏*，新山和人*，高附　巧，根本　了，佐々木久美子，米谷民雄：**LC/MSによる農産物中のドジンの分析法**

食品衛生学雑誌，**50**, 58-63(2009)

農産物中のドジンをLC/MS を用いて分析する方法を検討した. 試料からアセトニトリルおよび含水アセトニトリルで抽出した後, 酢酸エチルに転溶し，PSA ミニカラムで精製し，LC/MS のESl ポジティブモードで測定する方法を開発した．種実類には，転溶操作の前に塩酸添加アセトニトリル／ヘキサン分配による脱脂を，ほうれんそうなどにはグラファイトカーボンミニカラムによる精製を追加した．農産物16種類の試料からの回収率は80.3 ～ 100.0% (相対標準偏差0.3 ～ 6.4%)，検出限界は0.0006 mg/kg (S/N＝3) であった. 開発した方法は操作が簡便であり，また，一律基準値レベルの分析に対して十分な検出感度を有しており，農産物中のドジンの残留分析に有用と考えられた.

Keywords: dodine, fungicide, LC/MS

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* 北海道立衛生研究所

上野英二*, 椛島由佳*, 大島晴美*, 大野　勉*, 根本　了, 米谷民雄 : **LC-MSによる農産物中デメトン-S-メチル，オキシデメトンメチルおよびデメトン-S-メチルスルホンの分析**

食品衛生学雑誌，**50**, 64-69(2009)

農産物中のデメトン-S-メチル，オキシデメトンメチルおよびこれらの酸化生成物であるデメトン-S-メチルス

ルホンを定量するための同時分析法を検討した．抗酸化剤としてL-アスコルビン酸およびブチルヒドロキシトルエンを添加した試料から，アセトンで抽出し，多孔性ケイソウ土カラムを用いて酢酸エチルに転溶後，脂質の多い玄米，大豆はヘキサン／アセトニトリル分配により脱脂，次いで色素の少ない玄米，大豆，ばれいしょはPSAカラム，それ以外はグラファイトカーボン／PSA 連結カラムを用いて精製し，ESI-SIMモードLC-MSで測定した．玄米など10種類の試料からの平均回収率は73.8～102.5%（相対標準偏差≦5.7%）と良好であった．

Keywords: demeton-S-methyl, oxydemeton-methyl, demeton-S-methylsulfone

* 愛知県衛生研究所

長岡(浜野) 恵，松田りえ子，米谷民雄：**寒天中ホウ酸のICP-AES及びICP-MSによる試験法の開発とその評価**

食衛誌，**49**，333-338 (2008)

寒天中ホウ酸の新しい試験法を検討し，寒天を湿式灰化後，ホウ素濃度を内標を用いるICP-AES法またはICP-MS法で分析する方法を策定した．その方法につき，模擬試料として粉末寒天および標準試料（NIST SRM1570a）を用い，ICP-AES法5機関，ICP-MS法5機関による共同試験を実施した結果，策定した方法の真度・精度は非常に良好で，新規法として採用可能であると考えられた．

Keywords: agar，boric acid, validation

Takekawa, T.[*1], Koshikawa, T.[*2], Miyahara, M. : **Development of Microbiological Method（Heat Treatment Method）for Irradiated Food Detection and Blind Trial of Collaborative Laboratories**

Bokin. Bobai, **37**, 181-193(2009)

A microbiological detection method is needed as a practical screening method that each laboratory can distinguish easily and cheaply between the non-irradiated and irradiated foods. We carried out study last year based on the finding that sensitivity of bacteria to heat-treatment rose when bacteria received damage by the radiation, and the possibility that the irradiation could be detected in a certain processing condition. This method consists of the first judgment based on a general viable cell count and the second judgment based on the number of bacteria difference of before and after heat-treatment. A joint research between laboratories of the detection method by the presence of heat-treatment to viable cell that adhered to the spice was executed in this year. From the above results, we find that there are two kinds of spices as a result of this research, one should do first judgment and second judgment, and the other should do only first judgment. Therefore, the target spice should be classified into the one only of the first judgment and the one until judging the second. In that case, we were able to obtain a high correct answer rate.

Keywords : Food irradiation, Irradiated food detection, Irradiated spice, Microorganism method, Heat treatment method

[*1] Nuclear Fuel Industries, Ltd.
[*2] Koka Laboratory, Japan Radioisotope Association

Miyahara, M.， Furuta, M.[*1], Takekawa, T.[*2], Oda, S.[*3], Koshikawa, T.[*4], Akiba, T.[*5], Mori, T.[*6], Mimura, T.[*7], Sawada, C.[*8], Yamaguchi, T.[*9], Nishioka, S.[*10], Tada, M.[*11] : **Verification of the new detection method for irradiated spices based on microbial survival by collaborative blind trial**

Radiation Physics and Chemistry, Online April **10** 2009 **(doi:10.10.16/j: radphyschem.2009.0410)**

An irradiation detection method using the difference of the radiation sensitivity of the heat-treated microorganisms was developed as one of the microbiological detection methods of the irradiated foods.This detection method is based on the difference of the viable cell count before and after heat-treatment (70°C and 10 minutes).

The verification by collaborative blind trial of this method was done by nine inspecting agencies in Japan. The samples used for this trial were five kinds of spices consisting of non-irradiated, 5-kGy irradiated, and 7-kGy irradiated black pepper, allspice, oregano, sage, and paprika, respectively. As a result of this collaboration, a high percentage (80%) of the correct answers was obtained for irradiated back pepper and allspice. However, the method was less successful for irradiated oregano, sage and paprika. It might be possible to use this detection method for preliminary screening of the irradiated foods but further work is necessary to confirm these findings.

Key Words : food irradiation, microbial detection, heat treatment, blind trial, viable cell count

[*1] Nuclear Fuel Industries Ltd.
[*2] Osaka Prefecture University
[*3] Japan Food Research Laboratories
[*4] Japan Radio Isotope Association

*5 Japan Food Hygiene Association
*6 Tokyo Kenbikyo-In Foundation
*7 Japan Oilstuff Inspector's Corp.
*8 Japan Frozen Foods Inspection Corp.
*9 Japan Electron Beam Irradiation Service Co., Ltd.
*10 Mycotoxin Inspection Corp.
*11 Chugoku Gakuen University

Koshikawa, T. *1, Matsushima, M. *1, Hironiwa, T. *1, Takekawa, T. *2 and Miyahara, M. : **Improving the Determination of Irradiation Efficacy by the Identification of Surviving Bacteria from Irradiated Spices**

Bokin. Bobai, **37**, 15-20 (2009)

The identification of the surviving bacteria isolated from 5 kinds of irradiated spices (allspice,oregano,sage,paprica and Black pepper) was carried out to know whether these bacteria were marker organisms to determine the efficacy of the irradiation treatment.

Except in paprica, *B.megaterium* was detected.In allspice, paprica and black pepper *B.pumilus* was detected. *B.cereus* was detected in allspice, oOregano and black pepper. Gram negative bacteria such as *Methylobacterium* and *Enterobacter* genus were also detected in oregano, sage and paprica. These bacteria were strongly resistant to radiation, and can be used as marker organisms for the determination of the efficacy of the irradiation treatment of spices.

Keywords: Food irradiation, Irradiated food detection, Irradiated spices, Spore-forming bacteria, Identification of surviving bacteria

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*1 Koka Laboratory, Japan Radioisotope Association
*2 Nuclear Fuel Industries, Ltd.

Tsutsumi, T., Miyoshi, N., Sasaki, K., Maitani, T. : **Biosensor immunoassay for the screening of dioxin-like polychlorinated biphenyls in retail fish**

Anal. Chim. Acta., **617**, 177-183 (2008)

Dioxin-like polychlorinated biphenyls (DL-PCBs) often make up the majority of the toxic equivalent (TEQ) contribution of dioxins found in fish samples. For the purpose of making risk assessments, it is therefore important to develop screening methods for determining TEQ concentrations of DL-PCBs in retail fish. We have developed a rapid biosensor immunoassay (BIA) for DL-PCBs that uses a surface plasmon resonance sensor (Biacore 3000). The BIA is highly specific for 2,3',4,4',5-pentachlorobiphenyl (PCB 118) that is generally the most abundant DL-PCB isomer found in fish. The fish extracts were first cleaned up on a multilayer silica gel column followed by an alumina column, then subjected to the assay. The quantitative limit of the assay was 1 ng PCB 118 per gram of tested sample. Dilution and recovery tests using purified fish extracts suggested that the matrix effect was minimized in the assay by diluting the analyzed samples. The assay results for retail fish samples (n = 7) agreed well with those obtained by an enzyme-linked immunoassay (ELISA) using the same monoclonal antibody: ELISA has been already validated for determining DL-PCBs in fish samples, so BIA performs well in this analysis. Finally, BIA results for the TEQ concentrations of DL-PCBs in retail fish samples (n = 10) correlated well with those obtained by high-resolution gas chromatography coupled to high-resolution mass spectrometry (r = 0.89). Our method is therefore useful for screening retail fish to determine the TEQ concentrations of DL-PCBs.

Keywords: dioxin-like PCBs, Biacore, fish

Tsutsumi, T., Amakura, Y., Ashieda, K.*1, Okuyama, A.*2, Tanioka, Y.*3, Sakata, K.*3, Kobayashi, Y.*4, Sasaki, K., Maitani, T. : **PCB 118 and Aryl Hydrocarbon Receptor Immunoassays for Screening Dioxins in Retail Fish**

J. Agric. Food Chem., **56**, 2867-2874 (2008)

The efficacy of a combination of two enzyme-linked immunosorbent assay (ELISA) kits was examined for screening the toxic equivalent (TEQ) concentrations of dioxins in retail fish. The coplanar PCB EIA system (PCB-EIA), which is a competitive immunoassay specific for polychlorinated biphenyl (PCB) 118, was tested as a screening method for mono-*ortho* PCBs. The Ah-immunoassay (Ah-I), which is an ELISA-based aryl hydrocarbon receptor binding assay, was analyzed for its screening ability for non-*ortho* PCBs, polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs). Dilution and recovery tests using purified fish extracts revealed no major interference of the matrix in the PCB-EIA, and suggested that the matrix effect was minimized in the Ah-I. Finally, the results for the fish samples (n = 20) showed a strong correlation between this method and high-resolution gas chromatography coupled to high-resolution mass spectrometry for the determination of the TEQ concentrations of mono-*ortho* PCBs (r = 0.99), and

non-*ortho* PCBs and PCDD/Fs (r = 0.97). These data indicate that our method is suitable for screening retail fish to determine the TEQ concentrations of dioxins.
Keywords: ELISA, Ah receptor, dioxins

[*1] Nisshin Environmental Planning Inc.
[*2] EnBioTec Laboratories Co., Ltd.
[*3] Daiichi Fine Chemical Co., Ltd.
[*4] KUBOTA Corporation

Amakura, Y.[*1], Tsutsumi, T., Tanno, K.[*2], Nomura, K.[*2], Yanagi, T.[*2], Kono, Y.[*2], Yoshimura, M.[*1], Maitani, T., Matsuda, R., Yoshida, T.[*1] : **Dioxin concentrations in commercial health tea materials in Japan**
J. Health Sci., **55**, 290-293 (2009)

This study determined the concentrations of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dioxin-like PCBs) in five selected plant materials [dokudami (from houttuynia herb), rose hip (from rosa fruit), ebisugusa (from cassia seed), rooibos, and tochu (from eucommia leaf)] used as health teas in Japan. The toxic equivalent quantity (TEQ) levels for dioxins in the samples ranged from <0.001 to 0.27 pg-TEQ/g weight, when undetectable and trace amounts were taken as zero. The mean of total TEQ level in commercial tea materials was estimated as 0.08 pg-TEQ/g (n=5). The total TEQ in these samples was mainly dominated by the levels of PCDD/Fs (representing *ca*. 80% of the total TEQ).
Keywords: dioxin, health tea, food

[*1] Matsuyama University
[*2] Japan Food Research Laboratories

Amakura, Y.[*1], Tsutsumi, T., Sasaki, K., Nakamura, M.[*2], Yoshida, T.[*1], Maitani, T. : **Influence of food polyphenols on aryl hydrocarbon receptor-signaling pathway estimated by *in vitro* bioassay**
Phytochemistry, **69**, 3117-3130 (2008)

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the toxic and biological action of many aromatic environmental pollutants such as dioxins. We investigated the activation of the AhR by some vegetable constituents, including flavonoids, tannins, and related polyphenols, using the AhR-based in vitro bioassay for dioxins. Among tested compounds, isoflavones such as daidzein, resveratrol having a stilbene structure, some flavanones such as naringenin, and flavones such as baicalein, showed a marked AhR activation. On the other hand, some flavones such as apigenin, flavonols such as quercetin and anthraquinones such as emodin among vegetable polyphenols, showed notable inhibitory effects on the in vitro activation of AhR induced by the dioxin [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)]. Additionally, AhR-mediated interaction of some plant food extracts, including vegetables, fruits, herb and teas, were tested by using AhR-based bioassay. Of the tested samples, some green leafy vegetables, citrus fruits and herbs, containing food polyphenolics, showed AhR-based interactions at high concentrations. On the basis of these data, we discussed the implications of polyphenols on the AhR-signaling pathway.
Keywords: polyphenol, aryl hydrocarbon receptor, *in vitro* bioassay

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[*1] Matsuyama University
[*2] Hiyoshi Corporation

Kodama, T.[*1], Kuribara, H. [*1], Minegishi, Y. [*2], Futo, S.[*3], Watai, M.[*4], Sawada, C.[*5], Watanabe, T., Akiyama, H., Maitani, T., Teshima, R., Furui, S.[*6], Hino, A.[*6], Kitta, K.[*6] : **Evaluation of Modified PCR Quantitation of Genetically Modified Maize and Soybean Using Reference Molecules Interlaboratory Study**
J AOAC Int., **92**, 223-233 (2009)

Real-time polymerase chain reaction (PCR)-based quantitative methods were previously developed and validated for genetically modified (GM) maize or soy. In this study, the quantification step of the validated methods was modified, and an interlaboratory study was conducted. The modification included the introduction of the PCR system SSIIb 3 instead of SSIIb 1 for the detection of the taxon-specific sequence of maize, as well as the adoption of colE1 as a carrier included in a reference plasmid solution as a replacement for salmon testis. The interlaboratory study was conducted with the ABI PRISM 7700 and consisted of 2 separate stages: (1) the measurement of conversion factor (Cf) value, which is the ratio of recombinant DNA (r-DNA) sequence to taxon-specific sequence in each genuine GM seed, and (2) the quantification of blind samples. Additionally, Cf values of other instruments, such as the ABI PRISM 7900 and the ABI PRISM 7000, were measured in a multilaboratory trial. After outlier laboratories were eliminated, the repeatability and reproducibility for 5.0% samples were <15.8 and 20.6%, respectively. The

quantitation limits of these methods were 0.5% for Bt11, T25, and MON810, and 0.1% for GA21, Event176, and RR soy. The quantitation limits, trueness, and precision of the current modified methods were equivalent to those of the previous methods. Therefore, it was concluded that the modified methods would be a suitable replacement for the validated methods.

Keywords: polyphenol, aryl hydrocarbon receptor, in vitro bioassay

[*1] Food and Agricultural Materials Inspection Center
[*2] Nippon Gene Co., Ltd.
[*3] Fasmac Co., Ltd.
[*4] Japan Food Research Laboratories
[*5] Japan Frozen Foods Inspection Corp.
[*6] National Food Research Institute

渡邉敬浩，関野理子，白政優子，松田りえ子，米谷民雄：**安全性定量PCR法により得られる遺伝子組換えダイズ定量値に対するマトリクス品種の影響**

食品衛生学雑誌，**49**，294-302 (2008)

代表的な非遺伝子組換えダイズ10品種を用い，遺伝子組換えダイズ(RRS)を対象とした定量PCR法により得られる定量値への影響を調査し，さらにその要因について検討した．その結果，粉体重量混合率を真値とすると，マトリクスとなる非遺伝子組換えダイズの品種よって定量値との間にbiasが生じ，その大きさは品種依存的に変動することが明らかになった．一方で，DNA収量が品種により異なること，さらには，個別に抽出したDNAをその重量比として混合した試料を分析した場合には，混合率によく合致した定量値が得られることが明らかとなった．これらの結果から，定量PCRに供されるDNA溶液中に含まれるRRSならびに非遺伝子組換えダイズに由来するDNAの量比が，粉体重量混合率を保持しえない事が，粉体重量混合率と定量値との間に品種に依存したbiasを生じる大きな要因であることが強く示唆された．

Keywords: genetically modified soy, quantitative PCR method, PCR, testing method

渡邉敬浩，米谷民雄*，松田りえ子：**リアルタイムPCR法における検量線に基づき推定されるコピー数の変動要因**

食品衛生学雑誌，**50**，1-5 (2009)

リアルタイムPCR法により作成される検量線の信頼区間を，Ct値の変動から推定した．得られた結果は実際の検量線の変動範囲と良く一致した．検量線の95%信頼区間で認められた変動幅は最大で±1%程度であったが，個別の検量線から推定されるコピー数の変動は大きく，20コピーでは±40%の範囲に推定値が分布した．

各検量点におけるCt値の標準偏差は，20コピーの場合に特に大きく，これが原因となり検量線の変動が大きくなっていると考えられた．しかし，20コピーでのCt値のばらつきは測定系に入るコピーの確率分布に起因すると考えられ，分析法の改良により低減することは不可能である． 20コピーの検量点を除いて作成した検量線では，両端の変動幅は小さくなり測定精度が向上した．

Keywords: real-time PCR, calibration curve, precision

* 静岡県立大学

松田りえ子，渡邉敬浩，五十嵐敦子，白政優子，米谷民雄：**トータルダイエット試料の分析による硝酸塩の摂取量推定**

食品衛生学雑誌，**50**，29-33 (2009)

マーケットバスケット方式によって国内11カ所で調製したトータルダイエット試料を分析し，硝酸塩の摂取量を推定した．推定された摂取量は4.0 mg/kg/dayであり，JECFAの定めたADIを8%超過していた．硝酸塩の主要な摂取源は，7群(有色野菜)および8群(その他の野菜，漬け物，海草)であり，この２群からの摂取量が総摂取量の80%以上を占めていた．個別食品について報告された硝酸塩濃度と，食品の1日摂取量を勘案すると，ホウレンソウからADIの38%，白菜から10%，大根から20%を摂取していると推定された．

Keywords: nitrate, daily intake, total diet sample

河村葉子，六鹿元雄，山内朋子*，植田新二*，棚元憲一：**玩具塗膜からのカドミウムおよび鉛の溶出試験**

食品衛生学雑誌, **50**, 93-96 (2009)

玩具塗膜中のカドミウム（Cd）および鉛（Pb）の規格設定のため，食品衛生法とISO 8124-3の溶出試験法および規格値について比較検討した．塩化ビニル樹脂塗料とアクリル樹脂塗料にCdおよびPbを1,000 mg/kg添加し，ガラス板に塗布乾燥して塗膜を調製した．食品衛生法に従い塗膜1 cm^2あたり2 mLの水を用い40℃ 30分間静置したところ，いずれも溶出は認められなかった（定量限界 各0.1 μg/mL）．また溶媒を4%酢酸や0.07 mol/L塩酸に変更したところ，アクリル樹脂塗料では0.3 ～ 2.3 μg/mLの溶出がみられたが，塩化ビニル樹脂塗料では認められなかった．一方，ISO規格に従い塗膜を削り取って粉砕し，その50倍量の0.07 mol/L塩酸を加えて37℃で１時間振とうし１時間静置したところ，すべての試料で規格値を3.5 ～ 12倍超過する溶出が認められた．ISO規格の試験法は塗膜を粉砕したのち酸性溶媒で溶出するため

溶出力が強く，規格値が高いにもかかわらずより厳しい規格であることが確認された．

Keywords: baby toy, cadmium, lead

* （財）化学物質評価研究機構

Ohmori, K.*, Kawamura, Y. : **Cell transformation activities of abietic acid and dehydroabietic acid: safety assessment of possible contaminants in paper and paperboard for food contact use**

Food Additives and Contaminants Part A, **26**, 568-573 (2009)

Abietic acid (AA) and dehydroabietic acid (DHA) have been detected in virgin paper products and recycled paper products used for food packaging. In order to evaluate the cell transformation activities of AA and DHA, the Bhas 42 cell-transformation assay for initiation and promotion was carried out. Tested in the initiation stage, AA and DHA did not significantly increase transformation frequencies. On the other hand, both chemicals induced transformed foci dose dependently at the promotion stage. The highest transformed foci density induced by AA was about 13 foci/well at 60 nmol/mL, and that of DHA was about 16 foci/well at 40 nmol/mL (solvent control=2.3 ± 1.4 foci/well). The present results suggest that AA and DHA may have tumour-promoting potential.

Keywords: abietic acid, Bhas 42 cell transformation, genotoxicity

* Kanagawa Prefectural Institute of Public Health

建部千絵，河﨑裕美，杉本直樹，佐藤恭子，棚元憲一：**LC/MSによるポリソルベート類の分析**

日本食品化学学会誌, **15**, 129-134 (2008)

LC/MSを用いたポリソルベート（PS）類の新たな確認試験法を開発した．20ポリオキシエチレン（EO）ソルビタン（So）モノ，ジ，トリエステル（FA）（So-EO（20）-FA（1 ～ 3））のNa付加体からなる分子から計算された選択イオンを指標とし，LC/MSによる選択イオンモニタリング（SIM）モードで分析した．その結果，各PSのSIMピークは構成脂肪酸とPSのエステル化の度合いにより，異なる保持時間に溶出した．また，それぞれのSIMピークには異なる長さのEO（12 ～ 35）が確認された．この方法を用いて，PS40のTLC分析で検出されるスポット中の分子種の同定を試みた．分取TLCによって得られたTLCの2つの主スポットをLC/MSで分析したところ，So-EO（20）-モノパルミテートまたはSo-EO（20）-ジパルミテートを主成分として含むことが分かった．また，PS60が添加されたミルクココアの抽出液についてLC/MSによるSIM測定を行ったところ，So-EO（20）-モノステアレート，So-EO（20）-トリステアレートおよびSo-EO（20）-モノパルミテートが含まれていた．

Keyword: polysorbate, LC/MS, oxyethylene (EO)

河﨑裕美，建部千絵，高木繁行，川﨑有記*，原　貴彦*，飯塚太由*，杉本直樹，佐藤恭子，棚元憲一：**食品中のポリソルベートの分析**

日本食品化学学会誌, **15**, 122-128 (2008)

通知分析法を設定するため，食品中のポリソルベート（PS）類のTLCを用いた迅速なスクリーニング検査法および比色定量法を開発した．加工食品より，PSをメタノール含有アセトニトリルで抽出した．抽出液は，色素やその他の妨害物質を除くため，アルミナカラム（酸化アルミナ，塩基性，10 g）とシリカゲルカートリッジ（Sep-Pak Plus Silica，690 mg）を用いて精製した．TLCの最適条件は以下の通り．薄層板，シリカゲル；展開溶媒，ジクロロメタン・メタノール・アセトン・水混液（100 : 20 : 15 : 3）；発色試液，ドラーゲンドルフ試液．PS含量は，PS80を標準品としたチオシアン酸コバルト法により比色定量して求めた．11種類の食品にPS80を0.1 g/kgとなるように添加したときの回収率は，フリーズドライ製品で24.6%と低かったものを除き，48.8 ～ 76.2%であった．本法における定量下限は0.02 g/kgであった．PS60を含むミルクココアに本法を適用し，PS80として0.26 g/kgを検出した．

Keyword: polysorbate, processed food, TLC

* （財）食品環境検査協会

河﨑裕美，古庄紀子，建部千絵，久保田浩樹，柳　徳枝*1，安河内義和*2，森　曜子*2，山下靖子*3，飯塚太由*3，高畑　薫*4，高橋仁一*5，佐藤恭子，棚元憲一：**GC-ECDによる食用赤色104号（フロキシン）および105号（ローズベンガル）中のヘキサクロロベンゼンの分析**

食品衛生学雑誌, **50**, 6-9 (2009)

指定添加物として使用が認められている食用赤色104号（R104）および105号（R105）中のヘキサクロロベンゼン（HCB）の電子捕獲型検出器付きガスクロマトグラフ（GC/ECD）による分析法の検討を行った．色素を水に溶解後，ヘキサンで抽出し，無水硫酸ナトリウムで脱水したものを，試験溶液とした．R104，R105にHCBを2および5 μg/gとなるように添加し，5分析機関で添加回収試験を実施したところ，平均回収率は98.2%～

103.7%，RSD_tは2.9%～6.0%，RSD_Rは4.2%～9.3%であった．また，HORRAT値は1未満と室間再現性は良好であったことから，本分析法の妥当性が確認された．

Keywords: hexachlorobenzene, phloxine, rose bengal

[*1]（財）日本食品分析センター
[*2]（財）冷凍食品検査協会
[*3]（財）食品環境検査協会
[*4]（社）東京都食品衛生協会東京食品技術研究所
[*5] 日本食品添加物協会

多田敦子，杉本直樹，佐藤恭子，秋山卓美，麻野間正晴[*1]，尹　永淑[*2]，山崎　壮，棚元憲一：**既存添加物苦味料ジャマイカカッシア抽出物の成分組成に基づく基原植物の検討**

食品衛生学雑誌, **50**, 16-21 (2009)

ジャマイカカッシア抽出物製品間の成分組成の差異や副成分について検討した．4製品のLC/MS分析を行った結果，主成分に加え副成分の組成もよく一致した．4製品に共通の副成分4種を単離して11-dihydro-12-norneoquassin, canthin-6-one, 4-methoxy-1-vinyl-β-carboline および4,9-dimethoxy-1-vinyl-β-carbolineと同定した. 次に，アメリカニガキおよびニガキの枝から熱水抽出物を調製し，LC/MSにより本製品と成分組成を比較した．その結果，本製品はアメリカニガキ抽出物に類似していた．既存添加物名簿収載品目リストには，ジャマイカカッシア抽出物の基原としてジャマイカカッシアが当てられているが，国際自然保護連合により絶滅危惧種に指定されており，入手困難である.本研究から，我が国に流通する製品の基原はアメリカニガキであることが示唆された．

Keywords: Jamaica quassia extract, food additive, bittering agent

[*1] 名古屋市衛生研究所
[*2] 東京薬科大学

松藤　寛[*1]，佐々怜一郎[*1]，本間友輝[*1]，宮島拓臣[*1]，千野　誠[*1]，山崎　壮，島村智子[*2]，受田浩之[*2]，松井利郎[*3]，松本　清[*3]，山形一雄[*1]：**抗酸化物質の2成分混合系におけるDPPHラジカル消去活性**

日本食品科学工学会誌, **56**, 129-136 (2009)

DPPHラジカル消去活性測定法を用いて，2成分間の活性に及ぼす効果（相乗効果，相加効果，相殺効果）について検討した．11種の酸化防止剤55通りの組み合わせでは，36通りにおいて統計上相乗効果，1通りで相殺効果と判定される結果が得られた．一方，24種の化合物276通りの組み合わせ（うち15通りは重複）では，74通りにおいて相乗効果, 61通りで相殺効果が得られた．しかし，これらの多くの組み合わせによる効果は弱く，相加効果をわずかに上回る，あるいは下回る程度であり，2割以上の活性増強が認められた組み合わせは14通り，2割以下の活性低下が認められた組み合わせは33通りであった．一方，α-トコフェロールとの組み合わせのうち6通りで，*p*-クマル酸との組み合わせのうち4通りで2割以上の活性増強が観察され，バニリン酸との組み合わせのうち17通りで，*p*-クマル酸との組み合わせのうち12通りで2割以下の活性低下が観察された．

Keywords: antioxidant activity, synergy, antagonism

[*1] 日本大学
[*2] 高知大学
[*3] 九州大学

六鹿元雄，山口未来，河村葉子，棚元憲一：**瓶詰食品中のセミカルバジドの分析**

日本食品化学学会誌, **15**, 67-72 (2008)

食品中のセミカルバジド（SEM）の定量法を確立し，我が国で流通する瓶詰食品中のSEM含有量について調査を行った．併せてキャップシーリング中の含有量についても調査を行い，それらの関連性を検討した．瓶詰食品64検体についてSEM含有量を測定した結果，32検体から0.6 ～ 46.7 μg/kg検出された．そのうち，ベビーフードでは11検体中9検体から5.7 ～ 46.7 μg/kg検出され，欧州の調査結果ともよく一致していた．一般食品では53検体中23検体から0.6 ～ 45.2 μg/kg検出された．このレベルは欧州の調査結果と比べてやや高かった．SEMが検出されたすべての瓶詰食品では，シーリングからSEMまたはその親化合物であるヒドラゾジカルボンアミドが検出された．このことから，食品中のSEMはシーリングに添加されたアゾジカルボンアミド由来であることが確認された．

Keywords: sealing gasket, semicarbazide, azodicarbonamide

Mutsuga, M., Kawamura, Y., Tanamoto, K. : **Migration of lactic acid, lactide and oligomers from polylactide food contact materials**

Food Additives and Contaminants Part A, **25**, 1285-1292（2008）

Polylactide (PLA) is used for manufacturing lunch boxes and for packaging fresh food in Japan. PLA can be hydrolyzed relatively easily to produce lactic acid, lactide and oligomers. Different types of PLA sheet were subjected to migration tests under various conditions and the lactic acid, lactide and oligomers contents of the migration solutions

were determined using LC/MS. Furthermore, the change in molecular weight was determined by a migration test. PLA was stable at 40℃ for 180 days, the total of lactic acid, lactide and oligomers migration levels were 0.28–15.00 μg/cm^2. PLA decomposed clearly at 60℃ for only 10 days, the total migration levels were increased to 0.73–2840 μg/cm^2. PLA sheets with a high D-lactic acid content decomposed particularly rapidly. The amount of alkali decomposition products, based on the conversion of lactide and oligomers to lactic acid by alkali hydrolysis, corresponded with the total migration levels.

Keywords: polylactide, lactic acid, lactide

Boonmar, S.[*1], Markvichitr, K.[*1], Chaunchom, S.[*1], Chanda, C.[*2], Bangtrakulnonth, A.[*3], Pornrunangwong, S.[*3], Yamamoto, S., Suzuki , D.[*4], Kozawa, K.[*4], Kimura, H.[*5], and Morita, Y.[*4] : **_Salmonella_ prevalence in slaughtered buffaloes and pigs and antimicrobial susceptibility of isolates in Vientiane, Lao People's Democratic Republic**

J. Vet. Med. Sci., **70**(12), 1345-1348(2008)

This is the first report regarding isolation of *Salmonella* from cecum samples of buffaloes and pigs and characterization of the isolates in Laos. The organisms were isolated from 8% (4/50) of buffaloes and 76% (37/49) of pigs. In buffaloes, 3 animals harbored serotype 9, 12: -:1,5, and 1 animal harbored both S. Derby and S. Javiana. In pigs, the most predominant serotypes were S. Derby (51%) followed by S. Anatum (45%), S. Weltevrenden (15%) and S. Stanley (5%). The buffalo isolates were susceptible to the antimicrobials tested, whereas the pig isolates showed 10 resistance patterns to 1-5 antibiotics. Of the 59 pig isolates, the resistance rates to tetracycline, streptomycin, ampicillin, sulfamethoxazole-trimethoprim, chlorramphenicol, amoxicillin-clavulanic acid and malidixic acid were 24%, 22%, 14%, 5%, 2%, 2% and 2%, respectively. The results suggest that pigs and buffaloes harbor Salmonella, with a higher prevalence especially in pigs, and all the isolates showed sensitivity to cefotaxime, norfloxacin and ciprofloxacin.

Keywords:buffaloes, Lao People's Democratic Republic (Laos), *Salmonella*

[*1] カセサート大学（タイ）
[*2] ラオス国立大学
[*3] タイNIH
[*4] 群馬県環境衛生研究所
[*5] 国立感染症研究所

Boonmar, S.[*1], Chanda, C.[*2], Markvichitr, K.[*1], Chaunchom, S.[*1], Yingsakmongkon, S.[*1], Yamamoto, S., and Morita, Y[*3] : **Prevalence of *Campylobacter spp.* in slaughtered cattle and buffaloes in Vientiane, Lao People's Democratic Republic**

J. Vet. Med. Sci., **69**(8), 853-855(2007)

This is the first report regarding isolation of *Campylobacter* in caecum and bile samples obtained from ruminants in Vientiane, Lao PDR. Campylobacter was isolated from 3 (1.6%) of the 184 caecum samples and 1 (1.0%) of the 100 bile samples obtained from buffaloes. Three of the 4 isolates were determined to be *C. jejuni*, which was detected in 2 caecum samples and 1 bile sample; the other caecum sample contained *C. fetus*. *Campylobacter* was not isolated from any of the 82 cattle caecum samples. Our results suggest that cattle and buffaloes may not be important sources of *Campylobacter* food poisoning in Lao PDR.

Keywords : *Campylobacter*, Lao People's Democratic Republic (PDR), ruminant

[*1] カセサート大学
[*2] ラオス国立大学
[*3] 群馬県環境衛生研究所

Chandra, JHA, V.[*1], Morita, Y.[*2], Dhakal, M.[*1], Besnet, B.[*1], Sato, T.[*3], Nagai, A.[*2], Kato, M.[*2], Kozawa, K.[*2], Yamamoto, S., and Kimura, H.[*4]. : **Isolation of Mycobacterium spp. from milking buffaloes and cattle in Nepal**

J. Vet. Med. Sci., **69**(8), 819-825(2007)

In Nepal, mycobacterial isolates obtained from the milk and feces of buffaloes and cattle that were positive for the single intradermal cervical tuberculin (SICT) tests were genetically identified. A total of 36 mycobacterila strain were isolated from 39% of the buffaloes (14 of 36) and 34% of the cattle (11 of 32). Of the 36 strains, 13 were identified as *M. bovis*, and these strains were isolated and from 17% of the buffaloes (6 of 36) and 16% of the cattle (5 of 32). *M. bovis* was isolated from both the milk and feces of one buffalo and one cattle, the milk alone of three buffaloes and three cattle, and the feces alone of two buffaloes and one cattle. These results suggest that milking buffaloes and cattle infected with M. bovis exist in Nepal. The remaining 23 strains were atypical mycobacteria. A

program for the elimination of bovine tuberculosis should be implemented as soon as possible, and the public health education and proper hygienic practices may be required.

Keywords : buffalo, mycobacteria, Nepal

*1 ネパール農務省
*2 群馬県環境衛生研究所
*3 JICAネパール
*4 国立感染症研究所

Toyota-Hanatani, Y.*1, Inoue, M.*2, Ekawa, T.*1, Ohta, H.*1, Igimi, S., and Baba, E.*3 : **Importance of The Major Fli C Antigenic Site of *Salmonella* Enteritidis as A Subunit Vaccine Antigen**

Vaccine, **26**(33), 4135-4137 (2008)

Our previous study indicated that the antibody against the major antigenic site of SE Fli C (g.m. region) is characteristically produced after the application of SE bacterin, however, the antibody is not produced in chickens after SE infection. In the present study, we determined histologically if the major antigenic site could be a candidate antigen for SE subunit vaccine. When Layermune SE, a commercial SE bacterin, was injected subcutaneously into the shoulder region as a positive control, the following histological changes were observed: formation of epithelioid granuloma with epithelioid cells and multinuclear giant cells surrounding necrotic sites and oil cysts (Indicator 1); a perivascular accumulation of lymphocytes near the granulation tissue (Indicator 2); peripheral fibroplasia encapsulating the granulation tissue (Indicator 3). On the other hand, at the injection site from the incomplete Freund adjuvant as a negative control antigen, there was only hyperplasia of the connective tissues around oil cysts. By using these indicators, the histological changes induced by injection of major antigenic site (SEp9) of Fli C, Fli C, and SE somatic antigen were evaluated. Histological changes after the injection with SEp9 demonstrated Indicators 2 and 3. The injection with SE Fli C demonstrated all three indicators. Contrarily, de-flagellated SE antigen injection induced only Indicator 3. The present results suggest that the antigen g.m. site of SE Fli C (SEp9) may play an important role as a subunit vaccine not only for including continuous immunological reaction in SE infection in chickens but also for antigen presentation.

Keywords : *Salmonella*, vaccine antigen, flagella

*1 CAF Laboratories
*2 Yamaguchi University
*3 Osaka Prefecture University

Toyota-Hanatani, Y.*1, Ekawa, T.*1, Ohta, H.*1, Igimi, S., Hara-Kudo, Y., Sasai, K.*2, Baba, E.*2 : **An Assessment of Inactivated *Salmonella enterica* Serovar Enteritidis (SE) Vaccine Treatment in Layer Flocks with Regard to Public Health**

Appl. Environment. Microbiol., **75**, 1005-1010 (2009)

Although there have been several reports on the efficacy assessment of a *Salmonella enterica* serovar Enteritidis vaccine against intestinal and parenchymatous organ diseases of laying hens, no public health risk characterization of its long-term effect on eggs has been reported. In this study, we attempted to assess the public health effect of an inactivated *S. enterica* serovar Enteritidis vaccine against serovar Enteritidis contamination of chicken eggs. We analyzed serovar Enteritidis isolation test results from four windowless farms in which inactivated-vaccine administration was initiated based on the sanitary monitoring program of a farm. When flocks with and without *S. enterica* serovar Enteritidis vaccine treatments were mixed, the application of an inactivated serovar Enteritidis vaccine decreased the most probable number (MPN) of bacteria by at least 100-fold in broken (liquid) egg samples positive for serovar Enteritidis, although a statistical difference between those MPNs could not be obtained. The isolation frequency after the vaccine application was less than 1/10 ($P < 0.01$). No *S. enterica* serovar Enteritidis bacteria were isolated approximately 1 year after all of the chickens had received the inactivated serovar Enteritidis vaccine. It was suggested that an adequate administration of an inactivated serovar Enteritidis vaccine reduced the contamination risk of eggs (the number of isolated serovar Enteritidis cells and detection frequency) compared to the contamination risk of eggs laid by nonvaccinated hens.

Keywords : *Salmonella* Enteritidis, vaccine, layer flock

*1 CAF Laboratories
*2 Osaka Prefecture University

Takeshi, K.*1, Kitagawa, M.*2, Kadohira, M.*1, Igimi, S., and Makino, S.*1 : **Hazard analysis of *Listeria monocytogenes* contaminations in processing of salted roe from Walleye Pollock (Theragra chalcogramma) in Hokkaido, Japan**

J. Vet. Med. Sci., **71,** 87-91 (2009)

Hazard analysis of *Listeria monocytogenes* contamination during processing of salted walleye pollock (Theragra chalcogramma) roe was performed for a seafood plant in Japan from December 2005 to February 2006. As a result, *L. monocytogenes* number was detected on the pallet used for transport of barrels in the salting process and one of the rollers of the roller conveyor, which rotates while in contact with the bottoms of the barrels, but was not detected in any raw materials, interim products or final products. Thus, we believe that the pallet contamination initially occurred because of insufficient washing, that it was passed on to the bottoms of the barrels and that it was then passed on the roller of the roller conveyor by cross-contamination. Therefore, it is possible that interim and final products may become contaminated by processing devices and machinery. In addition, we conducted an inoculation study designed at the 1/20 actual factory scale using interim products with or without artificial color and seeded with *L. monocytogenes* to observe changes in its growth. In the inoculation study, multiplication of *L. monocytogenes* during the salting process was not confirmed in the samples with artificial color.

Keywords : *Listeria monocytogenes*, salted walleye pollock roe, hazard analysis

[*1] Obihiro University of Agriculture and Veterinary Medicine

[*2] Hokkaido Kushiro Fisheries Experiment Station

Kajikawa, A. and Igimi, S. : **Reduction of TNF-α Inducing Capacity of Recombinant *Lactobacillus casei* Caused by the Expression of Salmonella OmpC**

Appl. Environment. Microbiol., **75**(9), 2727-2734 (2009)

The insertion of a heterologous gene into commensal bacteria is a common technique to develop a delivery agent for vaccination and therapies, but the pleiotropic effects of genetic modifications need to be investigated before its use in practical applications. Although supplemental properties provided by the expression of heterologous antigens have been reported, the negative or side effects on the immune-modulating properties caused by recombination are barely understood. In the present study, we fortuitously found that the secretion of tumor necrosis factor alpha (TNF-α) from murine macrophages was reduced by recombinant *Lactobacillus casei* expressing *Salmonella* OmpC compared to the stimulation of TNF-α secretion by nonexpressing *L. casei*. This reduction could not be attributed to OmpC as a purified protein. The main component of the OmpCexpressing strain included in the attenuation of TNF-α release seemed to be the cell wall, which exhibited higher sensitivity against N-acetylmuramidase than that of nonexpressing strains. These results suggest that the recombinant strain expressing a specific heterologous antigen might be digested rapidly in macrophages and lose immune-stimulating capability at an early time point.

Keywords : recombinant, tumor necrosis factor alpha, *Lactobacillus casei*

松村浩介[*]，清水晃[*]，河野潤一[*]，五十君靜信：**畜水産食品からの黄色ブドウ球菌検出のための選択分離培地および選択増菌培地の検討**

日本食品微生物学会雑誌, **26**(1), 23-27 (2009)

わが国の黄色ブドウ球菌の試験法は，海外で広く用いられている試験法と比べると，用いられている選択分離培地の種類が異なっていることや，ISO法やBAM法のような規格化されたプロトコールとなっていないなどの指摘がある．食品流通がグローバル化する現状では，国際的に互換性がある黄色ブドウ球菌試験法を整備する必要がある．このため，筆者らは市販の食肉類およびその加工品と魚介類を用いて，直接平板培養法によるMSEY培地とBP培地の検出比較，選択増菌培養法における増菌培地の食塩添加濃度と培養時間について検討した．

Keywords : *Staphylococcus aureus*, selective media, meat

[*] 神戸大学

Asakura, H., Kawamoto, K[*]., Haishima, Y., Igimi, S., Yamamoto, S., and Makino, S.[*1] : **Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state**

Res. Microbiol., **159**, 709-717 (2008)

During an outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157, we showed previously that food isolates were resistant to oxidative stress, while patient isolates were sensitive to it. Because food isolates increased stress-sensitivity after mouse passage, this change most likely occurred during passage through patients. Here we demonstrate that the phenotypic change occurring during mouse passage correlates with the stress response of outer membrane protein W (OmpW) in EHEC O157 strains. Upon induction of oxidative stress, OmpW was highly

expressed only in the stress-sensitive MP37 strain, obtained by mouse passage of food strain F2, but not in the F2 strain. Western blotting confirmed that expression of OmpW was induced in the viable but non-culturable (VBNC) state. Deletion of ompW in the MP37 strain increased recovery from dormancy, while overexpression of OmpW in the F2 strain decreased recovery when exposed to oxidative stress, suggesting that high levels of OmpW sensitize the bacteria to stress. DNA alignment revealed that the class I integron (int1I) fragments flanking the ompW gene are oriented in opposite directions between stress-resistant and -sensitive strains. All stress-sensitive strains induced ompW under stress. We propose that the different stress response of OmpW was introduced by genetic alteration during in vivo passage.

Keywords: enterohemorrhagic *Escherichia coli*, outer membrane protein, stress response

* Obihiro University of Agriculture and Veterinary Medicine

Okada, Y., Makino, S.[*1], Okada, N.[*2], Asakura, H., Yamamoto, S., and Igimi, S. : **Identification and analysis of the osmotolerance associated genes in *Listeria monocytogenes***

Food Additives and Contaminants, **15**, 1-6 (2008)

Listeria monocytogenes, the causative agent of listeriosis, has strong osmotolerance and is able to grow in severe circumstance. Many studies about mechanisms of listerial osmotolerance were performed, however, there are some parts remaining unknown. In previous studies, we constructed two kinds of mutant in *L. monocytogenes* EGD strain to analyze the mechanisms of osmotolerance in *L. monocytogenes* by molecular genetical methods. In this paper, we summarized the genetical studies of osmotolerance in this bacterium by many researchers and ourselves, as a proceeding of UJNR symposium in last November. First, we constructed a transposon-insertional mutant strain that showed reduced growth in high osmotic agar compared with the parental strain. The results of cloning and sequencing analysis showed that *rel* gene, which encodes guanosine tetra- and pentaphosphate synthesis and hydrolysis protein involves in osmotolerance in *Listeria monocytogenes*. Next, we examined the expression levels of 5 sigma factor coding genes in *L. monocytogenes* using real-time PCR, and found that *rpoN* gene (the alternative sigma factor RpoN (sigma54) encoding gene) was activated under high osmotic condition. We constructed a deletion mutant of *rpoN* and analyzed its response to osmotic stress. In minimal medium with NaCl and carnitine, an osmoprotectant, the mutant showed deficient growth to that of the parental strain, when the starting optical density was high, though the expression level of carnitine transporter operon, *opuC*, and the rate of carnitine uptake in the mutant was similar to that of EGD. These results suggest that the *rpoN* mutant may needs larger amounts of carnitine might be needed for its growth under high osmolarity. Through the analysis of these mutants, we obtained the new insights of osmotolerance in *L. monocytogenes*.

Keywords: *Listeria monocytogenes,* osmotolerance

[*1] 帯広畜産大学
[*2] 北里大学

Suzuki, H. and Yamamoto, S. : **A Literature Survey of *Campylobacter* Contamination in Retail Poultry Meats and By-Products in the World**

Proceedings, The 15th Congress of FAVA FAVA-OIE Joint Symposium on Emerging Diseases, 51-52, (2008)

Campylobacter species are common bacterial pathogens associated with human gastroenteritis worldwide. In North America, Europe and Japan, campylobacteriosis is one of the leading food-borne bacterial illnesses and the consumption of poultry meats and/or by-products is suspected a major cause of the illness. In this study, we performed the literature survey of *Campylobacter* contamination in retail poultry meats and by-products worldwide for comparing the contamination levels among the areas or countries. In most of the countries, a majority of retail poultry meats and by-products were contaminated with *Campylobacter* spp. *C. jejuni* was usually the dominant *Campylobacter* species isolated from retail poultry and *C. coli* was less frequently isolated, although the ratio of *C. coli* to *C. jejuni* was considerably different among the countries. Especially in Thailand and South Africa, *C. coli* was the dominant *Campylobacter* species isolated from retail poultry, although the reasons are not certain. A large portion of retail poultry was contaminated with *Campylobacter* spp. in the world; therefore, proper countermeasures are required together with the sanitary handlings of poultry products.

Keywords:*Campylobacter*, poultry meats and by-products, world

Yamamoto, A.[*1], Iwahori, J.[*2], Vuddhakul, V.[*3], Charernjiratragul, W.[*3], Vose, D.[*4], Osaka, K.[*5], Shigematsu, M.[*6], Toyofuku, H., Yamamoto, S., Nishibuchi, M.[*7], and Kasuga, F. : **Quantitative modeling for risk assessment of Vibrio parahaemolyticus in bloody clams in southern Thailand**

Int. J. Food Microbiol., **124**, 70-8 (2008)

A risk assessment of *Vibrio parahaemolyticus* in bloody clams (Anadara granosa) consumed in southern Thailand was conducted. This study estimated the prevalence and concentration of pathogenic *V. parahaemolyticus* in bloody clams at harvest and retail stages; and during this process, methods to detect the total and pathogenic *V. parahaemolyticus* were investigated. Consumption of bloody clams and cooking efficiency were studied using interviews and onsite observation of consumers. A beta-Poisson dose–response model was used to estimate probability of illness applying estimation methods for the most likely parameter values presented by USFDA. Microbial and behavioral data were analyzed by developing a stochastic model and the simulation gave a mean number of times a person would get ill with *V. parahaemolyticus* by consuming bloody clams at 5.6×10^{-4}/person/year. Sensitivity analysis demonstrated the fraction of people who did not boil the clams properly was the primary factor in increasing risk. This study serves as an example of how a microbiological risk assessment with limited data collection and international cooperation leads to valuable local insight.

Keywords: quantitative microbiological risk assessment, *Vibrio parahaemolyticus*, bloody clams

[*1] Hyogo Prefectural Institute of Public Health and Environmental Sciences
[*2] Kochi Medical School
[*3] Prince of Songkla University
[*4] BVBA Vose Consulting
[*5] Tohoku University
[*6] National Institute of Infectious Diseases
[*7] Kyoto University

Kumagai, S.[*1], Nakajima, M.[*2], Tabata, S.[*3], Ishikuro, E.[*4], Tanaka, T.[*5], Norizuki, H.[*6], Itoh, Y., Aoyama, K.[*4], Fujita, K.[*7], Kai, S.[*8], Sato, T.[*9], Saito, S.[*1], Yoshiike, N.[*10], and Sugita-Konishi, Y. : **Aflatoxin and ochratoxin A contamination of retail foods and intake of these mycotoxins in Japan**

Food Additives & Contaminants: Part A, **25**(9), 1101-1106(2008)

A survey was undertaken of aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), ochratoxin A (OTA), and fumonisin B1 (FB1), B2 (FB2) and B3 (FB3) contamination of various retail foods in Japan during 2004-05. The mycotoxins were analysed by high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/ MS) or high-performance thin-layer chromatography (HPTLC). Aflatoxins (AFs) were detected in ten of 21 peanut butter and in 22 of 44 bitter chocolate samples; the highest level of AFB1, 2.59 μg kg-1, was found in peanut butter. Aflatoxin contamination was not observed in corn products (n = 55), corn (n = 110), peanuts (n = 120), buckwheat flour
(n = 23), dried buckwheat noodles (n = 59), rice (n = 83) or sesame oil (n = 20). OTA was detected in 120 out of 192 samples of oatmeal, wheat flour, rye, buckwheat flour, raw coffee, roasted coffee, raisin, beer, wine and bitter chocolate, but not in rice or corn products. OTA levels in the positive samples were below 13 μg kg-1. AFs and OTA intakes through the consumption of foods containing cacao were estimated using the data for mycotoxin contamination in bitter chocolate and those for the consumption of foods containing cacao in Japan.

Keywords: aflatoxin, ochratoxin A, retail foods

[*1] 東京大学大学院農学生命科学研究科
[*2] 名古屋市衛生研究所
[*3] 東京都立健康科学研究センター
[*4] （独）飼肥料検査所
[*5] 神戸市環境保健研究所
[*6] （財）日本穀物検定協会
[*7] （財）日本食品分析センター名古屋支所
[*8] 神奈川県衛生研究所
[*9] 北里大学
[*10]（独）国立健康・栄養研研究所

Shinkawa, N.[*1], Noda, M., Yoshizumi, S.[*2], Tokutake, Y.[*3], Shiraishi, T.[*4], Arita-Nishida, T.[*4], Nishio, O.[*4], Oka, T.[*5], Hansman, G.S.[*5], Takeda, N.[*5], and Kimura, H. [*4] : **Molecular Epidemiology of noroviruses detected in food handler-associated outbreaks of gastroenteritis in Japan**

Intervirol., **51**, 422-426 (2008)

Twelve outbreaks of food handler-associated gastroenteritis between November 2002 and March 2006 in Japan were examined for norovirus (NoV) using RT-PCR and

sequence analysis. NoV was detected in 77 of 81 customers and 45 of 104 food handlers. Identical NoV sequences were detected in patients and food handlers in each outbreak.

Keywords: norovirus, foodborne outbreak, food handler

[*1] Kagoshima Prefectural Institute for Environmental Research and Public Health
[*2] Hokkaido Institute of Public Health
[*3] Nagano Environmental Conservation Research Center
[*4] Infectious Disease Surveillance Center, National Institute of Infectious Diseases
[*5] Department of Virology II, National Institute of Infectious Diseases

Hansman, G.S.[*1], Oka, T.[*1], Li, T.C.[*1], Nishio, O.[*2], Noda, M., and Takeda, N.[*1] : **Detection of Human Enteric Viruses in Japanese Clams**

J. Food Protect., **71**, 1689-1695 (2008)

A total of 57 clam packages that were collected from supermarkets and fish markets from 11 different sites in western Japan between 8 December 2005 and 6 September 2006 were examined for human enteric viruses (i.e., norovirus, Aichi virus, rotavirus, adenovirus, hepatitis A virus, and astrovirus), using PCR and reverse transcription PCR. Sixty-one percent of the packages were contaminated with one type of virus, 9% had two different types of viruses, 28% had three different types of viruses, and 9% had at least four different types of viruses. Thirty-one (54%) of 57 packages were contaminated with noroviruses. Norovirus genogroup I and genogroup II sequences were detected in 24 and 23 packages, respectively, and these sequences belonged to nine genogroup I and eight genogroup II genotypes. Aichi viruses were found in 19 (33%) of 57 packages, and these belonged to genogroup A. Rotaviruses (group A) were detected in 14 (42%) of 33 of packages and 9 of 14 rotavirus-positive packages contained two or more rotavirus genogroup types. Adenoviruses (Ad40 and Ad41) were detected in 17 (52%) of 33 packages. One of the 57 (2%) packages was positive with hepatitis A virus (subtype IA). Astrovirus was not detected in any of the packages. This is the first study to detect such a high level of contamination in Japanese clams. These results represent an important finding because the Japanese clams were considered suitable for human consumption. Further studies are needed to determine the health risks associated with eating these highly contaminated clams.

Keywords: human enteric ciruses, clam, PCR

[*1] Department of Virology II, National Institute of Infectious Diseases
[*2] Infectious Disease Surveillance Center, National Institute of Infectious Diseases

有田知子*，木村博一*，野田衛，西尾治*：**パンに含まれるノロウイルスの回収法の検討**

感染症学雑誌, **82**, 473-475 (2008)

学校給食におけるノロウイルス食中毒事件では，疫学的に原因食がパンと推定される事例が認められるがパン自体からノロウイルスを検出した事例は稀である．本研究では，パンからのノロウイルス検出法を確立することを目的として，学校給食で提供される機会が最も多いコッペパンを用いてαアミラーゼで消化することで，付着しているノロウイルスを回収する実験系を検討し，回収率を向上させることができた．

Keywords: norovirus，bread，PCR

* 国立感染症研究所感染症情報センター

阿部勝彦[*1]，国寄勝也[*1]，島本琢士[*2]，国井悦子[*1]，山本美和子[*1]，伊藤文明[*1]，野田　衛，池田義文[*1]，笠間良雄[*1]：**2006年5月～2008年4月に広島市で流行したノロウイルスGII/4の分子疫学について**

広島市衛研年報, **27**, 35-40 (2008)

2006 年5 月～2008 年4 月に発生した食中毒，有症苦情事例，集団感染症事例等52 事例からノロウイルスが検出され，そのうちGII/4 は45件であった．NV の遺伝子解析にはこれまで厚労省通知にあるORF2 上流の保存領域での解析を行ってきた．今回，ORF2 の可変領域の解析を行ったところ，保存領域では同一配列株と思われたGII/4 が可変領域では異なるクラスターに分類される株であることがわかった．また，流行株は3～4 ヶ月の間で移り変わっていた．

Keywords: norovirus，GII/4，molecular epidemiology

[*1] 広島市衛生研究所
[*2] 広島市水道局

Yamamoto, M.[*], Abe, K.[*], Kuniyori, K.[*], Kunii, E.[*], Ito, F.[*], Kasama, Y.[*], Yoshioka, Y.[*], Noda, M. : **Epidemic of human parechovirus type 3 in Hiroshima City, Japan in 2008**

Jpn. J. Infect. Dis., **62**, 244-245 (2009)

In the summer of 2008, an epidemic of human parecho-

virus type 3 (HPeV-3) was observed in Hiroshima City, Japan. HPeV-3 was isolated from 40 patients associated with various kinds of diseases. Thirty-two patients (80%) were infants under the age of 4 months. The main clinical symptom was fever and 15 patients (38%) had only fever without other apparent symptoms. The HPeV-3 isolates were genetically close to each other (99.3 to 99.7% homologies) in the VP1 region compared and they showed 95.0 to 96.0% homologies to the HPeV-3 reference strain used.

Keywords: human parechovirus type 3, infant infection, fever

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* Hiroshima City Institute of Public Health

Mochizuki, N*., Hoshino, M*., Suga, K*., Sugita-Konishi, Y. : **Identification of an interfering substrate in apple juice and improvement for determination of patulin with high-performance liquid chromatography analyses**

J Food Prot., **72**, 805-9 (2009)

An interfering substance that is not 5-hydroxymethylfurfural appears in some apple juices during high-performance liquid chromatography (HPLC) analysis of patulin based on the AOAC 995.10 method. Because this interfering substance could cause the overestimation of patulin in the apple juices, we tried to identify the substance and to develop an improved method of analyzing patulin free from the influence of this substance. We isolated the substance from the apple juice and identified it as adenosine based on its mass spectrometry, proton nuclear magnetic resonance, and photo diode array spectra. Because of the chemical properties of adenosine, changes in the extraction method under acidic conditions and the HPLC conditions (wavelength and analytical column) were effective for avoiding the influence of adenosine and more specifically for analyzing the patulin. The most effective and simple improvement of the official method was the use of column in-point carbon contents greater than 15.5%.

Keywords: HPLC, apple juice, patulin, AOAC 995.10 method

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* アサヒビール（株）

Tamura, N*., Yoshida, T*., Miyaji, K*., Sugita-Konishi, Y., Hattori, M*. : **Inhibition of infectious diseases by components from Aloe vera**

Biosci Biotechnol Biochem ., **73**, 950-953 (2009)

The ability to eliminate Escherichia coli K-12 from the peritoneal cavity in the early stage of infection (48 h) was improved by the pre-administration of an aloe sample to BALB/c mice. Our results suggest that the aloe sample could inhibit infectious diseases by stimulating the host defense mechanism, especially the phagocytic and killing activities of macrophages.

Keywords: Escherichia coli K-12, aloe, phagocytic, killing activities, macrophages

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* 東京農工大学農学部

Yaguchi, A*1., Yoshinari, T*1., Tsuyuki, R*1., Takahashi, H*2., Nakajima, T*3., Sugita-Konishi, Y., Nagasawa, H*1., Sakuda, S*1. : **Isolation and identification of precocenes and piperitone from essential oils as specific inhibitors of trichothecene production by *Fusarium graminearum***

J Agric Food Chem., **57**, 846-851 (2009)

Inhibitors of deoxynivalenol production by *Fusarium graminearum* are useful for protecting crops from deoxynivalenol contamination. We isolated precocenes and piperitone from the essential oils of Matricaria recutita and Eucalyptus dives, respectively, as specific inhibitors of the production of 3-acetyldeoxynivalenol, a biosynthetic precursor of deoxynivalenol. Precocenes I and II and piperitone inhibited 3-acetyldeoxynivalenol production by F. graminearum in a liquid culture with IC(50) values of 16.6, 1.2, and 306 microM, respectively, without inhibiting fungal growth. Precocene II also inhibited deoxynivalenol production by the fungus in a solid culture on rice with an IC(50) value of 2.0 ppm. Precocene II and piperitone decreased the mRNA levels of Tri4, Tri5, Tri6, and Tri10 encoding proteins required for deoxynivalenol biosynthesis.

Keywords: deoxynivalenol, 3-acetyldeoxynivalenol, biosynthetic precursor, Precocenes I and II

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*1 東京大学大学院
*2 千葉市衛生研究所
*3 九州沖縄農研

Poapolathep, A*1., Poapolathep, S*1., Jermnak, U*1., Imsilp, K*1., Wannapat, N*1., Sugita-Konishi, Y., Kumagai, S*2. : **Muscle tissue kinetics of oxytetracycline following intramuscular and oral administration at two dosages to giant freshwater shrimp (*Macro-***

***brachium rosenbergii*)**

J Vet Pharmacol Ther., **31**, 517-22 (2008)

The giant river shrimp (*Macrobrachium rosenbergii*), a native species of Thailand, is either exported for commercial purposes or supplied to meet the local requirements in Thailand. Limited pharmacokinetic information of the major antibiotic, oxytetracycline (OTC), is available for this freshwater shrimp. The purpose of the present study was to investigate the muscle tissue kinetics of OTC in M. rosenbergii following either intramuscular (i.m.) or oral (p.o.) administration at two dosages of 11 and 22 mg/kg body weight (b.w.). The concentration of OTC in shrimp tissues was measured using high-performance liquid chromatography (HPLC) equipped with a fluorescence detector. Muscle tissue concentrations were below the detection limit (LOD, 0.1 microg/g) after 96 and 120 h, following i.m. and p.o. administration, respectively. Peak muscle concentrations (C(max)) were 3.47 and 1.73 microg/g after i.m. and p.o. administration at a single dose of 11 mg/kg b.w. whereas they were 6.03 and 2.51 microg/g at a single dose of 22 mg/kg b.w., respectively. A noncompartment model was developed to describe the pharmacokinetics of OTC in the giant freshwater shrimp. The terminal half-lives of OTC were 28.68 and 28.09 h after i.m. and p.o. administration at a single dose of 11 mg/kg b.w., but 29.95 and 27.03 h at a single dose of 22 mg/kg b.w., respectively. The relative bioavailability was 82.32 and 64.67% following i.m. and p.o. administration, respectively. Based on the pharmacokinetic data, i.m. and p.o. administration with OTC at a dose of 11 mg/kg b.w. would be appropriate for use in giant freshwater shrimp farming. To avoid the OTC residue in shrimp muscle, it should take at least seven half-lives (8 days) to wash out the drug from the muscle of *M. rosenbergii*.

Keywords: *Macrobrachium rosenbergii*, shrimp,oxytetracycline

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[*1] カセサート大学

[*2] 東京大学大学院

Poapolathep, A[*1]., Poapolathep,S [*1]., Klangkaew, N[*1]., Sugita-Konishi, Y., Kumagai, S[*2]. : **Detection of deoxynivalenol contamination in wheat products in Thailand**

J Food Prot.. **71**, 1931-1933 (2008)

A total of ninety samples in three kinds of wheat products (30 noodle, 30 bread, and 30 cereal samples) were collected from the supermarkets in Bangkok, Thailand, from February to April 2007. The occurrence of deoxynivalenol (DON) contamination in wheat products was investigated using high-performance liquid chromatography equipped with a UV light detector. The extraction method was performed using a multifunctional cleanup column. The limit of quantification was 0.10 microg x g(-1) from the range obtained in a linear calibration. The survey found almost 94% of the DON-contaminated samples below 1 microg x g(-1), which corresponds to the U.S. Food and Drug Administration advisory level. DON was detected in 18.9% (17 of 90) of all samples, in 6.67% (2 of 30) and 16.67% (5 of 30) of noodle and bread samples at levels from 0.17 to 0.35 and 0.14 to 1.13 microg x g(-1), respectively, while it was in 33.33% (10 of 30) of cereal samples at levels from 0.13 to 0.39 microg x g(-1). The results suggest that the exposure to DON from the consumption of wheat products, especially noodles, bread, and cereal, is at a very low risk level.

Keywords: occurrence, wheat products , noodle, bread, cereal, Thailand, deoxynivalenol

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[*1] カセサート大学

[*2] 東京大学大学院

Sugita-Konishi, Y., Kubosaki, A., Takahashi, M., Park, BJ., Tanaka, T[*]., Takatori, K., Hirose, M., Shibutani, M. : **Nivalenol and the targeting of the female reproductive system as well as haematopoietic and immune systems in rats after 90-day exposure through the diet**

Food Addit Contam Part A Chem Anal Control Expo Risk Assess., **25**, 1118-1127 (2008)

Nivalenol (NIV) is considered to be an important trichothecene mycotoxin produced by Fusarium species because of its frequent contamination in wheat and barley worldwide. The present study examined the subchronic toxicity of NIV in male and female F344 rats fed diets containing 0, 6.25, 25 and 100 mg kg(-1) of the toxin for 90 days. During the experimental period there was a decrease in the white blood cell count at 100 mg kg(-1) in males and at > or =6.25 mg kg(-1) in females. Histopathologically, treatment-related changes were observed in the haematopoietic and immune systems in both sexes and in the female reproductive system at 100 mg kg(-1). Flow cytometric analysis of splenic cells revealed an elevation in the ratio of helper/cytotoxic T-lymphocytes at 100 mg kg(-1). In summary, NIV targets the female reproductive system

as well as haematopoietic and immune systems in rats fed NIV for 90 days. Based on a significant decrease in white blood cells in female rats relative to controls, the lowest observable effect level was calculated as 0.4 mg kg(-1) body weight day(-1).

Keywords: Nivalenol, female reproductive system, haematopoietic, immune systems

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* 神戸市環境保健研究所

Poapolathep, A[*1]., Poapolathep, S[*1]., Sugita-Konishi, Y., Imsilp, K[*1]., Tassanawat, T[*1]., Sinthusing [*1]., Itoh, Y., Kumagai, S[*2]. : **Fate of fusarenon-X in broilers and ducks**

Poult Sci., **87**, 1510-1515 (2008)

In order to investigate the comparative fates and dispositions of fusarenon-X (FX) in broilers and ducks, FX was administered i.v. or orally (p.o.) to broilers and ducks. The FX and its metabolite (nivalenol, NIV) were determined in plasma and excreta using gas chromatography-mass spectrometry. The plasma concentrations of FX were determined up to 180 and 120 min in broilers and ducks, respectively, after i.v. and p.o. administration. The NIV was eliminated more slowly than its parent compound. The FX disposition fit an open 2-compartment pharmacokinetic model in broilers and ducks. The elimination half-life (t(1/2beta)) of FX was longer in ducks than in broilers. The elimination rate constant (kel) was higher in broilers than in ducks, whereas the oral bioavailability of FX was higher in ducks than in broilers. The gas chromatography-mass spectrometry profile in plasma showed that a large proportion of FX was recovered as NIV after administration of FX in both broilers and ducks. In vitro incubation of liver microsomal and cytosolic fractions with FX demonstrated that the liver and kidney are capable of the FX-to-NIV conversion. Thus, this study demonstrated that FX is absorbed more efficiently in ducks than in broilers, whereas it is eliminated more slowly in ducks than in broiler chickens. Consequently, the toxicity would have more serious consequences in ducks rather than broilers.

Keywords: fusarenon-X, broilers, ducks, liver, kidney

[*1] カセサート大学
[*2] 東京大学大学院

Mizutani K[*1]., Hirasawa Y[*2]., Sugita-Konishi Y., Mochizuki N[*1]., Morita H[*2]. : **Structural and conformational analysis of hydroxycyclochlorotine and cyclochlorotine, chlorinated cyclic peptides from *Penicillium islandicum***

J Nat Prod., **71**, 1297-1300(2008)

A new chlorinated cyclic pentapeptide, hydroxycyclochlorotine (1), has been isolated from Penicillium islandicum, and the structure including absolute stereochemistry of 1 and conformational properties of 1 and cyclochlorotine (2) in DMSO-d6 were elucidated by using extensive 2D NMR and chemical means. Hydroxycyclochlorotine (1) and astin B (3) from Aster tataricus, each containing an allo threonine at residue 2, have a cis proline configuration, whereas cyclochlorotine (2) has two conformational states in solution, which may be produced from cis-trans isomerization of the proline amide bond. The presence of an intramolecular hydrogen bond between Ser (3)-NH and a hydroxyl oxygen atom of alloThr (2) may serve to maintain the backbone conformation with a cis proline amide bond.

Keywords: hydroxycyclochlorotine, astin B, Aster tataricus

[*1] アサヒビール（株）
[*2] 星薬科大学

Yoshinari T[*1]., Yaguchi A[*1]., Takahashi-Ando N[*1]., Kimura M[*2]., Takahashi H[*3]., Nakajima T[*4]., Sugita-Konishi Y., Nagasawa H[*1]., Sakuda S[*1]. : **Spiroethers of German chamomile inhibit production of aflatoxin G and trichothecene mycotoxin by inhibiting cytochrome P450 monooxygenases involved in their biosynthesis**

FEMS Microbiol Let., **284**, 184-190 (2008)

The essential oil of German chamomile showed specific inhibition toward aflatoxin G(1) (AFG(1)) production, and (E)- and (Z)-spiroethers were isolated as the active compounds from the oil. The (E)- and (Z)-spiroethers inhibited AFG(1) production of Aspergillus parasiticus with inhibitory concentration 50% (IC(50)) values of 2.8 and 20.8 microM, respectively, without inhibiting fungal growth. Results of an O-methylsterigmatocystin (OMST) conversion study indicated that the spiroethers specifically inhibited the OMST to AFG(1) pathway. A cytochrome P450 monooxygenase, CYPA, is known as an essential enzyme for this pathway. Because CYPA has homology with TRI4, a key enzyme catalyzing early steps in the biosynthesis of trichothecenes, the inhibitory actions of the two spiroethers against TRI4 reactions and 3-acetyldeoxynivalenol (3-ADON) production were tested. (E)- and (Z)-spiroethers

inhibited the enzymatic activity of TRI4 dose-dependently and interfered with 3-ADON production by Fusarium graminearum, with IC(50) values of 27.1 and 103 microM, respectively. Our results suggest that the spiroethers inhibited AFG(1) and 3-ADON production by inhibiting CYPA and TRI4, respectively.

Keywords: essential oil of German chamomile,aflatoxin G(1), 3-acetyldeoxynivalenol

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*1 東京大学大学院
*2 理化学研究所
*3 千葉市衛生研究所
*4 九州沖縄農研

Mino Y*1., Amano F*1., Yoshioka T*2., Konishi Y. : **Determination of Organothins in human breast milk by gas chromatograpy with flame photometric detection**

J.Health Science, **54**, 224-228 (2008)

An analytical method for the quantitative determination of monobutyltin (MBT), dibutyltin (DBT), pounds in human breast milk is described. After the addition of surrogates (deuterium derivatives), milk samples were extracted with hexane-diethy ether (4:6) in the presence of HCl and NaCl. Each extract was purified by cation exchange chromatography and treated with Grignard reagent to yield ethyl derivatives, which were determined by gas chromatography (GC) with flame photometric detectionoperated in the tin mode (610 nm). This analytical method was used to determine organotins in about 70 breast milk samples obtained from mothers who had given birth within the previous week. DBT dichloride levels varied from undetectable to 9.5 ng/ml in human milk from mothers who habitually ate fish, however the other organotins were not detectable.

Keywords: organotins, dibutyltin, breast milk, Gas chromatography

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*1 大阪薬科大学
*2 倉敷市民病院

Hatao F*1., Yamamoto M., Muroi M., Kaminishi M*1., Tanamoto K. : **MyD88-induced downregulation of IRAK-4 and its structural requirements**

FEMS Immunol. Med. Microbiol., **53**, 260-264 (2008)

We show that expression of MyD88 leads to downregulation of endogenous as well as exogenously expressed IRAK-4 protein in HEK293 cells. Expression of TRIF did not cause IRAK-4 downregulation although it induced NF-κB activation. Expression of either a deletion mutant of MyD88 lacking its death domain or MyD88s, neither of which induced NF-κB activation, did not lead to IRAK-4 downregulation. MyD88-induced downregulation was observed in an IRAK-4 mutant lacking the kinase domain, but not in another mutant lacking the death domain. These results demonstrate that downregulation of IRAK-4 requires activation of the MyD88-dependent pathway and that the death domains of both MyD88 and IRAK-4 are important for this downregulation.

Keywords: Toll-like receptor, IRAK, lipopolysaccharide

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*1 東京大学

Tanamoto K., Muroi M., Nakagawa Y., Shima K., Ichimura K. : **日本薬局方指定菌株の特性と保存管理法に関する研究**, *Pharm. Regul. Sci*., **39**, 309-312 (2008)

菌の特性を捉える新たな方法としてMALDI-TOFMSを利用する方法を検討した．*E. coli* (NBRC3972), *B. subtilis* (NBRC3134), *P. aeruginosa* (NBRC13275), *S. aureus* subsp. *aureus* (NBRC13276), *S. enterica* subsp. *enterica* (NBRC100797)といった菌株についてMALDI-TOFMSプロファイルを検討し，それぞれの菌株ではまったく異なるプロファイルを示すこと，*B. subtilis*以外の菌株では5継代までほとんどパターンに変化がなく，安定したプロファイルが得られること，異なる2種類の培地で培養した場合や，異なる2種類の凍結法により凍結した場合でも同様のプロファイルが得られることを確認した．これらの結果から，MALDI-TOFMSが再現性よく菌株の特性を捉えることが可能であり，菌株の特性指標として有用であることを示した．

Keywords: MALDI-TOF，菌

宮原美知子，荒川英二*：**食品からの腸炎ビブリオ迅速検出法の検討**

防菌防黴誌，**10**，669-675 (2008)

腸炎ビブリオは日本では食中毒発生の主な原因物質となっている．日本では生鮮魚介類の微生物状態は腸炎ビブリオでMPN　100／g以下に設定されている．アルカリ性ペプトン水は増菌培地として使われ，選択分離培地にはTCBS，クロモアガービブリオとX-ビブリオ寒天が使われている．これらの培地上で*Vibrio alginolyticus*（アルギノリティカス）は腸炎ビブリオと同様な性状が見られる．生鮮魚介類はしばしば両菌に汚染される．我々はPCRでの食品中の腸炎ビブリオの新検出法を検討した．腸炎ビブリオの6株とアルギノリティカスの13株の*toxRS*

遺伝子の塩基配列のシークエンスを行い，また，生化学的な性状も検討した．*toxRS*配列の分析を行った．腸炎ビブリオ検出のプライマーを設定し，腸炎ビブリオ検出を検討した．新プライマーによるPCRで38株の腸炎ビブリオと33株のアルギノリティカスが検討された．すべての腸炎ビブリオは検出され，すべてのアルギノリティカスは検出されなかった．他のビブリオもこのPCRで検出されなかった．イカに腸炎ビブリオと過剰のアルギノリティカスを接種し，培養後に検出を行ったが，腸炎ビブリオをこのPCRによりうまく検出することができた．この方法を迅速法に応用した．腸炎ビブリオがグラムあたり100を超える接種検体は4時間の培養でPCRにより腸炎ビブリオを検出できることが分かった．

Keywords: *Vibrio parahaemolyticus, Vibrio alginolyticus, PCR detection*

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* 国立感染研

Masashi Kanki*, Junko Sakata*, Masumi Taguchi*, Yuko Kumeda*, Masanori Ishibashi*, Takao Kawai*, Kentaro Kawatsu*, Wataru Yamasaki*, Kiyoshi Inoue*, Michiko Miyahara : **Effect of sample prepararion and bacterial concentration on Salmonella enterica detection in poultry meat using culture methods and PCR assaying of preenrichment broths**

Food Microbiol., **26**, 1-3 (2009)

我々は鶏肉からのサルモネラの検出を検討した．培養とPCR検出の比較を行った．培養と同等の検出はPCRではできなかった．PCRによって，MPN（最確法）では1.0 CFU/gまた，培養液では10^3 CFU/mlが最小の検出菌数と考えられた．鶏肉のサルモネラ検査においてPCRでの検査は，鶏肉を汚染しているサルモネラの菌数が少ないこと，阻害物質が鶏肉に存在していることから，通常検査に採り入れるのは困難だ．

Keywords: *Salmonella*, PCR, Chicken

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* 大阪公衛研

渡辺麻衣子，加藤裕子*1，戸上敬子*2，山中実喜子*2，若林佳子*1，小川裕由*1，稙田裕子*1，後藤慶一*1，工藤由起子，天野典英*2，横田　明*3：***Byssochlamys spp.*同定のための遺伝子指標の評価**

食品衛生学雑誌，**49**，82-87(2008)

Byssochlamys spp.について，簡便，迅速かつ正確に種を同定するために有効な遺伝子指標を評価する目的で，26株の*Byssochlamys* spp.および関連菌種の18SrDNA，26/28SrRNA遺伝子D2領域および*lys2*の塩基配列を決定し，分子系統解析および相同性解析を行った．その結果，いずれの遺伝子を用いても，その塩基配列の相同性を指標として，それぞれの菌種あるいはグループを識別することができた．3種類の遺伝子のうち，最も優れた解像度を有するのは*lys2*であったが，最も簡便に結果を得ることができたのは26/28SrRNA遺伝子D2領域であった．また，分子系統解析の結果，*Byssochlamys* spp.とその関連菌種は再分類の必要性があることが示唆された．

Keywords: heat resistant molds, identification, molecular phylogenetic analysis

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*1 三井農林（株）食品総合研究所
*2 サントリー（株）安全性科学センター
*3 東京大学分子細胞生物学研究所

Hayashidani, H.*1, Iwata, T.*1, Yamaguchi, S.*1, Hara-Kudo, Y., Okatani, T. A.*1, Watanabe, M., Lee, K.*1, Kumagai, S.*2 : **Survival of Pathogenic Yersinia enterocolitica in vacuum-packed or non-vacuum-packed pork at low temperature**

Biocontrol Sci., **13**, 139-44 (2008)

Pathogenic *Yersinia enterocolitica* serotypes O:3, O:5,27, O:8 and O:9 were inoculated into sliced and ground pork, and the samples were stored under vacuum or aerobic conditions at 2 and degrees C. All serotypes survived for 5 weeks in pork with or without vacuum packing without any discernable increases in their population. In sterilized pork with or without vacuum packing, there was no evident growth of *Y. enterocolitica*. In pork broth in which the pH had been artificially adjusted to 6.8, the growth of *Y. enterocolitica* was faster than that at 5.7. It is suggested that the growth of *Y. enterocolitica* in pork with or without vacuum packing may be inhibited by pH but not by the microflora or lactic acid bacteria in pork.

Keywords: *Yersinia enterocolitica*, pork, vacuum packing

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*1 東京農工大学
*2 東京大学

占部友理恵*1，薬袋裕二*1，芳賀実*1，小西良子，石黒厚*2，工藤由起子：**香辛料におけるサルモネラの生残性と調理食品中での増殖性**

食品衛生学雑誌，**49**，70-75 (2008)

本研究では，香辛料におけるサルモネラ汚染があった場合，汚染品の使用が食中毒発生につながる可能性があるかを検討するために，香辛料中でのサルモネラの生残性およびそれらを添加した調理食品中での増殖性につい

て調べた．ブラックペッパーおよびレッドペッパーにおいて，それら香辛料由来のS. WeltevredenおよびS. SenftenbergはS. Enteritidis よりも生残性が高かった．タマゴサラダおよびナムル中で，サルモネラは10℃保存では増殖しなかったが, 30℃保存では24時間で著しく増殖した．今回の結果から，加熱調理後の食材を和える際に香辛料を添加する調理食品の保存は，温度や時間に注意が必要であることが明らかになった．

Keywords: 香辛料, サルモネラ, 生残, 増殖, 調理食品

[*1] 玉川大学
[*2] ドンク

Kamio, A.[*1], Hara-Kudo, Y., Miyasaka, J.[*2], Yahiro, T.[*2] and Konuma, H.[*1] : **Efficiency of real-time polymerase chain reaction assay to detect *Vibrio vulnificus* in seawater.**

International Journal of Hygiene and Environmental Microbiology, **211**(5-6),518-523 (2008)

The growth of *Vibrio vulnificus* in an enriched culture of seawater during the summer in Japan was monitored by a plating technique used as the culture method and a real-time polymerase chain reaction (PCR) assay as the molecular method. *V. vulnificus* was detected by the real-time PCR assay in the samples of August and September but not by the culture method. *V. parahaemolyticus*, however, was detected among all of the samples with both the culture method and real-time PCR assay. In the analysis of the bacterial populations in enrichment culture, it was demonstrated that the growth of *V. vulnificus* on agar media was inhibited by the rapid growth of *V. parahaemolyticus* after 4 h of incubation and the 100 times larger initial populations of bacteria other than *V. vulnificus* and *V. parahaemolyticus*. These findings demonstrate that *V. vulnificus* detection by culture methods is a failure, and molecular methods are effective and detect *V. vulnificus* accurately.

Keywords: *Vibrio vulnificus*, real-time PCR, detection

[*1] 東海大学
[*2] 熊本県保健環境科学研究所

Asai, Y.[*1], Kaneko, M.[*2], Ohtsuka, K.[*3], Morita, Y.[*4], Kaneko, S.[*5], Noda, H.[*2], Furukawa, I.[*1], Takatori, K. and Hara-Kudo, Y. : ***Salmonella prevalence* in seafood imported into Japan**

J. Food Prot, **71**, 1460-1464(2008)

A total of 353 samples of 29 types of seafood were tested for *Salmonella* prevalence and total microbial population. *Salmonella* enterica serotype Weltevreden was isolated from two of 47 black tiger prawn samples. The contamination levels of *Salmonella* were in a range of <30 to 40 MPN/100g. In addition, one sample of black tiger prawns and two samples of white shrimp were positive for *Salmonella* invA gene on PCR assay. Although the mean aerobic bacterial count was greater than 4.0 log CFU/g in most of the sample types, those in the two *Salmonella*-isolated samples of black tiger prawn were 7.48 and 5.18 log CFU/g, respectively. These results indicate the possibility that shrimp and prawns are related to food borne infections, and the improvement of seafood quality in an important issue, and information on contamination by pathogens should be provided as feedback to the original country with the aim of increasing safety.

Keywords: *Salmonella*, seafood, bacteriological quality, contamination, shrimp, prawn

[*1] 神奈川県衛生研究所
[*2] 山梨県衛生公害研究所
[*3] 埼玉県衛生研究所
[*4] 群馬県衛生環境研究所
[*5] 東京都健康安全研究センター

Hidaka, A. [*1], Hokyo, T., Arikawa[*1], T., Fujiwara, S. [*2], Ogasawara, J. [*3], Hase, A. [*3], Hara-Kudo, Y., and Nishikawa, Y. : **Multiplex real-time PCR for exhaustive detection of diarrhoeagenic *Escherichia coli***

J. Appl. Microbiol. **106**, 410-420 (2009)

Multiplex real-time PCR for exhaustive detection of diarrhoeagenic *Escherichia coli*.

Aims: The source and routes of diarrhoeagenic *Escherichia coli* (DEC) have not been clarified because it is difficult to detect these organisms in samples with numerous coliform bacteria. We have developed multiplex real-time PCR assays for exhaustive detection of DEC. Methods and Results: Primers and TaqMan probes were designed to amplify and quantify one gene (eae, stx1, stx2, elt, est, virB, aggR, astA, and afaB) from each of seven pathotypes of DEC, in duplex or triplex reactions under the same PCR cycling conditions. Specificity was confirmed using 860 strains including 88 DEC strains. The fluorescence threshold cycle and DNA concentrations correlated with decision coefficients of more than 0.99. Subsequently, meat samples and enrichment broths were spiked with DEC and the assays used to detect the genes. The detection limits varied

from 7.1 x 10(2) to 1.1 x 10(4) CFU ml(-1), depending on the target genes. All meat samples spiked with a variety of DEC (more than 10 CFU 10 g(-1)) were found to be positive by the method. Conclusions: The present system allows for the efficient and simultaneous determination of various DEC pathotypes. Significance and Impact of the Study: This system makes epidemiological investigations for DEC sensitive and quick, and is a useful tool to clarify the source and routes of DEC.

keywords: detection, environmental health, food, identification, PCR, rapid techniques, virulence

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[*1] 大阪市立大学大学院
[*2] 大阪生活衛生協会
[*3] 大阪市環境科学研究所

Kobayashi, H.[*1], Kanazaki, M.[*1], Ogawa, T.[*1,2], Iyoda, S.[*3], Hara-Kudo, Y. : **Changing prevalence of O-serogroups and antimicrobial susceptibility among STEC strains isolated from healthy dairy cows over a decade in Japan between 1998 and 2007**

J. Vet. Med. Sci. , **71**, 363-366 (2009)

The prevalence of STEC in Japan was examined using rectal stool samples taken from 932 healthy dairy cows from 123 farms in 11 prefectures between 2006 and 2007. Screening with stx-PCRs revealed the prevalence to be 30.4% (283 animals), and STEC strains were isolated from 111 animals. Although ten O-serogroups (O8, O22, O84, O103, O111, O113, O116, O136, O153 and O157) were the major O-serogroup in healthy dairy cows in Japan in 1998, half of the 118 selected STEC strains were serotyped as O2, O8, O26, O153, or O163 in this study. Twenty-eight of the 118 STEC strains (24%) showed resistance to some conventional drugs, such as dihydrostreptomycin, oxytetracycline and aminobenzylpenicillin. Although STEC prevalence in cows decreased from 17% to 12%, the antimicrobial resistance ratio increased from 8.7% to 24% in the past decade in Japan.

keywords: O-serogroups, STEC, Cows

[*1]（独）農業・食品産業技術総合研究機構・動物衛生研究所
[*2] 福岡県家畜保健所
[*3] 国立感染症研究所

Hara-Kudo, Y. and Takatori, K. : **Microbial quality of liquid egg and *Salmonella* infection status in Japan**

J. Food Hyg. Soc. Japan, **50**, 34-40 (2009)

Aerobic bacteria counts and contamination with Salmonella were investigated in a total of 1,327 samples of commercial liquid egg in 1992-2002. Salmonella was isolated from 8.1 % of the samples, and *Salmonella* contamination in 1.7 % of even the pasteurized liquid egg was revealed. The major *Salmonella* serotype was Enteritidis from more than 50 % of the cases of liquid egg. In addition the aerobic bacteria counts in *Salmonella*-positive liquid eggs were significantly higher than those of Salmonella-negative samples. However Salmonella was detected in liquid egg in which the aerobic bacteria counts are in a range of 102 to 106 cfu/g. Furthermore, foodborne outbreaks of *Salmonella* infections associated with liquid egg were analyzed. Liquid eggs should be carefully treated for the possibility of *Salmonella* contamination. Supply of pasteurized liquid eggs and control of re-contamination are needed.

Keywords: liquid eggs, aerobic bacteria count, *Salmonella*

Sakai, A., Ozeki, Y.[*1], Sasaki, Y.[*2], Aihara, M., Kikuchi, Y., Takatori,K. : **Utilization of DNA sequences for identifying Fusarium species isolated from rice**

Mycotoxins, **57** Supplement, 171-176 (2007)

We attempted the identification of *Fusarium* species by a DNA sequence analysis to practically validate the utility of a molecular approach for fungal identification and reveal its limitations. We sequenced three regions, the 5'end of the large-subunit (D2 region) and the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions, in the rRNA genes. The DNA sequences of 38 *Fusarium* strains isolated from unpolished rice harvested in Japan were analyzed and compared for similarity to entries in the GenBank. Based on this comparison, it was estimated that all these three regions, as a minimum, must be compared with the database to identify *Fusarium* fungi at the species level. According to the sequence differences in the three regions, the 38 isolates were classified into 13 groups. Out of the 13 groups, 6 groups (20 isolates in total) were able to be identified as the definite species based only on the sequence data. For the other 6 groups (17 isolates in total), the candidate species were restricted on the basis of the sequence similarity. The restriction of candidate species rendered it easy to identify the *Fusarium* isolates at the species level with the aid of their morphologies. Only one isolate could not be identified. It was verified in this study that a DNA sequence comparison with the GenBank database was useful and reliable for the identification of the *Fusarium* species.

Keywords: identification, DNA sequence, *Fusarium*, rRNA gene

*1 Saitama Institute of Public Health
*2 Nippon Kaiji Kentei Kyokai

Saka,M.*, Tada,N.*, Kamata,Y : **Cross-reactivity of a polyclonal antibody against *Chinemys reevesii* vitellogenin with the vitellogenins of other turtle species:*Chelydra serpentina, Macrochelys temminckii,* and *Pelodiscus sinensis***

Zoological Aci., **25**, 907-911(2008)

ビテロジェニンは卵黄の前駆体タンパク質で，環境を汚染する女性ホルモンに対するバイオマーカーになっている．ヌマガメのビテロジェニンの抗体を作製し，スッポン，ニシキガメ，カミツギガメのビテロジェニンに対する交差性を調べた．形態学的特徴からの系統的距離と，抗体の交差性に相関性があった．

Keywords: Turtle vitellogenin, Cross-reactivity, Polyclonal antibody, Enzyme-linked immunosorbent assay

* Kyoto Prefectural Institute of Public Health and Environment

Sugiyama, K., Muroi, M. and Tanamoto, K. : **A novel TLR4-binding peptide that inhibits LPS-induced activation of NF-κB and in vivo toxicity**

Eur. J. Pharmacol., **594**, 152-156 (2008)

We screened for peptides that associate with TLR4 with a yeast two-hybrid screen using the human TLR4 extracellular domain as bait. A peptide (STM28) isolated from the screen inhibited LPS-induced nuclear factor-κB (NF-κB) activation in human and mouse macrophage cells and interacted with TLR4 in yeast and mammalian cells. STM28 showed no inhibitory effects against NF-κB activation induced by TLR1/2, TLR3 and TLR9 ligands in a mouse macrophage cell line, RAW 264. In addition, STM28 suppressed LPS-induced tumor necrosis factor-α production by differentiated THP-1 cells. Moreover, LPS-induced lethality in $_{D}$-galactosamine-sensitized mice was significantly repressed by STM28 in a dose-dependent manner. These results demonstrate that STM28 selectivity inhibits TLR4-induced macrophage activation, and suggest that STM28 may have utility as a novel therapeutic agent for Gram-negative bacterial sepsis.

Keywords: Yeast two-hybrid, Toll-like receptor 4, Endotoxin shock

Sugiyama, K., Hiraoka, H.* and Sugita-Konishi, Y. : **Aflatoxin M_1 contamination in raw bulk milk and the presence of aflatoxin B_1 in corn supplied to dairy cattle in Japan**

J. Food Hyg. Soc. Japan, **49**, 352-355 (2008)

Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of aflatoxin B_1 (AFB_1), which has been found in the milk of dairy cattle fed AFB_1 contaminated feeds. To evaluate the risk of AFM_1 contamination in milk, it is necessary to analyze the risk factors of AFB_1 contamination in corn provided for concentrated feed in Japan. The AFM_1 level in domestic raw bulk milk was measured at three sampling times, January, February and June in 2004. The AFB_1 contamination in corn supplied to cow was determined at the same time as the sampling of raw milk. The AFM_1 contamination levels in milk in January, February and June 2004 was 0.011, 0.007 and 0.005 ng/g, respectively. The AFB_1 contamination level in the corn of the concentrated feed was higher from October of 2003 to February of 2004 than from April to June in 2004. This study showed evidence that AFM_1 contamination level in milk is parallel to that of AFB_1 in corn of concentrated feed, thus showing that the monitoring of the AFB_1 level in corn is important to prevent the risk of AFM_1 contamination in milk in Japan.

Keywords: Aflatoxin M_1, Aflatoxin B_1, Milk

*（独）農林水産消費安全技術センター

Sugiyama, K., Tanaka, H.*1, Kamata, Y., Tanaka, T.*2 and Sugita-Konishi, Y. : **A reduced rate of deoxynivalenol and nivalenol during bread production from wheat flour in Japan**

Mycotoxins, **59**, 1-6 (2009)

Deoxynivalenol (DON) and nivalenol (NIV) are among the mycotoxins known as trichothecenes and they naturally occur in cereal grains of bread making wheat. In this study, contamination levels of these mycotoxins in various wheat flour samples containing domestic flour used for the mass production of bread and related products were collected and analyzed in Japan. Samples of flour and bread were collected from nine prefectures, and their trichothecene levels were measured by a validated High Performance Liquid Chromatography-Mass Spectrometry system. The average concentrations of DON and NIV in flour samples collected were 31.3 ± 28.9 and 8.5 ± 3.7 μg/kg, whereas those in bread samples were 8.6 ± 5.1 μg/kg and 3.4 ± 2.0 μg/

kg, respectively. These results suggest that the percentage of DON and NIV remaining after converting flour into bread using industrial equipment and baking yeast were estimated as approximately 74.4 and 65.8%, respectively.

Keywords: Deoxynivalenol, Nivalenol, Wheat flour, Bread

*1 サントリー㈱
*2 神戸市環境保健研究所

Shoda, T., Fukuhara, K., Goda, Y., Okuda, H. : **4-Hydroxy-3-methoxymethamphetamine Glucuronide as a Phase II Metabolite of 3,4-Methylenedioxymethamphetamine: Enzyme-Assisted Synthesis and Involvement of Human Hepatic Uridine 5'-Diphosphate-Glucuronosyltransferase 2B15 in the Glucuronidation**

Chem. Pharm. Bull., **57**, 472-475 (2009)

3,4-Methylenedioxymethamphetamine (MDMA), one of the most popular illicit recreational drugs, is metabolized primarily into 4-hydroxy-3-methoxymethamphetamine (HMMA) by drug-metabolizing enzymes. HMMA is further metabolized by phase II enzymes to give the glucuronide or sulfate which is excreted into urine. In the present study, enzyme kinetic studies with various microsomes showed that rat liver microsomes pretreated with Aroclor 1254 were most suitable for the enzyme-assisted synthesis of the glucuronide (HMMAGluc). This method selectively produced the b-anomer of HMMA-Gluc in a very high, isolated yield (71%), and with a purity that was sufficient for use in an analysis of MDMA intake and for enzyme kinetic studies. We also identified, by an LC-MS method, the human uridine 5'-diphosphate-glucuronosyltransferase (UGT) isoforms that catalyze the glucuronidation of HMMA. Among 12 isoforms of human recombinant UGT expressed in insect cells, UGT2B15 was the only isoform that showed adequate enzymatic activity in catalyzing HMMA glucuronidation with *K*m and *V*max values of 3.8 mM and 1.6 nmol/min/mg protein, respectively. The finding that UGT2B15 is capable of HMMA glucuronidation suggests this isoform may have an important *in vivo* role in human MDMA metabolism.

Keywords: 3,4-Methylenedioxymethamphetamine, enzyme-assisted synthesis, glucuronide

Hishikawa, K.*, Nakagawa, H.*, Furuta, T.*, Fukuhara, K., Tsumoto, H.*, Suzuki, T.*, Miyata, N.* : **Photoinduced nitric oxide release from a hindered nitrobenzene derivative by two-photon excitation**

J. Am. Chem. Soc., **131**, 7488-7489(2009)

We demonstrated photoinduced NO generation from a 2,6-dimethylnitrobenzene-based compound (Flu-DNB) via a two-photon excitation (TPE) process. After pulse laser irradiation to a solution of Flu-DNB, oxidation products of NO were observed. This is the first account of a non-nitrosyl-chelated metal ion containing NO donor which can be controlled by the TPE technique.

keywords: nitric oxide, NO donor, two-photon excitation process

* 名古屋市立大学大学院

Kobayashi, H.*, Fukuhara, K., Tada-Oikawa S.*, Yada, Y.*, Hiraku, M.*, Oikawa, S.* : **The mechanisms of oxidative DNA damage and apoptosis induced by norsalsolinol, an endogenous tetrahydroisoquinoline derivative associated with Parkinson's disease**

J. Neurochem., **108**, 397-407 (2009)

Tetrahydroisoquinoline (TIQ) derivatives are putative neurotoxins that may contribute to the degeneration of dopaminergic neurons in Parkinson's disease. One TIQ, norsalsolinol (NorSAL), is present in dopamine-rich areas of human brain, including the substantia nigra. Here, we demonstrate that NorSAL reduces cell viability and induces apoptosis via cytochrome c release and caspase 3 activation in SH-SY5Y human neuroblastoma cells. Cytochrome c release, caspase 3 activation, and apoptosis induction were all inhibited by the antioxidant N-acetylcysteine. Thus, reactive oxygen species (ROS) contribute to apoptosis induced by NorSAL. Treatment with NorSAL also increased levels of oxidative damage to DNA, a stimulus for apoptosis, in SH-SY5Y. To clarify the mechanism of intracellular DNA damage, we examined the DNA damage caused by NorSAL using ^{32}P-5'-end-labeled isolated DNA fragments. NorSAL induced DNA damage in the presence of Cu(II). Catalase and bathocuproine, a Cu(I) chelator, inhibited this DNA damage, suggesting that ROS such as the Cu(I)-hydroperoxo complex derived from the reaction of H_2O_2 with Cu(I), promote DNA damage by NorSAL. In summary, NorSAL-generated ROS induced oxidative DNA damage, which led to caspase-dependent apoptosis in neuronal cells.

Keywords: norsalsolinol, Perkinson's disease, reactive oxygen species

* 三重大学大学院

Kakuda, S.[*1], Okada, K.[*1] Eguchi, H.[*1] Takenouchi, K.,[*1] Hakamata, W., Kurihara, M., M. Takimoto-Kamimura, M.[*1] : **Structure of the ligand-binding domain of rat VDR in complex with a nonsecosteroidal vitamin D_3 analogue YR301**

Acta Crystallogr. F, **64**, 970-973(2008)

YR301 is the only one of the four evaluated stereoisomers of LG190178 to have strong activity. To understand the strong activity of YR301, the crystal structure of YR301 complexed with the rat VDR ligand-binding domain (VDR LBD) was solved at 2.0 A resolution and compared with the structure of the VDR LBD-1alpha,25(OH)(2)D(3) complex. YR301 and 1α,25(OH)(2)D(3) share the same position and the diethylmethyl group occupies a similar space to the C and D rings of 1alpha,25(OH)(2)D(3). YR301 has two characteristic hydroxyl groups which contribute to its potent activity. The first is 2'-OH, which forms hydrogen bonds to the NE2 atoms of both His301 and His393. The other is 2-OH, which interacts with Ser233 OG and Arg270 NH1. These two hydroxyl groups of YR301 correspond exactly to 25-OH and 1-OH, respectively, of 1alpha,25(OH)(2)D(3). The terminal hydroxyl group (3-OH) of YR301 is directly hydrogen bonded to Arg270 and also interacts indirectly with Tyr232 OH and the backbone NH of Asp144 via water molecules. Additional derivatization of the terminal hydroxyl group using the positions of the water molecules might be useful for the design of more potent compounds.

Keywords: VDR, crystal structure, YR301

[*1] 帝人ファーマ生医研

Satoh, T.[*1], Cowieson, N. P.[*2], Hakamata, W., Ideo, H.[*3], Fukushima, K.[*3], Kurihara, M., Kato, K.[*1], Yamashita, K.[*3], Wakatsuki, S.[*1] : **Structure Basis for Recognition of High Mannose Type Glycan Transport Lectin VIP36**

PF NEWS, **25**, 17-22(2008)

We report the crystal structure of VIP36 exoplasmic/luminal domain comprising a carbohydrate recognition domain and a stalk domain. The structures of VIP36 in complex with Ca^{2+} and mannosyl ligands are also described. The carbohydrate recognition domain is composed of a 17-stranded antiparallel β-sandwich and binds one Ca^{2+} adjoining the carbohydrate-binding site. The structure reveals that a coordinated Ca^{2+} ion orients the side chains of Asp131, Asn166, and His190 for carbohydrate binding. This result explains the Ca^{2+}-dependent carbohydrate binding of this protein. The Man-α-1,2-Man-α-1,2-Man, which corresponds to the D1 arm of high mannose type glycan, is recognized by eight residues through extensive hydrogen bonds. The complex structures reveal the structural basis for high mannose type glycoprotein recognition by VIP36 in a Ca^{2+}-dependent and D1 arm-specific manner.

Keywords: lectin, VIP36, glycoprotein

[*1] 高エネルギー加速器研究機構
[*2] University of Queensland
[*3] 東京工業大学

Nagano, M.[*1], Tanaka, M.[*1], Doi, M.[*2], Demizu, Y., Kurihara, M., Suemune, H.[*1] : **Helical-Screw Directions of Diastereoisomeric Cyclic α-Amino Acid Oligomers**

Org. Lett., **11**, 1135-1137(2009)

Two series of homooligomers composed of diastereoisomeric cyclic α-amino acids having two chiral centers at the α-carbon and the side chain were synthesized, and their preferred secondary structures were studied in solution and in the crystal state. The oligomers are a new class of helical-foldamers possessing two kinds of chiral centers on the helical backbone and at the lateral surface of the helix.

Keywords: α, α-disubstituted α-amino acid, peptide, cyclic α-amino acid oligomer

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[*1] 九州大学大学院
[*2] 大阪薬科大学

Hakamata, W.[*1], Kurihara, M., Okuda, H., Nishio, T.[*1], Oku, T.[*1] : **Design and Screening Strategies for α-Glucosidase Inhibitors Based on Enzymological Information**

Curr. Top. Med. Chem., **9**, 3-12 (2009)

Alpha-glucosidase inhibitors are marketed as therapeutic drugs for diabetes that act through the inhibition of carbohydrate metabolism. Inhibitors of the alpha-glucosidases that are involved in the biosynthesis of N-linked oligosaccharide chains have been reported to have antitumor, antiviral, and apoptosis-inducing activities, and some have been used clinically. alpha-Glucosidase inhibitors have interesting biological activities, and their design,

synthesis, and screening are being actively performed. In quite a few reports, however, alpha-glucosidases with different origins than the target alpha-glucosidases, have been used to evaluate inhibitory activities. There might be confusion regarding the naming of alpha-glucosidases. For example, the term alpha-glucosidase is sometimes used as a generic name for alpha-glucoside hydrolases. Moreover, IUBMB recommends the use of "alpha-glucosidase" (EC 3.2.1.20) for exo-alpha-1,4-glucosidases, which are further classified into four families based on amino acid sequence similarities. Accordingly, substrate specificity and susceptibility to inhibitors varies markedly among enzymes in the IUBMB alpha-glucosidases. The design and screening of inhibitors without consideration of these differences is not efficient. For the development of a practical inhibitor that is operational in cells, HTS using the target alpha-glucosidase and the computer-aided design of inhibitors based on enzymatic information concerning the same alpha-glucosidase are essential.

Keywords: α-glucosidase, α-glucosidase inhibitor, enzymological information

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[*1] 日本大学生物資源

Kurihara, M., Sato, Y., Yamagata, N., Okuda, H., Nagano, M.[*1], Demizu, Y., Doi, M.[*2], Tanaka, M.[*1], Suemune, H.[*1] : **Computational Study on Helical Structure of α,α−Disubstituted Oligopeptides Containing Chiral α-Amino Acids**

Peptide Science 2008, 149-150(2009)

Computational simulation using conformational search calculations with AMBER* force field is most useful for conformational analysis of oligopeptides containing α,α-disubstituted a-amino acids. The results were in agreement with those of x-ray and were most stable conformation evaluated by molecular orbital calculation.

Keywords: α, α-disubstituted α-amino acid, conformational search, 3_{10}-helix

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[*1] 九州大学大学院
[*2] 大阪薬科大学

Takazaki, H.[*1], Tanaka, M.[*1], Kawabe, N.[*1], Nagano, M.[*1], Doi, M.[*2], Kurihara, M., Suemune, H.[*1] : **Design and synthesis of chiral cyclic α,α-disubstituted amino acid having azido functions and its oligopeptides**

Peptide Science 2008, 159-160(2009)

We designed and synthesized a chiral cyclic α,α-disubstituted α-amino acid having azido functions: {(3R,4R)-l-amino-3,4-diazidocyclopentanecarboxylic acid; (R,R)-Ac_5c^{dN3}), and studied the preferred secondary structure of its homopeptides. Furthermore, the azido junctions in the cyclic amino acid (R,R)-Ac_5c^{dN3}) could be converted into various functional groups.

Keywords: α, α-disubstituted α-amino acid, secondary structure, 3_{10}-helix

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[*1] 九州大学大学院
[*2] 大阪薬科大学

Sugiyama, T.[*1], Imamura, Y.[*2], Kurihara, M., Kittaka, A.[*3] : **Cooperative Strand Invasion by Peptide Nucleic Acid**

Peptide Science 2008, 481-282(2009)

Peptide nucleic acid (PNA) is a synthetic DNA/RNA mimic in which the sugar-phosphate backbone is replaced by a peptide backbone. A remarkable feature of PNA is its ability to recognize sequences within duplex DNA by strand invasion. In order to improve DNA binding properties of PNA, we tested the effect of cooperativity on triplex invasion. We here demonstrate that a PNA targeting six bases cooperatively strand invades into duplex DNA with excellent sequence specificity.

Keywords: peptide nucleic acid (PNA), cooperative strand invasion

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[*1] 東京大学大学院
[*2] 工学院大学
[*3] 帝京大学

Suzuki, N.[*1], Suzuki, T.[*1], Ota, Y.[*1], Nakano, T., Kurihara, M., Okuda, H., Yamori, T.[*2], Tsumoto, H.[*1], Nakagawa, H.[*1], Miyata, N.[*1] : **Design, Synthesis, and Biological Activity of Boronic acid-Based Histone Deacetylase Inhibitors**

J. Med. Chem., **52**, 2909-2922(2009)

Guided by the proposed catalytic mechanism of histone deacetylases (HDACs), we designed and synthesized a series of boronic acid-based HDAC inhibitors bearing an alpha-amino acid moiety. In this series, compounds (S)-18, 20, and 21 showed potent HDAC-inhibitory activity, highlighting the significance of the (S)-amino acid moiety. In cancer cell growth inhibition assays, compounds (S)-18, 20, and 21 exerted strong activity, and the values of the

ratio of the concentration causing 50% growth inhibition (GI(50)) to the concentration causing 50% enzyme inhibition (IC(50)), i.e., GI(50)/IC(50), were low. The potency of these compounds was similar to that of clinically used suberoylanilide hydroxamic acid (SAHA) (2). The results of Western blot analysis indicated that the cancer cell growth-inhibitory activity of compounds (S)-18, 20, and 21 is the result of HDAC inhibition. A molecular modeling study suggested that the hydrated boronic acid interacts with zinc ion, Tyr residue, and His residue in the active site of HDACs. Our findings indicate that these boronic acid derivatives represent an entry into a new class of HDAC inhibitors.

Keywords: histone Deacetylase Inhibitors, design, synthesis

[*1] 名古屋市立大
[*2] 癌化学療法センター

Nagano, M.[*1], Tanaka, M.[*1], Doi, M.[*2], Kurihara, M., Suemune, H.[*1] : **Helical-screw handedness of peptides composed of diastereoisomeric cyclic amino acids**

Peptide 2008, 106-107(2009)

We designed two new diastereomeric cyclic α,α-disubstituted amino acids; (1S,3S)- and (1R,3S)- 1-amino-3-(methoxy)cyclopentanecarboxylic acid [Ac(5)c(OM)] having chiral centers both at the α-carbon atom and at the side chain, and studied the preferred conformation of their homopeptides.

Keywords: helical-screw handedness, peptide, cyclic amino acids

[*1] 九州大学大学院
[*2] 大阪薬科大学

Tanaka, M.[*1], Nagano, M.[*1], Demizu, Y., Doi, M.[*2], Kurihara, M., Suemune, H.[*1] : **Controlling α-helical secondary structure of oligopeptides and its use as a chiral catalyst**

Peptide 2008, 104-105(2009)

When the cyclic amino acid (S,S)-Ac(5)c(dOM) was incorporated into L-Leu-hexapeptide, the hexapeptide Cbz-{L-Leu-LLeu-(S,S)-Ac(5)c(dOM)}2-OMe preferentially formed right-handed α-helices in the crystal state, whereas the hexapeptide Cbz-{L-Leu-L-Leu-Aib}2-OMe having Aib formed a right-handed 310-helix [3]. The fi nding that the propensity of cyclic amino acid Ac(5)c(dOM) is to form α-helix over 310-helix, encouraged us to use the cyclic amino acid containing α-helical oligomer as an asymmetric catalyst for epoxidation.

Keywords: α-helical secondary structure, peptide, chiral catalyst

[*1] 九州大学大学院
[*2] 大阪薬科大学

Nishimaki-Mogami, T., Tamehiro, N., Sato, Y., Okuhira, K., Sai, K., Kagechika, H.[*1], Shudo, K., Abe-Dohmae, S.[*2], Yokoyama, S.[*2], Inoue, K. and Sawada, J. : **The RXR agonists PA024 and HX630 have different abilities to activate LXR/RXR and to induce ABCA1 expression in macrophage cell lines**

Biochem. Pharmacol., **76**, 1006-1013 (2008)

Release of cellular cholesterol by ATP-binding cassette transporter (ABC)A1 and apolipoproteins is a major source of plasma high-density lipoprotein (HDL). Expression of ABC transporter A1 (ABCA1) is directly stimulated by liver X receptor (LXR)/retinoid X receptor (RXR) activation. We evaluated the abilities of two RXR agonists, PA024 and HX630, to increase ABCA1 expression. In differentiated THP-1 cells, the two agonists efficiently enhanced ABCA1 mRNA expression and apoA-I-dependent cellular cholesterol release. However, in RAW264 cells and undifferentiated THP-1 cells, PA024 was highly effective while HX630 was inactive in increasing ABCA1 mRNA. In parallel, the two agonists had different abilities to activate ABCA1 promoter in an LXR-responsive-element (LXRE)-dependent manner and to directly stimulate LXRalpha/RXR transactivation. The ability of HX630 to enhance ABCA1 expression was correlated closely with the cellular PPARgamma mRNA level. Moreover, HX630 was able to activate PPARgamma/RXR. Transfection of PPARgamma in RAW264 cells induced HX630-mediated activation of LXRE-dependent transcription and ABCA1 promoter, suggesting the ability of HX630 to activate PPARgamma-LXR-ABCA1 pathway. We conclude that RXR agonist PA024 and HX630 have different abilities to activate LXR/RXR, and that the cell-type-dependent effect of HX630 on ABCA1 expression and HDL generation is closely associated with this defect.

Keywords: ABCA1, RXR, LXR

[*1] 東京医科歯科大学大学院
[*2] 名古屋市立大学医学研究科

Hioki, H.*, Shima, N.*, Kawaguchi, H.*, Harada, K.*, Kubo, M.*, Esumi, T.*, Nishimaki-Mogami, T., Sawada, J., Hashimoto, T.*, Asakawa, Y.*and Fukuyama, Y.*
: **Synthesis of riccardin C and its seven analogues. Part 1: The role of their phenolic hydroxy groups as LXRalpha agonists**

Bioorg. Med. Chem. Lett., **19**, 738-741 (2009)

Riccardin C, a nuclear receptor LXRalpha selective agonist, is an 18-membered macrocyclic bisbibenzyl isolated from several liverworts. Synthesis of riccardin C and its seven O-methylated derivatives was accomplished. The synthetic sequence highlights an intramolecular Suzuki-Miyaura coupling in the formation of the 18-membered biaryl linkage present in riccardin C. The structure-activity relationship of these compounds suggests that all of the phenolic hydroxy groups present in riccardin C are essential for the activation of LXRalpha.

Keywords: LXR, agonist, synthesis

* 徳島文理大学薬学部

Inoue, J.*, Satoh, S.*, Kita, M.*, Nakahara, M.*, Hachimura, S.*, Miyata, M.*, Nishimaki-Mogami, T. and Sato, R*. **PPARalpha gene expression is up-regulated by LXR and PXR activators in the small intestine**

Biochem. Biophys. Res. Commun., **371**, 675-678 (2008)

LXR, PXR, and PPARalpha are members of a nuclear receptor family which regulate the expression of genes involved in lipid metabolism. Here, we show the administration of T0901317 stimulates PPARalpha gene expression in the small intestine but not in the liver of both normal and FXR-null mice. The administration of LXR specific ligand GW3965, or PXR specific ligand PCN has the same effect, indicating that ligand-dependent activation of LXR and PXR, but not FXR, is responsible for the increased gene expression of PPARalpha in the mouse small intestine.

Keywords: PPARalpha, LXR, mRNA

* 東京大学大学院農学生命科学研究科

Tamehiro, N.*1, Zhou, S.*1, Okuhira, K., Benita, Y.*1, Brown, C.E.*1, Zhuang, D.Z.*1, Latz, E.*2, Hornemann, T.*3, von Eckardstein, A.*3, Xavier, R.J.*1, Freeman, M.W.*1 and Fitzgerald, M.L.*1 : **SPTLC1 binds ABCA1 to negatively regulate trafficking and cholesterol efflux activity of the transporter**

Biochemistry, **47**, 6138-6147 (2008)

ABCA1 transport of cholesterol and phospholipids to nascent HDL particles plays a central role in lipoprotein metabolism and macrophage cholesterol homeostasis. ABCA1 activity is regulated both at the transcriptional level and at the post-translational level. To explore mechanisms involved in the post-translational regulation of the transporter, we have used affinity purification and mass spectrometry to identify proteins that bind ABCA1 and influence its activity. Previously, we demonstrated that an interaction between beta1-syntrophin stimulated ABCA1 activity, at least in part, be slowing the degradation of the transporter. This work demonstrates that one subunit of the serine palmitoyltransferase enzyme, SPTLC1, but not subunit 2 (SPTLC2), is copurified with ABCA1 and negatively regulates its function. In human THP-I macrophages and in mouse liver, the ABCA1-SPTLC1 complex was detected by co-immunoprecipitation, demonstrating that the interaction occurs in cellular settings where ABCA1 activity is critical for HDL genesis. Pharmacologic inhibition of SPTLC1 with myriocin, which resulted in the disruption of the SPTLC1-ABCA1 complex, and siRNA knockdown of SPTLC1 expression both stimulated ABCA1 efflux by nearly 60% ($p < 0.05$). In contrast, dominant-negative mutants of SPTLC1 inhibited ABCA1 efflux, indicating that a reduced level of sphingomyelin synthesis could not explain the effect of myriocin on ABCA1 activity. In 293 cells, the SPTLC1 inhibition of ABCA1 activity led to the blockade of the exit of ABCA1 from the endoplasmic reticulum. In contrast, myriocin treatment of macrophages increased the level of cell surface ABCA1. In composite, these results indicate that the physical interaction of ABCA1 and SPTLC1 results in reduction of ABCA1 activity and that inhibition of this interaction produces enhanced cholesterol efflux.

Keywords: ABCA1, SPTLC1, HDL

*1 Massachusetts General Hospital, Harvard Medical School
*2 University of Massachusetts Medical School.
*3 University Hospital Zurich.

Yin, T.*, Maekawa, K., Kamide, K.*, Saito, Y., Hanada, H.*, Miyashita, K.*, Kokubo, Y.*, Akaiwa, Y.*, Otsubo, R.*, Nagatsuka, K.*, Otsuki, T.*, Horio, T.*, Takiuchi, S.*, Kawano, Y.*, Minematsu, K.*, Naritomi, H.*, Tomoike, H.*, Sawada, J. and Miyata, T.* : **Genetic variations of *CYP2C9* in 724 Japanese individuals and their**

impact on the antihypertensive effects of losartan
Hypertens. Res., **31**, 1549-1557 (2008)

CYP2C9, a drug-metabolizing enzyme, converts the angiotensin II receptor blocker losartan to its active form, which is responsible for its antihypertensive effect. We resequenced CYP2C9 in 724 Japanese individuals, including 39 hypertensive patients under treatment with losartan. Of two novel missense mutations identified, the Arg132Gln variant showed a fivefold lower intrinsic clearance toward diclofenac when expressed in a baculovirus-insect cell system, while the Arg335Gln variant had no substantial effect. Several known missense variations were also found, and approximately 7% of the Japanese individuals (53 out of 724) carried one of the deleterious alleles (*CYP2C9 *3*, **13*, **14*, **30*, and Arg132Gln) as heterozygotes. After 3 months of losartan treatment, systolic blood pressure was not lowered in two patients with *CYP2C9 * 1/*30*, suggesting that they exhibited impaired in vivo CYP2C9 activity. *CYP2C9 *30* might be associated with a diminished response to the antihypertensive effects of losartan.

Keywords: genetic polymorphism, CYP2C9, function

* 国立循環器病センター

Kim, S.R., Saito, Y., Maekawa, K., Sugiyama, E., Kaniwa, N., Ueno, H.[*1], Okusaka, T.[*1], Ikeda, M.[*1], Morizane, C.[*1], Yamamoto, N.[*1], Yoshida, T.[*1], Kamatani, N.[*2], Furuse, J.[*1], Ishii, H.[*1], Saijo, N.[*1], Ozawa, S. and Sawada, J. : **Twenty novel genetic variations and haplotype structures of the *DCK* gene encoding human deoxycytidine kinase (dCK)**
Drug Metab. Pharmacokinet., **23**, 379-384 (2008)

Deoxycytidine kinase (dCK) is a rate-limiting enzyme in the activation of nucleoside anticancer drugs, such as gemcitabine and cytarabine (Ara-C), to their active metabolites. In this study, the 5'-flanking region, 7 exons and their flanking introns of *DCK* were comprehensively screened for genetic variations in 256 Japanese cancer patients administered gemcitabine. Twenty-nine genetic variations, including twenty novel ones, were found: 11 in the 5'-flanking region, 1 in the 5'-untranslated region (UTR), 1 in the coding exon, 9 in the 3'-UTR, and 7 in the introns. The novel variations included -1110C>T, -757G>A, -639C>T, -465G>A, -402T>C, -224C>A, -199C>G, IVS1+38G>T, IVS2+78_+83delTTTTTC, IVS3-9C>T, IVS4+12T>C, IVS5+39T>C, 1357A>G, 1545A>T, 1572delA, 1736G>A, 1749G>A, 1838T>C, 1889G>A, and 2048A>T. The frequencies were 0.01 for IVS2+78_ +83delTTTTTC, 0.008 for -402T>C, 0.006 for -639C>T and IVS4+12T>C, 0.004 for -757G>A and 1572delA, and 0.002 for the other 14 variations. A known nonsynonymous SNP 364C>T (Pro122Ser) was detected at a 0.061 frequency. Using the detected polymorphisms, linkage disequilibrium analysis was performed, and 24 haplotypes were identified or inferred. Our findings suggest considerable ethnic differences in genetic variations of *DCK* and provide fundamental and useful information for genotyping *DCK* in the Japanese and probably other Asian populations.

Keywords: genetic polymorphism, *DCK*, gemcitabine

[*1] 国立がんセンター
[*2] 東京女子医科大学

Saito, Y., Sai, K., Maekawa, K., Kaniwa, N., Shirao, K.[*], Hamaguchi, T.[*], Yamamoto, N.[*], Kunitoh, H.[*], Ohe, Y.[*], Yamada, Y.[*], Tamura, T.[*], Yoshida, T.[*], Minami, H.[*], Ohtsu, A.[*], Matsumura, Y.[*], Saijo, N.[*] and Sawada, J. : **Close association of *UGT1A9* IVS1+399C>T with *UGT1A1*28*, **6*, or **60* haplotype and its apparent influence on 7-ethyl-10-hydroxycamptothecin (SN-38) glucuronidation in Japanese**
Drug Metab. Dispos., **37**, 272-276 (2009)

The anticancer prodrug, irinotecan, is converted to its active form 7-ethyl-10-hydroxycamptothecin (SN-38) by carboxylesterases, and SN-38 is inactivated by UDP-glucuronosyltransferase (UGT)1A1-mediated glucuronidation. UGT1A9 also mediates this reaction. In a recent study, it was reported that the *UGT1A9* IVS1+399 (I399)C>T polymorphism is associated with increased SN-38 glucuronidation both in vitro and in vivo. However, its role in UGT1A9 expression levels and activity is controversial. Thus, we evaluated the role of I399C>T in SN-38 glucuronidation using 177 Japanese cancer patients administered irinotecan. I399C>T was detected at a 0.636 allele frequency. This polymorphism was in strong linkage disequilibrium (LD) with *UGT1A9*1b* (-126_-118T_9>T_{10}, |D'| = 0.99) and *UGT1A1*6* (211G>A, 0.86), in moderate LD with *UGT1A1*60* (-3279T>G, 0.55), but weakly associated with *UGT1A1*28* (-54_-39A$(TA)_{(6)}$TAA>A$(TA)_{(7)}$TAA, 0.25). Haplotype analysis showed that 98% of the I399C alleles were linked with low-activity haplotypes, either *UGT1A1*6*, **28*, or **60*. On the other hand, 85%

of the T alleles were linked with the *UGT1A1* wild-type haplotype **1*. Although I399T-dependent increases in SN-38 glucuronide/SN-38 area under concentration-time curve (AUC) ratio (an in vivo marker for UGT1A activity) and decreases in SN-38 AUC/dose were apparent ($P < 0.0001$), these effects were no longer observed after stratified patients by *UGT1A1*6*, **28*, or **60* haplotype. Thus, at least in Japanese populations, influence of I399C>T on SN-38 glucuronidation is attributable to its close association with either *UGT1A1*6*, **28*, or **60*.

Keywords: genetic polymorphism, *UGT1A9*, irinotecan

* 国立がんセンター

Tatewaki, N., Maekawa, K., Katori, N., Kurose, K., Kaniwa, N., Yamamoto, N.*, Kunitoh, H.*, Ohe, Y.*, Nokihara, H.*, Sekine, I.*, Tamura, T.*, Yoshida, T.*, Saijo, N.*, Saito, Y. and Sawada, J. : **Genetic variations and haplotype structures of the glutathione S-transferase genes, *GSTT1* and *GSTM1*, in a Japanese patient population**

Drug Metab. Pharmacokinet., **24**, 118-126 (2009)

Glutathione S-transferases (GSTs) play a vital role in phase II biotransformation of many synthetic chemicals including anticancer drugs. Deletion polymorphisms in *GSTT1* and *GSTM1* are reportedly associated, albeit controversial, with an increased risk in cancer as well as with altered responses to chemotherapeutic drugs. In this study, to elucidate the haplotype structures of *GSTT1* and *GSTM1*, genetic variations were identified in 194 Japanese cancer patients who received platinum-based chemotherapy. Homozygotes for deletion of *GSTT1* (*GSTT1*0/*0* or null) and *GSTM1* (*GSTM1*0/*0* or null) were found in 47.4% and 47.9% of the patients, respectively, while 23.2% of the patients had both *GSTT1* null and *GSTM1* null genotypes. From homozygous (+/+) and heterozygous (**0*/+) patients bearing *GSTT1* and *GSTM1* genes, six single nucleotide polymorphisms (SNPs) for *GSTT1* and 23 SNPs for *GSTM1* were identified. A novel SNP in *GSTT1*, 226C>A (Arg76Ser), and the known SNP in *GSTM1*, 519C>G (Asn173Lys, **B*), were found at frequencies of 0.003 and 0.077, respectively. Using the detected variations, *GSTT1* and *GSTM1* haplotypes were identified/inferred. Three and six common haplotypes (N> or =10) in *GSTT1* and *GSTM1*, respectively, accounted for most (>95%) inferred haplotypes. This information would be useful in pharmacogenomic studies of xenobiotics including anticancer drugs.

Keywords: genetic polymorphism, *GSTM1*, *GSTT1*

* 国立がんセンター

Maekawa, K., Yoshimura, T.*1, Saito, Y., Fujimura, Y.*1, Aohara, F.*1, Emoto, C.*2, Iwasaki, K.*2,3, Hanioka, N.*4, Narimatsu, S.*4, Niwa, T.*1 and Sawada, J. : **Functional characterization of CYP3A4.16: catalytic activities toward midazolam and carbamazepine**

Xenobiotica. **39**, 140-147 (2009)

To assess the substrate-dependent effects of the low-activity allele of human *CYP3A4*, *CYP3A4*16* (Thr185Ser), a recombinant wild-type (CYP3A4.1) or variant (CYP3A4.16) protein was co-expressed with human NADPH-P450 reductase in Sf21 insect cells using a baculovirus-insect cell system. The holo-CYP3A4 protein level of CYP3A4.16 in insect microsomes was slightly higher than that of CYP3A4.1, while no difference in total (apo- and holo-) CYP3A4 protein levels was observed between them. When midazolam was used as a substrate, *K*m and *V*max for 1'-hydroxylation in CYP3A4.16 were significantly higher and lower, respectively, than those in the wild-type, resulting in a 50% decrease in intrinsic clearance (*V*max / *K*m) of the variant. In contrast, intrinsic clearance for 4-hydroxylation of the variant was decreased by 30% due to a significant increase in *K*m without a difference in *V*max. Both the wild-type and variant exhibited sigmoidal kinetic profiles for carbamazepine 10,11-epoxide formation. When the modified two-site equation was applied for the analysis of kinetic parameters, Km_2 and $Vmax_2$ of CYP3A4.16 were approximately two times higher and lower than those of the wild-type, resulting in a 74% decrease in intrinsic clearance. These results demonstrated that CYP3A4.16 shows the substrate-dependent altered kinetics compared with CYP3A4.1.

Keywords: genetic polymorphism, CYP3A4, function

*1 田辺三菱製薬（株）
*2 ファイザー（株）
*3 （株）新日本科学
*4 岡山大学大学院医歯薬学総合研究科

Sai, K., Saito, Y., Fukushima-Uesaka, H., Kurose, K., Kaniwa, N., Kamatani, N.*1, Shirao, K.*2, Yamamoto, N.*2, Hamaguchi, T.*2, Kunitoh, H.*2, Ohe, Y.*2, Tamura, T.*2, Yamada, Y.*2, Minami, H.*2, Ohtsu, A.*2, Yoshida, T.*2,

Saijo, N.[*2] and Sawada, J. : **Impact of CYP3A4 haplotypes on irinotecan pharmacokinetics in Japanese cancer patients**

Cancer Chemother. Pharmacol., **62**, 529-537(2008)

Cytochrome P450 3A4 (CYP3A4) converts an anticancer prodrug, irinotecan, to inactive metabolites such as APC. However, the contribution of *CYP3A4* genetic polymorphisms to irinotecan pharmacokinetics (PK) and pharmacodynamics (PD) is not fully elucidated. In this study, the effects of *CYP3A4* haplotypes on irinotecan PK/PD were investigated in 177 Japanese cancer patients who received irinotecan. Area under the concentration-time curve ratios of APC/irinotecan, an in vivo parameter for CYP3A4 activity, were significantly higher in females than in males. The male patients with **16B* showed significantly decreased AUC ratios (APC/irinotecan) with 50% of the median value of the non-**16B* male patients (no **16B*-bearing female patients in this study). A slight trend toward increasing AUC ratios (20%) was detected in both male and female patients bearing **1G*. Multivariate analysis confirmed contributions of *CYP3A4*16B* and **1G* to the AUC ratio. However, no significant association was observed between the *CYP3A4* genotypes and total clearance of irinotecan or toxicities (severe diarrhea and neutropenia). This study suggested that *CYP3A4*16B* was associated with decreased metabolism of irinotecan to APC. However, the clinical impact of *CYP3A4* genotypes on total clearance and irinotecan toxicities was not significant.

Keywords: *CYP3A4*, irinotecan, genetic polymorphism

[*1] 東京女子医科大学

[*2] 国立がんセンター

Upham, B.L.[*], Park, J.S.[*], Babica, P.[*], Sovadinova, I.[*], Rummel, A.M.[*], Trosko, J.E.[*], Hirose, A., Hasegawa, R., Kanno, J. and Sai, K. : **Structure-activity-dependent regulation of cell communication by perfluorinated fatty acids using in vivo and in vitro model systems**

Environ. Health Perspect., **117**, 545-551(2009)

Perfluoroalkanoates, [e.g., perfluorooctanoate (PFOA)], are known peroxisome proliferators that induce hepatomegaly and hepatocarcinogenesis in rodents, and are classic non-genotoxic carcinogens that inhibit in vitro gap-junctional intercellular communication (GJIC). This inhibition of GJIC is known to be a function of perfluorinated carbon lengths ranging from 7 to 10. The aim of this study was to determine if the inhibition of GJIC by PFOA but not perfluoropentanoate (PFPeA) observed in F344 rat liver cells in vitro also occurs in F344 rats in vivo and to determine mechanisms of PFOA dysregulation of GJIC using in vitro assay systems. PFOA inhibited GJIC and induced hepatomegaly in rat livers, whereas PFPeA had no effect on either end point. Serum biochemistry of liver enzymes indicated no cytotoxic response to these compounds. In vitro analysis of mitogen-activated protein kinase (MAPK) indicated that PFOA, but not PFPeA, can activate the extracellular receptor kinase (ERK). Inhibition of GJIC, in vitro, by PFOA depended on the activation of both ERK and phosphatidylcholine-specific phospholipase C (PC-PLC) in the dysregulation of GJIC in an oxidative-dependent mechanism. The in vitro analysis of GJIC, an epigenetic marker of tumor promoters, can also predict the in vivo activity of PFOA, which dysregulated GJIC via ERK and PC-PLC.

Keywords: GJIC, PFOA, MAPK

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[*] Michigan State University

Satoh, R., Koyano, S., Takagi, K., Nakamura, R, Teshima, R., Sawada, J. : **Immunological Characterization and mutational analysis of the recombinant protein BWp16, a major allergen buckwheat**

Biol.Pharm. Bull. **31**, 1079-1085 (2008)

Buckwheat allergy is one of the most critical diseases manifested by severe and dangerous symptoms in Japan and other countries. We previously isolated the cDNA encoding protein BWp16, a member of the 2S albumin family with a conserved motif of 8 cysteine (Cys) residues. Comparison of the deduced amino acid sequences of BWp16 and related proteins in the 2S albumin family showed similarities between BWp16 and BW 8-kDa from buckwheat, Ara h 6 from peanuts and Ric c 1 from castor bean. Purified recombinant BWp16 (rBWp16) expressed in Escherichia coli was recognized by >80% of sera from patients with positive for IgE binding to buckwheat. Mutational analysis of rBWp16 revealed that 7 out of 10 mutants in the Cys residues showed weaker IgE binding to patient's serum than wild-type rBWp16 (rBWp16 WT). Mutations of Cys65 and Cys66 in rBWp16 decreased the pepsin digestibility of the protein, and an ELISA inhibition assay revealed a weaker inhibitory effect of rBWp16 C65S than that of rBWp16 WT. These results suggest that the Cys residues, especially Cys65, are involved in

the allergenicity of rBWp16. Our findings provide new evidence for the role of Cys residues in 2S albumin family proteins and open the door to the production of hypoallergens and application to safe diagnostic methods and allergen-specific immunotherapy of buckwheat allergy.

Keywords: buckwheat allergen, IgE, hypoallergen

Teshima, R., Nakamura, R., Nakamura, R., Hachisuka, A., Sawada, J., Shibutani, M.* : **Effects of Exposure to Decabromodiphenyl Ether on the Development of the Immune System in Rats**

J.Health Sci., **54**(4), 382-389 (2008)

To evaluate the developmental toxicity of decabromodiphenylether (DBDE) after exposure during the period from late gestation to after lactation, maternal Sprague-Dawley rats were given DBDE at dietary concentrations of 0, 10, 100, and 1000 ppm from gestational day 10 (GD 10) to postnatal day (PND) 21. On PND 21 and 77, lymphocytes (Lymph) in the spleen, thymus, and peripheral blood of male pups were subjected to flow cytometric analyses for expression of surface markers [CD3, CD4, CD8a, CD25, CD45RA, CD71, and CD161(NKRP1A)]. On PND 21, the proportions of splenic CD4+ T cells in the 10-ppm group, activated B (CD45RA+CD71+) cells in the 100- to 1000-ppm groups, and activated T cells (CD3+CD71+) in the 1000-ppm group were significantly decreased, and the population of peripheral CD161+ natural kiler cells on PND 21 and 77 had decreased in the 100-to 1000-ppm groups. In the 1000-ppm group, the serum T3 level was significantly decreased on PND 21 and the serum T4 level was decreased on PND 77. The body, spleen, and thymus weights were not significantly decreased, but liver weight was significantly increased on PND 21 in the 10- to 1000-ppm groups. These results suggest that on PND 21, developmental exposure to the highest dose of DBDE had a weak immunomodulatory effect. Although the most of the immunomodulatory effect had recovered to normal levels on PND 77, a decreasing effect on the natural killer (NK) cell population remained.

Keywords: decabromodiphenylether (DBDE), flow cytometry, immunotoxicity

* Tokyo University of Agriculture and Technology

Nakamura, R., Hachisuka, A., Sato, Y., Nakamura, R., Shibutani, M.*1, Sawada, J., Teshima, R. : **Effect of perinatal exposure to the flame-retardant tetrabromobisphenol A on the developing immune system of rats**

Bulletin of National Institute of Health Sciences, **126**, 65-70 (2008)

Tetrabromobisphenol A (TBBPA) is very popular flame retardant, that is often used as an industrial laminate for printed circuit boards. TBBPA is widely detected in the environment and is known to be transferred from dams to fetuses and offspring through the placenta and milk, respectively. Recent studies have also shown that TBBPA might modulate the thyroid hormone axis; however, the relation between the perinatal exposure of dams to TBBPA and the developing immune system of offspring has not yet been investigated. Here, we exposed maternal rats to TBBPA (0, 100, 1000, and 10000 ppm) in their diet beginning on gestational day 10. The exposure of the offspring was ceased by weaning at postnatal week (PNW) 3, and the subpopulational changes in the immune cells of the offspring were analyzed by flowcytometry at PNW3 and PNW11. The T3 hormone levels of the offspring were slightly decreased from 1.31 ng/ml to 1.13 ng/ml when their mother was exposed to 100 ppm TBBPA. We found that perinatal exposure to a high-dose (> 1000 ppm) of TBBPA caused a decrease in T cells and increases in regulatory T cells and NK cells in the spleens of the offspring at PNW11. We also found that increases in T cells and Treg cells in the peripheral blood at PNW11. However, body weight, immune-related organ weight, and the production of an antibody against KLH was not affected by exposure to TBBPA.

Keywords: brominated flame retardant, tetrabromobisphenol A, developing immune system, T cell subpopulation, thyroid hormone

*1 Tokyo University of Agriculture and Technology

Yoshioka, Y., Akiyama, H., Nakano, M.*1, Shoji, T.*2, Kanda, T.*2, Ohtake, Y.*2, Takita, T.*1, Matsuda, R., Maitani, T. : **Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice**

Int Immunopharmacol. **20**, 1802-1807 (2008)

Apple procyanidins (ACT) is a natural biologically active compound extracted from apple. Our recent studies have shown that ACT ameliorates the symptoms of atopic dermatitis and inhibits food-allergen-induced oral sensitiza-

tion. The aim of this study was to investigate the potential protective effect and mechanism of action of ACT in a murine model of inflammatory bowel disease. We investigated the preventive effects of ACT in experimental models of colitis induced by dextran sulfate sodium (DSS) or oxazolone. Oral administration of ACT before DSS treatment attenuated the DSS-induced mortality rate and decreased body weight loss. ACT also prevented the body weight loss associated with oxazolone-induced colitis. Next we examined the effect of ACT on intraepithelial lymphocytes (IEL), which is a major T cell population in the intestine. Oral administration of ACT increased the proportions of TCRγδ and TCRαβ-CD8αα T cells in IEL and suppressed interferon γ synthesis in stimulated IEL. In addition, ACT inhibited phorbol 12-myristate 13-acetate-induced secretion of interleukin 8 (IL-8) in intestinal epithelial cells. The combined anti-inflammatory and immunomodulatory effects of ACT on intestinal epithelial cells and IEL suggest that it may be an effective oral preventive agent for inflammatory bowel diseases.

Keywords: Colitis, Intestinal epithelial cell, Intraepithelial lymphocyte

[*1] Department of Nutritional Science, Faculty of Applied Biological Science, Tokyo University of Agriculture
[*2] Fundamental Research Laboratory, Asahi Breweries

Shimizu, E.[*1], Kato, H.[*2], Nakagawa, Y.[*2], Kodama, T.[*3], Futo, S.[*1], Minegishi, Y.[*4], Watanabe, T., Akiyama, H., Teshima, R., Furui, S.[*5], Hino, A.[*5], Kitta, K.[*5] : **Development of a screening method for genetically modified soybean by plasmid-based quantitative competitive polymerase chain reaction**

J. Agric. Food Chem. **56**, 5521-5527 (2008)

A novel type of quantitative competitive polymerase chain reaction (QC-PCR) system for the detection and quantification of the Roundup Ready® soybean (RRS) was developed. This system was designed based on the advantage of a fully validated real-time PCR method for the quantification of RRS used in Japan. A plasmid was constructed as competitor plasmid for the detection and quantification of the GM soy, RRS. The plasmid contained the construct specific sequence of RRS and the taxon specific sequence of lectin1 (Le1), and both had 21 bp oligonucleotide insertion in the sequences. The plasmid DNA as a reference molecule was used in place of ground raw materials, which enabled us to adjust the copy number of targets precisely and stably. The present study indicated that the novel PQC-PCR method could be a simple, feasible and the alternative way to real-time PCR method for the quantification of GMO contents.

Keywords: quantitative competitive-PCR (QC-PCR), Roundup Ready® soybean (RRS), plasmid based QC-PCR (PQC-PCR)

[*1] Fasmac Co., Ltd.
[*2] Showa Sangyo Co., Ltd.
[*3] Food and Agricultural Materials Inspection Center
[*4] Nippon Gene Co., Ltd.
[*5] National Food Research Institute

Mano, J.[*1], Oguchi, T.[*1], Akiyama, H., Teshima, R., Hino, A.[*1], Furui, S.[*1], Kitta K.[*1] : **Simultaneous Detection of Recombinant DNA Segments Introduced into Genetically Modified Crops with Multiplex Ligase Chain Reaction Coupled with Multiplex Polymerase Chain Reaction**

J. Agric. Food Chem., **57**, 2640-2646 (2009)

We developed a multiplex polymerase chain reaction (PCR)-multiplex ligase chain reaction (LCR) (MPCR-MLCR) technique as a novel approach for the simultaneous detection of recombinant DNA segments (e.g., promoters, trait genes, and terminators) of genetically modified (GM) crops. With this technique, target DNA regions were amplified by multiplex PCR, the PCR products were subjected to the following multiplex LCR as template DNAs, and the LCR products were then analyzed by polyacrylamide gel electrophoresis and subsequent fluorescent scanning. Seven recombinant DNA segments commonly introduced into some GM crop lines were selected as target DNA regions. In addition, another MPCR-MLCR system for the simultaneous detection of three endogenous DNA segments was designed as a positive control test. The specificity and sensitivity of the method were examined. The method allowed us to detect GM crops comprehensively and is expected to be utilized for efficient screening of GM crops into which any one of the seven recombinant DNA segments have been introduced, and for profiling the segments.

Keywords: genetically modified (GM), real-time PCR array, TaqMan® assay

[*1] National Food Research Institute

Mano, J.[*1], Shigemitsu, N.[*1], Futo, S.[*2], Akiyama, H., Teshima, R., Hino, A.[*1], Furui, S.[*1], Kitta K.[*1] : **Real-**

time PCR array as a universal platform for the detection of genetically modified crops and its application in identifying unapproved genetically modified crops in Japan

J. Agric. Food Chem., **57**, 26-37 (2009)

We developed a novel type of real-time polymerase chain reaction (PCR) array with TaqMan® chemistry as a universal platform for the comprehensive and semiquantitative detection of genetically modified (GM) crops. Thirty primer-probe sets for the specific detection of GM lines, recombinant DNA (r-DNA) segments, endogenous reference genes and donor organisms were synthesized and a 96-well PCR plate was prepared with a different primer-probe in each well as the real-time PCR array. The specificity and sensitivity of the array were evaluated. A comparative analysis with the data and publicly available information on GM crops approved in Japan allowed us to assume the possibility of unapproved GM crop contamination. Furthermore, we designed a Microsoft Excel® spreadsheet application, Unapproved GMO Checker version 2.01, which helps process all the data of real-time PCR arrays for the easy assumption of unapproved GM crop contamination.

Keywords: multiplex PCR, genetically modified (GM), ligase chain reaction (LCR)

[*1] National Food Research Institute

[*2] FASMAC Co., Ltd.

Yamakawa, H.[*1], Akiyama, H., Endo, Y.[*1], Miyatake, K.[*1], Sakata, K., Sakai, S., Toyoda, M.[*2] Urisu, A.[*3], Teshima, R. : **Specific Detection of Buckwheat Residues in Processed Foods by Polymerase Chain Reaction**

Biosci. Biotech. Biochem., **72**, 312-316 (2008)

A sensitive qualitative detection method for buckwheat in foods using polymerase chain reaction (PCR) was developed. Trace amounts of buckwheat in commercial food products could be qualitatively detected by this method. The sensitivity of the proposed PCR method appears to be similar to that of ELISA. The present method should be reliable for detecting buckwheat residues in processed foods and practical for monitoring the labeling system for allergenic food material.

Keywords: buckwheat, *Fagopyrum esculentum*, polymerase chain reaction (PCR)

[*1] Nisshin Seifun Group Inc.

[*2] Jissen Woman's University

[*3] Fujita Health University The Second Teaching Hospital

Morishita, N.[*1], Kamiya, K.[*1], Matsumoto, T.[*1], Sakai, S., Teshima, R., Urisu, A.[*2], Moriyama, T.[*3], Ogawa, T. [*4], Akiyama, H., Morimatsu, F.[*1] : **A Reliable Enzyme-Linked Immunosorbent Assay for Determination of Soybean Proteins in Processed Foods**

J. Agric. Food Chem. **56**, 6818-6824 (2008)

Among allergenic foods, soybean is known as a food causing adverse reactions in allergenic patients. To clarify the validity of labeling, the specific and sensitive detection method for the analysis of the soybean protein would be necessary. The p34 protein, originally characterized to be p34 as an oil-body associated protein in soybean, has been identified one of the major allergenic proteins and named as Gly m Bd 30K. A novel sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of the soybean protein in processed foods was developed using polyclonal antibodies raised against p34 as a soybean marker protein and the specific extraction buffer for extract. The developed sandwich ELISA method was highly specific for the soybean protein. The limit of detection (LOD) and the limit of quantification (LOQ) of the developed ELISA were 0.47 ng/mL (equivalent to 0.19 μg/g in foods) and 0.94 ng/mL (equivalent to 0.38 μg/ in foods), respectively. The recovery ranged from 87.7 to 98.7 %, while the intra- and inter-assay coefficients of variation were less than 4.2 % and 7.5 %, respectively. This study showed that the developed ELISA method is a specific, precise and reliable tool for the quantitative analysis of the soybean protein in processed foods.

Keywords: enzyme-linked immunosorbent assay (ELISA), Gly m Bd 30K, allergen

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[*1] R&D Center Nippon Meat Packers, Inc.

[*2] The Second Teaching Hospital, Fujita Health University

[*3] Kinki University .

[*4] Kansai University of Welfare Sciences

Doi, H.[*1], Shibata, H.[*1], Shoji, M.[*1], Sakai, S., Urisu, A.[*2], Akiyama, H., Teshima, R. : **A Reliable Enzyme-Linked Immunosorbent Assay for the Determination of Walnut Proteins in Processed Foods**

J. Agric. Food Chem. **56**, 7625-7630 (2008)

Among food allergens of tree nuts, walnuts are a frequent cause of adverse food reactions in allergic patients. A novel sandwich enzyme linked immunosorbent assay

(ELISA) for the detection and the quantification of walnut soluble proteins in processed foods was developed. The sandwich ELISA method is highly specific for walnut soluble proteins. The recovery ranged from 83.4 to 123 %, while the intra- and interassay coefficients of variation were less than 8.8 % and 7.2 %, respectively. This study showed that the proposed method is a reliable tool for detection in the presence of hidden walnut proteins in processed foods.

Keywords: enzyme-linked immunosorbent assay (ELISA), 2S-Albumin, food allergy

*1 Morinaga Institute of biological Science Inc..
*2 The Second Teaching Hospital, Fujita Health University

Harikai, N.*1, Saito, S.*1, Abe, M.*1, Kondo, K., Kitta, K.*2, Akiyama, H., Teshima, R., Kinoshita, K.*1 : **A Real-Time PCR Method Using Capture Oligo-Immobilized PCR Tubes to Determine the Specific Gene for Soybean and Genetically Modified Soybean in Food Matrices**

Biosci. Biotech. Biochem., **72**, 2953-2958 (2008)

A new single-tube real-time PCR method using capture oligo immobilized PCR tube was developed to determine the amount of soybean and genetically modified (GM) soybean in foods. Hybridization conditions, such as location, length and amount of capture oligo and incubation time and temperature, were examined using soybean genomic DNA in the tube immobilized a capture oligo for lectin gene (*Le1*). Under optimized conditions, the copy number of *Le1* was dose-dependently determined from soybean genomic DNA and soybean lysate (DNA 10-1000 ng: r = 0.99, lysate 1-100%: r = 0.99). Using PCR tube immobilized a capture oligo for transgene in Roundup Ready soybean (*RRS*), the copy number of *RRS* in GM soybean lysate (1-100%: r = 0.99) was dose-dependently determined . The present studies suggest that the proposed method is rapid and simple way to determine the amount of soybean and GM soybean.

Keywords: DNA extraction method, genetically modified organism, hybridization, polymerase chain reaction

*1 Mukogawa Women's University
*2 National Food Research Institute

Oguchi,T.*1, Onishi,M.*2, Chikagawa, Y.*2, Kodama,T.*3, Suzuki, E.*1, Kasahara, M.*3, Akiyama, H., Teshima, R., Futo, S.*2, Hino, A.*1, Furui, S.*1, Kitta, K.*1 : **Investigation of Residual DNAs in Sugar from Sugar Beet (*Beta vulgaris L.*)**

J. Food Hyg. Soc. Japan, **50**, 41-46 (2009)

Genetically modified (GM) sugar beets have been bred to use for food and feed. To evaluate applicability of GM analyses on the processed foods of sugar beets, we investigated the residual DNA in the eight sorts of in-process beet sugar samples and the commercialized beet sugar products. Polymerase chain reaction (PCR) analyses with the taxonomic-specific primers indicated that sugar beet DNA were degraded at the early stage of the purification process of sugar and no detectable DNA remained in the investigated sugar products.

Keywords: genetically modified (GM), sugar beet (*Beta vulgaris L.*), deoxyribonucleic acid (DNA)

*1 National Food Research Institute
*2 Fasmac Co., Ltd.
*3 Food and Agricultural Materials Inspection Center

Toida, T.*1, Sato, K.*1, Sakamoto, N.*1, Sakai, S., Hosoyama, S.*1, Linhardt, R. J.*2 : **Solvolytic depolymerization of chondroitin and dermatan sulfates**

Carbohydr. Res., **344**, 888-893 (2009)

It is essential to establish a library of glycosaminoglycan oligosaccharides from the chondroitin and dermatan sulfates to investigate their biological functions and structure-activity relationships (SARs). There are several approaches to obtain oligosaccharides using chemical and enzymatic degradation procedures; however, purification of each resulting oligosaccharide is complicated because of the diversity of sulfonation patterns present in these oligosaccharides. We have developed a new method for the solvolytic degradation for chondroitin and dermatan sulfates to obtain an oligosaccharide mixture that can be easily purified into chondro/dermato oligosaccharides for characterization by both ^{1}H NMR and MALDI-TOFMS. These oligosaccharides have a methyl-esterified uronate residue and a methyl 2-acetamido-2-deoxy-D-galactofuranoside at the nonreducing and reducing ends, respectively. All other internal repeating disaccharide units were desulfonated, but maintained their core carbohydrate structures.

Keywords: chondroitin sulfate, dermatan sulfate, oligosaccharide depolymerization, solvolysis, sulfonation

*1 Graduate School of Pharmaceutical Sciences, Chiba University
*2 Rensselaer Polytechnic Institute

酒井信夫，松田りえ子，杉本敏明[*1]，米谷民雄[*2]：**野菜及び野菜加工食品に含まれる硝酸塩について**

日本食品化学学会誌，**15**，110-115 (2008)

国産野菜中の硝酸塩濃度についてHPLCを用いて定量した．夏季に収穫したホウレンソウは，冬季に収穫したものよりも硝酸塩濃度が有意に高かったのに対し，夏季に収穫したレタス，サニーレタス，及びダイコンは，冬季に収穫したものよりも硝酸塩濃度が有意に低かった．次に我々は，市販59加工食品中に含まれる硝酸塩濃度を定量した．それらに含まれる硝酸塩濃度は以下の通りである．ジュース (n=15)，5.9 ～ 652.5 μg/mL; スープ (n=13)，12.1 ～ 437.9 μg/mL; ペースト (n=3)，46.1 ～ 888.8 μg/mL; 錠剤 (n=13)，4.0 ～ 1373.5 μg/g; 顆粒粉末 (n=6)，37.1 ～ 309.7 μg/g; 菓子 (n=5)，3.1 ～ 72.6 μg/g; ふりかけ(n=2)，699.2 ～ 1147.4 μg/g; パスタ (n=2), 36.9 ～ 45.6 μg/g．これらの食品の過剰摂取により，JECFAの定める硝酸塩の一日許容摂取量を超過することが懸念されることから，我々は，健康危害を未然に防ぐために硝酸塩摂取に関する情報提供が必要であると考える．

Keywords: vegetables, processed foods, nitrate, high performance liquid chromatography

[*1] 財団法人日本食品分析センター
[*2] 静岡県立大学

Kubota, K., Iwasaki, E.[*1], Inagaki, S.[*1], Nokubo, T.[*1], Sakurai, Y[*2], Komatsu, M[*2], Toyofuku, H., Kasuga, F., Angulo, F. J.[*3], Morikawa, K. : **The Human Health Burden of Foodborne Infections Caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* in Miyagi Prefecture, Japan**

Foodborne Pathog Dis., **5**(5), 641-8 (2008)

To estimate the human health burden of foodborne infections caused by *Campylobacter, Salmonella*, and *Vibrio parahaemolyticus* in Japan, an epidemiological study was conducted in Miyagi Prefecture. Laboratoryconfirmed infections among patients with diarrhea caused by the three pathogens were ascertained from two clinical laboratories in the prefecture from April 2005 to March 2006. To estimate the number of ill persons who were not laboratory-confirmed, we estimated physician-consultation rates for patients with acute diarrhea by analyzing foodborne outbreak investigation data for each pathogen and the frequency at which stool specimens were submitted from a physician survey. Each factor was added to a Monte-Carlo simulation model as a probability distribution, and the number of laboratory-confirmed cases was extrapolated to estimate the total number of ill persons. The estimated incidence of foodborne infections per 100,000 per year in this region estimated by this model was 237 cases for *Campylobacter,* 32 cases for *Salmonella*, and 15 cases for *V. parahaemolyticus*. Simulated results indicate a significant difference between our estimated incidence and the reported cases of food poisoning in this region. An enhanced surveillance system is needed to complement the present passive surveillance on foodborne illnesses in Japan to identify food safety issues more precisely, and to monitor the effectiveness of risk management options.

Keywords: Burden, diarrhea, *Campylobacter, Salmonella*, *Vibrio parahaemolyticus*

[*1] Sendai Quarantine Station, Miyagi, Japan
[*2] Miyagi Medical Association, Miyagi, Japan
[*3] Centers for Disease Control and Prevention, Atlanta, Georgia, U.S.A.

Sato, S.[*1], Shirakawa, H.[*1], Tomita, S.[*2], Ohsaki, Y.[*1], Haketa, K.[*1], Tooi, O.[*3], Santo, N.[*4,5], Tohkin, M., Furukawa, Y.[*1], Gonzalez, F.J.[*6], Komai, M.[*1]. : **Low-dose dioxins alter gene expression related to cholesterol biosynthesis, lipogenesis, and glucose metabolism through the aryl hydrocarbon receptor-mediated pathway in mouse liver**

Toxicol. Appl. Pharmacol., **229**, 10-19 (2008)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a common environmental contaminant. TCDD binds and activates the transcription factor aryl hydrocarbon receptor (AHR), leading to adverse biological responses via the alteration of the expression of various AHR target genes. Although small amounts of TCDD are consumed via contaminated daily foodstuffs and environmental exposures, the effects of low-dose TCDD on gene expression in animal tissues have not been clarified, while a number of genes affected by high-dose TCDD were reported. In this study, we comprehensively analyzed gene expression profiles in livers of C57BL/6N mice that were orally administered relatively low doses of TCDD (5, 50, or 500 ng/kg body weight (bw) day^{-1}) for 18 days. The hepatic TCDD concentrations, measured by gas chromatography-mass spectrometry, were 1.2, 17, and 1063 pg toxicity equivalent quantity (TEQ)/g, respectively. The mRNA level of the cytochrome P450 CYP1A1 was significantly increased by treatment with only TCDD 500 ng/kg bw day^{-1}. DNA microarray and

quantitative RT-PCR analyses revealed changes in the expression of genes involved in the circadian rhythm, cholesterol biosynthesis, fatty acid synthesis, and glucose metabolism in the liver with at all doses of TCDD employed. However, repression of expression of genes involved in energy metabolism was not observed in the livers of Ahr-null mice that were administered the same dose of TCDD. These results indicate that changes in gene expression by TCDD are mediated by AHR and that exposure to low-dose TCDD could affect energy metabolism via alterations of gene expression.

Keywords: Dioxin, Mouse liver, DNA microarray

[*1] Laboratory of Nutrition, Graduate School of Agricultural Science, Tohoku University

[*2] Division of Experimental Immunology, Institute for Genome Research, University of Tokushima

[*3] Biotechnology Research Laboratory, Towa Environmental Science Co., Ltd.,

[*4] Medical and Environment Technology Co., Ltd.,

[*5] Research Center for Green Science

[*6] Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health

Kurose, K., Saeki, M., Tohkin, M. Hasegawa, R. : **Thyroid hormone receptor mediates human MDR1 gene expression−Identification of the response region essential for gene expression**

Arch. Biochem. Biophys., **474**, 82-90 (2008)

P-glycoprotein, encoded by the MDR1 gene, is a drug efflux transporter that is expressed in various tissues and plays an important role in the absorption and elimination of many drugs and xenobiotics. Induction of the MDR1 gene affects drug disposition and the efficacy of drug treatment. In this study, we demonstrated that the thyroid hormone receptor (TR) induces MDR1 gene expression in a thyroid hormone

(TH)-dependent manner. The 5'-upstream region of the human MDR1 gene was examined for the presence of TH-responsive elements. Luciferase-reporter gene assays revealed that the TH response region is located between -7.9 and -7.8 kb upstream from the transcription start site of MDR1. The region contains two TH response clusters, one of which includes a direct repeat with a three-nucleotide spacer (DR3) and a four-nucleotide spacer DR4(I), and the other of which includes two DR4s (II and III). Mutation analyses indicated that every direct repeat has a unique contribution to the TH response. In particular, DR4(I) was shown to be the most important element. Chromatin immunoprecipitation assays revealed that TR and retinoid X receptor (RXR) bind to the TH response region, and gel mobility shift assays confirmed that one molecule of TR/RXR heterodimer binds to each of the clusters in this region, with preferential binding to the upstream one. We furthermore demonstrated that two molecules of TR/RXR could bind simultaneously to the TH response region. The order of binding affinity to the direct repeats was DR4(I) > DR4(II) > DR4(III) ≈ DR3. Our results indicate that these two closely spaced TR/RXR-binding clusters are both required for the maximal induction of MDR1 gene expression mediated by TR.

Keywords: P-glycoprotein (P-gp), TR, Triiodothyronine (T3), Thyroxine (T4), Thyroid hormone response element (TRE), ABCB1

Saeki, M., Kurose, K., Tohkin, M., Hasegawa, R. : **Identification of the functional vitamin D response elements in the human MDR1 gene**

Biochem. Pharmacol., **76**, 531-342 (2008)

P-glycoprotein, encoded by the multidrug resistance 1 (*MDR1*) gene, is an efflux transporter and plays an important role in pharmacokinetics. The expression of MDR1 is induced by a variety of compounds, of which 1α,25-dihydroxyvitamin D3 is known to be an effective inducer. However, it remains unclear how 1α,25-dihydroxyvitamin D3 regulates the expression of *MDR1*. In this study, we demonstrated that the vitamin D receptor (VDR) induces *MDR1* expression in a 1α,25-dihydroxyvitamin D3-dependent manner. Luciferase assays revealed that the region between −7.9 and −7.8 kbp upstream from the transcription start site of the MDR1 is responsible for the induction by 1α,25-dihydroxyvitamin D3. Electrophoretic mobility shift assays revealed that several binding sites for the VDR/retinoid X receptor α (RXRα) heterodimer are located between the −7880 and −7810 bp region, to which the three molecules of VDR/RXRα are able to simultaneously bind with different affinities. Luciferase assays using mutated constructs revealed that the VDR-binding sites of DR3, DR4(I), MdC3, and DR4(III) contribute to the induction, indicating that these binding sites act as vitamin D response elements (VDREs). The contribution of each VDRE to the inducibility was different for each response element. An additive effect of the individual VDREs on induced luciferase activity by 1α,25-dihydroxyvitamin D3 was also

observed. These results indicate that the induction of *MDR1* by 1α,25-dihydroxyvitamin D3 is mediated by VDR/RXRα binding to several VDREs located between −7880 and −7810 bp, in which every VDRE additively contributes to the 1 α,25-dihydroxyvitamin D3 response.

Keywords: Multidrug resistance 1, P-glycoprotein (P-gp), Vitamin D receptor (VDR), 1α,25-Dihydroxyvitamin D3, Vitamin D response element (VDRE)

Ueno[*1], H., Kaniwa, N., Okusaka[*1], T., Ikeda[*1], M., Morizane[*1], C., Kondo[*1], S., Sugiyama, E., Kim, S. R., Hasegawa, R., Saito, Y., Yoshida[*2], T., Saijo[*3] N., and Sawada, J. : **Homozygous CDA*3 is a major cause of life-threatening toxicities in gemcitabine-treated Japanese cancer patients**

Br. J. Cancer **100**, 870 – 873, (2009)

ゲムシタビンの投与を受けた膵がん患者242名のうち，好中球減少症を含む重篤な副作用を示した3人の患者について，*CDA*遺伝子の多型，血漿CDA活性及び可能な場合には薬物動態を測定した．その結果，2人の患者が*CDA*3*（*CDA*208G>A）をホモ接合で保有しており，血漿中CDAの著しい低下，また，薬物動態が測定できた1名では，著しいゲムシタビンのクリアランスの低下が観測され，この2名の患者では，*CDA*3*によるCDAの活性低下が重篤な副作用の原因であることが判明した．残りの1名の患者は*CDA*3*を保有しておらず，重篤副作用の発現にCDAの活性低下が関与していないことが判明した．日本人においては，*CDA*3*ホモ接合がゲムシタビンによる重篤な副作用の発現の重要な要因の一つであることが明らかとなった．

Keywords: gemcitabine, cytidine deaminase, genetic polymorphism

[*1] 国立がんセンター東病院
[*2] 国立がんセンター研究所
[*3] 国立がんセンター中央病院

Kaniwa, N., Saito, Y., Aihara, M., Matsunaga, K., Tohkin, M., Kurose, K., Sawada, J., Furuya[*1], H., Takahashi[*1], Y., Muramatsu[*1], M., Kinoshita[*1], S., Abe[*1], M., Ikeda[*1], H., Kashiwagi[*1], M., Song[*1], Y., Ueta[*1], M., Sotozono[*1], C., Ikezawa[*1] Z., and Hasegawa, R. : **HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis**

Pharmacogenomics, **9**, 1617-1622 (2008)

スティーブンス・ジョンソン症候群（SJS）及び中毒性表皮壊死融解症（TEN）は，発生頻度は希であるが重篤な副作用である．近年，カルバマゼピン誘因性のSJS/TEN及びアロプリノール誘因性の重症薬疹と，HLAB*1502及びHLA-B*5801とが，それぞれ，非常に強い関連があることが台湾の漢民族を対象として研究で示された．しかし，このような関連には民族依存性があることも報告されている．そこで，58名の日本人患者を対象として，これら2つのHLAタイプと重症薬疹との関連を，ケース・コントロール研究により調べた．58名の症例の中で，HLAB*1502の保有者は検出されず，日本人において，カルバマゼピンや芳香族系抗てんかん薬誘因性のSJS/TENの発症との関連は認められなかった．一方，アロプリノール誘因性のSJS/TENの患者では，複数のHLA-B*5801の保有者がおり，日本人においても，アロプリノール誘因性のSJS/TENとHLA-B*5801との関連は認められた．

Keywords: Stevens-Johnson syndrome, toxic epidermal necrolysis, HLA-B locus

[*1] SJS/TEN遺伝子多型研究班

Sato[*1, 2], Y., Laird[*1], N. M., Nagashima[*3], K., Kato[*3], R., Hamano[*4], T., Yafune[*5], A., Kaniwa, N., Saito, Y., Sugiyama, E., Kim, S-R., Furuse[*6], J ., Ishii[*6], H., Ueno[*7], H., Okusaka[*7], T., Saijo[*6], N., Sawada J., and Yoshida[*2], T. : **A new statistical screening approach for finding pharmacokinetics-related genes in genome-wide studies**

Pharmacogenomics J., **9**, 137-146 (2009)

Biomedical researchers usually test the null hypothesis that there is no difference of the population mean of pharmacokinetics (PK) parameters between genotypes by the Kruskal–Wallis test. Although a monotone increasing pattern with a number of alleles is expected for PK-related genes, the Kruskal–Wallis test does not consider a monotonic response pattern. For detecting such patterns in clinical and toxicological trials, a maximum contrast method has been proposed. We show how that method can be used with pharmacogenomics data to a develop test of association. Further, using simulation studies, we compare the power of the modified maximum contrast method to those of the maximum contrast method and the Kruskal–Wallis test. On the basis of the results of those studies, we suggest rules of thumb for which statistics to use in a given situation. An application of all three methods to an actual genome-wide pharmacogenomics study illustrates the practical relevance of our discussion.

Keywords: genome-wide study, maximum contrast method, statistical screening method

[*1] ハーバード大学
[*2] 国立がんセンター研究所
[*3] 東京理科大学
[*4] Hamano Statistical Analysis Ltd.
[*5] クリニック千駄ヶ谷
[*6] 国立がんセンター東病院
[*7] 国立がんセンター中央病院

Matsubara[*1,5], J., Ono[*1], M., Negishi[*1], A., Ueno[*2], H., Okusaka[*2], T., Furuse[*3], J., Furuta[*3], K., Sugiyama, E., Saito, Y., Kaniwa, N., Sawada, J., Honda[*1], K., Sakuma[*4], T., Chiba[*5], T., Saijo[*3], N., Hirohashi[*1], S., Yamada[*1], T. : **Identification of a predictive biomarker for hematologic toxicities of gemcitabine**

J. Clin. Oncol., **27**, 2261-2268 (2009)

PURPOSE: Gemcitabine monotherapy is the current standard for patients with advanced pancreatic cancer, but the occurrence of severe neutropenia and thrombocytopenia can sometimes be life threatening. This study aimed to discover a new diagnostic method for predicting the hematologic toxicities of gemcitabine. PATIENTS AND METHODS: Using quantitative mass spectrometry (MS), we compared the baseline plasma proteomes of 25 patients who had developed severe hematologic adverse events (grade 3 to 4 neutropenia and/or grade 2 to 4 thrombocytopenia) within the first two cycles of gemcitabine with those of 22 patients who had not (grade 0). RESULTS: We identified 757 peptide peaks whose intensities were significantly different ($P < .001$, Welch t test) among a total of 60,888. The MS peak with the highest statistical significance ($P = .0000282$) was revealed to be derived from haptoglobin by tandem MS. A scoring system (nomogram) based on the values of haptoglobin, haptoglobin phenotype, neutrophil count, platelet count, and body-surface area was constructed to estimate the risk of hematologic adverse events (grade 3 to 4 neutropenia and/or grade 2 to 4 thrombocytopenia) with an area under curve value of 0.782 in a cohort of 166 patients with pancreatic cancer. Predictive ability of the system was confirmed in two independent validation cohorts consisting of 87 and 52 patients with area under the curve values of 0.655 and 0.747, respectively. CONCLUSION: Although the precise mechanism responsible for the correlation of haptoglobin with the future onset of hematologic toxicities remains to be clarified, our prediction model seems to have high practical utility for tailoring the treatment of patients receiving gemcitabine.

Keywords: gemcitabine, proteomics, neutropenia

[*1] 国立がんセンター研究所
[*2] 国立がんセンター中央病院
[*3] 国立がんセンター東病院
[*4] 三井情報科学株式会社.
[*5] 京都大学

伊集院一成[*1]，岩木和夫[*2]，林　譲，矢島毅彦[*3]：**薬局の在庫管理に対するFUMI理論の応用に関する研究**

社会薬学，**27**，7-18 (2008)

薬局間をインターネットでつなぐ薬局情報ネットワークを解説した.

Keywords: health vigilance, drug, pharmacy, FUMI theory

[*1] 田無薬品
[*2] 奥羽大学
[*3] 東邦大学

Ito, M.[*1], Fukuzawa, K.[*2], Mochizuki, Y.[*3], Nakano, T., Tanaka, S.[*1] : **Ab Initio Fragment Molecular Orbital Study of Molecular Interactions between Liganded Retinoid X Receptor and Its Coactivator; Part II: Influence of Mutations in Transcriptional Activation Function 2 Activating Domain Core on the Molecular Interactions**

J. Phys. Chem. A, **112**, 1986-1998 (2008)

The ab initio fragment molecular orbital (FMO) calculations were performed for retinoid X receptor (RXR) complexes with its ligand 9-cis retinoic acid (9cRA) and steroid receptor coactivator-1 (SRC1) to examine the influence of mutations in transcriptional activation function 2 activating domain core (AF2C) of RXR on molecular interactions between 9cRA liganded RXR and SRC1 coactivator.

Keywords: FMO, Retinoid X receptor, coactivator

[*1] 神戸大学
[*2] みずほ情報総研
[*3] 立教大学

Sato, M.[*1], Yamataka, H.[*1], Komeiji, Y.[*2], Mochizuki, Y.[*1], Ishikawa, T.[*1], Nakano, T. : **How Does an S_N2 Reaction Take Place in Solution? Full Ab Initio MD**

Simulations for the Hydrolysis of the Methyl Diazonium Ion

J. Am. Chem. Soc., **130**, 2396-2397 (2008)

FMO-MD法を用い，水溶液中のメチルジアゾニウムイオンのS_N2反応のシミュレーションを行った.

Keywords: FMO-MD, S_N2 reaction, Methyl Diazonium Ion

[*1] 立教大学
[*2] 産業技術総合研究所

Koyano, K.[*1], Nakano, T. : **Interaction of HIV-1 aspartic protease with its inhibitor, by molecular dynamics and ab initio fragment molecular orbital method**

J. Synchrotron Rad. 15, 239–242 (2008)

HIV-1プロテアーゼについて，古典MDとFMO法を用いた分子シミュレーションを行い，阻害剤との相互作用について解析を行った.

Keywords: FMO, HIV-1 protease, inhibitor

[*1] 東京大学

Mochizuki, Y.[*1], Yamashita, K.[*1], Murase, T.[*2], Nakano, T., Fukuzawa, K.[*3], Takematsu, K.[*4], Watanabe, H.[*4], Tanaka, S.[*4] : **Large scale FMO-MP2 calculations on a massively parallel-vector computer**

Chem. Phys. Lett. **457**, 396-403 (2008)

The fragment molecular orbital (FMO) calculations have been successfully applied to a variety of realistic biochemical problems, by using our original ABINIT-MP program. In these applications, the inclusion of electron correlation through the second-order Møller-Plesset perturbation (MP2) was demonstrated to be essential to obtain qualitatively correct descriptions. Recently, the FMO calculations in ABINIT-MP were tuned for a massively parallel-vector processing. A series of FMO-MP2/6-31G calculations were performed on the Earth Simulator by which up to 4,096 vector processors are available. The largest FMO-MP2 computation was carried out for an influenza hemagglutinin antigen–antibody system consisting of 921 residues, which was completed within one hour with 4,096 processors.

Keywords: FMO, earth simulator, parallel-vector computer

[*1] 立教大学
[*2] ＮＥＣソフト
[*3] みずほ情報総研
[*4] 神戸大学

Harada, T.[*1], Yamagishi, K.[*1], Nakano, T., Kitaura, K.[*2], Tokiwa, H.[*1] : **Ab initio fragment molecular orbital study of ligand binding to human progesterone receptor ligand-binding domain**

Naunyn-Schmiedeberg's Arch. Pharmacol., **377**, 607-615 (2008)

We applied the fragment molecular orbital (FMO) method, which enables total electronic calculations of large molecules at ab initio level, to the evaluation of binding affinities between the human progesterone receptor ligand-binding domain (PR LBD) and various steroidal ligands.

Keywords: Progesterone receptor, Binding affinity, FMO

[*1] 立教大学
[*2] 産業技術総合研究所

Ito, M.[*1], Fukuzawa, K.[*2], Ishikawa, T.[*3], Mochizuki, Y.[*3], Nakano, T., Tanaka, S.[*1] : **Ab Initio Fragment Molecular Orbital Study of Molecular Interactions in Liganded Retinoid X Receptor: Specification of Residues Associated with Ligand Inducible Information Transmission**

J. Phys. Chem. B, **112**, 12081-12094 (2008)

The ab initio fragment molecular orbital calculations were performed for the α-subtype of the human retinoid X receptor (hRXRα) complex with its natural ligand 9-cis retinoic acid (9cRA) to quantitatively specify the key residues with important roles for the ligand inducible information transmission of RXR.

Keywords: FMO, retinoid X receptor, information transmission

[*1] 神戸大学
[*2] みずほ情報総研
[*3] 立教大学

Ishikawa, T.[*1], Mochizuki, Y.[*1], Amari, S.[*2], Nakano, T., Tanaka, S.[*3], Tanaka, K.[*4] : **An application of fragment interaction analysis based on local MP2**

Chem. Phys. Lett. **463**, 189-194 (2008)

We have developed a method named 'fragment interaction analysis based on local MP2' (abbreviated as FILM). This method enables us to decompose the interaction energy associated with dispersion interactions into contributions of localized occupied orbitals. In this study, the basis set dependence of the results derived from FILM was examined. The results suggested that the individual ratio of pair correlation energies of selected

orbital pairs to the total dispersion interaction was almost independent of the basis set size. As an illustrative example, detailed analysis was performed on the human immunodeficiency virus type 1 protease complexed with lopinavir molecule.

Keywords: FMO, FILM, local MP2

[*1] 立教大学
[*2] 菱化システム
[*3] 神戸大学
[*4] 東京大学

Ishimaru, N.[*1], Takagi, A., Kohashi, M.[*1], Yamada, A.[*1], Arakaki, R.[*1], Kanno, J., Hayashi, Y.[*1]. : **Neonatal exposure to low-dose 2,3,7,8-tetrachlorodibenzo-p-dioxin causes autoimmunity due to the disruption of T cell tolerance**

J Immunol., **182** (10), 6576-6586 (2009)

Although 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to influence immune responses, the effects of low-dose TCDD on the development of autoimmunity are unclear. In this study, using NFS/sld mice as a model for human Sjögren's syndrome, in which the lesions are induced by the thymectomy on day 3 after birth, the autoimmune lesions in the salivary glands, and in later phase, inflammatory cell infiltrations in the other organs were developed by neonatal exposure to non apoptotic dosage of TCDD without thymectomy on day 3 after birth. We found disruption of thymic selection, but not thymic atrophy, in TCDD-administered mice. The endogenous expression of aryl hydrocarbon receptor in the neonatal thymus was significantly higher than that in the adult thymus, suggesting that the neonatal thymus may be much more sensitive to TCDD compared with the adult thymus. In addition, the production of T(H)1 cytokines such as IL-2 and IFN-gamma from splenic CD4(+) T cells and the auto antibodies relevant for Sjögren's syndrome in the sera from TCDD-exposed mice were significantly increased compared with those in control mice. These results suggest that TCDD/aryl hydrocarbon receptor signaling in the neonatal thymus plays an important role in the early thymic differentiation related to autoimmunity.

Keywords: TCDD, autoimmunity, NFS mouse

[*1] Department of Oral Molecular Pathology, Institute of Health Biosciences, University of Tokushima Graduate School

Hirabayashi, Y., Tsuboi, I., Kitada, K.[*1], Igarashi, K., Kodama, Y., Kanno, J., Yoshida, K.[*2], Dainiak, N.[*3], and Inoue, T. : **Comparison of murine gene expression profiles between spontaneous and radiation-induced myelogenous leukemias: Stochastic and probabilistic expression variances in the former vs. radiation-specific expression commonalities in the latter**

Exp Hematol., **37**, 195-205 (2009)

Objective. To elucidate the common characteristics of murine radiation-induced myelogenous leukemias, global gene-chip expression profiles were compared with age-matched steady-state bone marrow tissue profiles and spontaneous myelogenous leukemia profiles.

Materials and Methods. Six each of C3H/He mice-derived radiation-induced and spontaneously developed myelogenous leukemias were analyzed. Bone marrow cells from five each of 2- and 21-month old mice were used to subtract non-leukemic information in the analysis. mRNAs from individual mice were analyzed separately using 45,101 gene chips followed by computational biological analysis.

Results. First, principal component analysis (PCA) was performed to discriminate the gene expression profiles of individual mice with radiation-induced myelogenous leukemia from those of bone marrow cells from 2- or 21-month-old mice. Discriminant union genes for individual leukemias were then selected, which finally yielded 242 genes, among which six are radiation-related genes including *Hus-1*, *Edf1a2*, and *Vegf-c*; 16 are apoptosis/cell-death-related genes, 13 are cell-cycle/cell-growth-related genes, and 50 are suppressor/promoter genes. PCA of these 242 genes consistently enabled the discrimination of the radiation-induced leukemias from the spontaneous leukemias. Second, the other components of the same PCA provided four different eigenvector clusters in an unsupervised manner representing four histopathological findings, with which the differential diagnosis in molecular taxonomy was significant as determined by ANOVA of the global gene expression profiles.

Conclusion. Discriminant union genes in radiation-induced myelogenous leukemias against spontaneous myelogenous leukemias and age-matched nonleukemic bone marrow profilings generated by unsupervised computational analysis essentially represent probabilistic biomarkers for radiation-induced myelogenous leukemias, which may contribute to developing a model for risk of secondary carcinogenesis in patients treated by whole-body irradiation.

Keywords: myelogenous leukemia, global gene-chip expression profiles, principal component analysis (PCA)

*1 Kamakura Research Laboratories, Chugai Pharmaceutical Co. Ltd.
*2 National Institute of Radiological Sciences
*3 Yale University School of Medicine

Hirabayashi, Y., Yoon, B.I., Li, G.X., Fujii-Kuriyama, Y.*, Kaneko, T., Kanno, J., Inoue, T. : **Benzene-induced hematopoietic toxicity transmitted by AhR in wild-type mouse and nullified by repopulation with AhR-deficient bone marrow cells: time after benzene treatment and recovery**

Chemosphere, **73**, S290-294 (2008)

Previously, we found an aryl hydrocarbon receptor (AhR)-transmitted benzene-induced hematotoxicity; that is, AhR-knockout (KO) mice did not show any hematotoxicity after benzene exposure (Yoon et al., 2002). Furthermore, our preliminary study showed a significant attenuation of benzene-induced hemopoietic toxicity by AhR expression, when the bone marrow (BM) cells of mice were repopulated with AhR-KO BM cells (Hirabayashi et al., 2005a). In this study, benzene-induced hematotoxicity and its nullification by AhR-KO BM cells were further precisely reevaluated including the duration of the effect after benzene treatment and recovery after the cessation of exposure. Exposure routes, namely, intraperitoneal (*i.p.*) injection used in our previous study, and intragastric (*i.g.*) administration used in this study, were also compared in terms of their toxicologic outcomes. From the results of this study, mice that had been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not show any benzene-induced hematotoxicity. The AhR-KO BM cells nullified benzene-induced toxicities in notably different hemopoietic endpoints between the *i.p.* treatment and the *i.g.* treatment; however, the number of granulo-macrophage colony forming unit *in vitro* (CFU-GM) was a common target parameter, the benzene-induced toxicity of which was nullified by the AhR-KO BM cells.

Keywords: Aryl hydrocarbon receptor, hematopoietic stem/progenitor cell, benzene-induced hematopoietic impairment

* University of Tsukuba

Hirabayashi, Y., Yoon, B.I., Li, G.X., Fujii-Kuriyama, Y.*, Kanno, J., Inoue, T. : **AhR-mediated benzene-induced hematopoietic toxicities: differential toxicities between one from AhR in the hematopoietic stem cells for the bone marrow and the other from possible hepatic- AhR for the peripheral blood**

Organohalogen Compounds, **70**, 287-290 (2008)

The elucidation of the signal transduction mechanism associated with the aryl hydrocarbon receptor (AhR) after benzene exposure may contribute to understanding the physiological role of the AhR during xenobiotic responses mediated by the AhR. Recently, we have found that the benzene-induced hematopoietic toxicity was transduced via the AhR, and the toxicity was attenuated when the AhR-knockout (KO) mouse was exposed to benzene. Consequently, a question is raised on whether the benzene-mediated hematotoxicity is derived from the primitive hematopoietic progenitor cells in the bone marrow (BM) or from classic hepatic xenobiotic metabolism, because the AhR expression was found to be high in the primitive hematopoietic progenitor cells, which were reported to express CYP450-2E1. To answer the question, BM transplantation assays were; first, BM cells from wild-type mice or AhR-KO mice were utilized to repopulate lethally irradiated wild-type mice, and second, the wild-type BM cells were transplanted into lethally irradiated wild-type or AhR-KO mice, then, the possible changes in the steady state among the groups were compared one month after repopulation followed by benzene exposure.

First, as mentioned above, the benzene-induced hematopoietic impairment in the progenitor cell level was attenuated in the AhR-KO mice. Also, lethally irradiated wild-type mice repopulated with BM cells from AhR-KO mice showed similar attenuation of benzene-induced hematopoietic impairment in the progenitor cell level. Next, the AhR-KO mice were repopulated with wild-type BM cells. Namely, a question in the next study is to see whether the benzene-induced hematopoietic impairment in the level of progenitor cells would be observed in the lethally irradiated AhR-KO mice repopulated with wild-type BM cells. As results, the impairment of hematopoietic progenitor cells in the BM after benzene exposure was dependent on the hematopoietic progenitor cells themselves with their own simultaneous expressions of AhR and CYP2E1. Interestingly, the number of WBCs in wild-type mice repopulated with wild-type BM cells was kept significantly lower than those of the AhR-KO mice repopulated with wild-type BM cells throughout the benzene

exposure period. In this regard, the significant decreases in the number of WBCs observed in previous studies as well as in the similar reports are presumably based not on the BM-AhR but on the AhR possibly derived from hepatic tissue or other visceral organs.

In conclusion, the benzene-induced hematopoietic impairment was primarily found to be dependent on the AhR in the hematopoietic progenitor cells of the BM; however, interestingly, the benzene-induced hematopoietic impairment in the peripheral blood was found to be dependent on the AhR other than in the BM, possibly in the hepatic tissue; thus, the benzene-mediated toxicity in peripheral blood was attenuated in the lethally irradiated AhR-KO mice repopulated with wild-type BM cells, although the benzene-mediated toxicity in the hematopoietic progenitor cells was clearly reappeared.

Keywords: Aryl hydrocarbon receptor, hematopoietic stem/progenitor cell, benzene-induced hematopoietic impairment

* University of Tsukuba

Tsuboi, I.*, Hirabayashi, Y., Harada, T.*, Hiramoto, M.*, Kanno, J., Inoue, T., and Aizawa, S.* : **Predominant regeneration of B-cell lineage, instead of myeloid lineage, of the bone marrow after 1 Gy whole-body irradiation in mice: role of differential cytokine expression between B-cell stimulation by IL10, Flt3 ligand and IL7 and myeloid suppression by GM-CSF and SCF**

Radiat Res., **170**, 15-22 (2008)

Irradiation of mice at doses of 1-1.5 Gy induced a predominant regeneration of the B-cell lineage but suppressed the regeneration of the myeloid lineage. The mechanisms underlying such reciprocal regulation of regeneration and the relationship between the two lineages remain unclear. Because the predominant regeneration of the B-cell lineage observed is considered to depend on the stromal cell function, and because the impairment of such stromal function may nullify such reciprocal responses, mouse models of senescent stromal cell impairment (SCI) and the less senescent stage of SCI (non-SCI) were compared to elucidate the mechanisms underlying the reciprocal regulation of both lineages after radiation exposure. In non-SCI mice irradiated with 1 Gy, the numbers of B-lymphocyte progenitor (CFU-preB) and granulocyte-macrophage progenitor (CFU-GM) cells in the bone marrow decreased rapidly during the first 24 h. Then the number of CFU-preB cells in the bone marrow promptly recovered from the nadir and exceeded the pretreatment level, whereas that of CFU-GM cells remained lower than the pretreatment level. The expression of genes encoding positive regulators of the B-lymphoid lineage [interleukin (IL)10, Flt3 ligand and IL7] was up-regulated; in contrast, expression of the positive regulators of the myeloid lineage [granulocyte macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF)] was down-regulated. In SCI mice irradiated with 1 Gy, the oscillatory changes in the numbers of femoral CFU-preB and CFU-GM cells and in the expression levels of cytokine genes were less marked than those in the non-SCI mice. These results thus imply that the reciprocal regeneration depends on the up-regulation of IL10, Flt3 ligand and IL7 expression and the down-regulation of GM-CSF and SCF expression in the bone marrow, possibly depending on the hematopoietic microenvironment.

Keywords: Cytokines, Gene Expression, SAMP1

* Nihon University School of Medicine

Tsuboi, I.*, Hirabayashi, Y., Harada, T.*, Koshinaga, M.*, Kawamata, T.*, Kanno, J., Inoue, T., and Aizawa, S.* : **Role of hematopoietic microenvironment in prolonged impairment of B cell regeneration in age-related stromal-cell-impaired SAMP1 mouse: effects of a single dose of 5-fluorouracil**

J Appl Toxicol., **28**, 797-805 (2008)

In this study, we examined the age-associated defect of stromal cells, which support B cell development, treated with 5-fluorouracil (5-FU) to induce severe perturbation of hematopoiesis, including B lymphocyte development, using SAMP1 mice exhibiting senescence-mimicking stromal-cell impairment after 30 weeks of age. Significant findings of this study are as follows: first, a marked and prolonged decrease in number of CFU-preB cells in non-SCI mice (58% of the steady-state level) associated with more markedly depressed number of CFU-preB cells in SCI mice (20% of the steady-state level), despite the absence of difference in the number of CFU-GMs during the period; second, in the non-SCI mice, a significant and prolonged up-regulation of GM-CSF and IL-6, positive regulators of myelopoiesis and suppressive factors of B lymphopoiesis, was observed. In SCI mice, greater and prolonged suppression of B lymphopoiesis was clearly demonstrated by the significant up-regulation of the negative regulator

TNF-alpha associated with the concomitant marked down-regulation of the positive regulator SDF-1, although the increases of GM-CSF and IL-6 were limited. That is, 'negative complementation' makes preB recovery after 5-FU treatment impaired and prolonged. Principal component analysis clearly showed differences in the cytokine expression patterns in both early and later phases and the time course of the expression pattern of each cytokine between SCI and non-SCI mice without supervising information. An impaired regulation of the expressions of not only positive but also negative regulators after 5-FU treatment was, in part, the cause of the impaired regeneration of CFU-preB cells in SCI mice.

Keywords: Cytokines, Gene Expression, SAMP1

* Nihon University School of Medicine

Sanosaka, T.*, Namihira, M.*, Asano, H.*, Kohyama, J.*, Aisaki, K., Igarashi, K., Kanno, J., Nakashima, K.* : **Identification of genes that restrict astrocyte differentiation of midgestational neural precursor cells**

Neuroscience, **155**, 780-788 (2008)

During development of the mammalian CNS, neurons and glial cells (astrocytes and oligodendrocytes) are generated from common neural precursor cells (NPCs). However, neurogenesis precedes gliogenesis, which normally commences at later stages of fetal telencephalic development. Astrocyte differentiation of mouse NPCs at embryonic day (E) 14.5 (relatively late gestation) is induced by activation of the transcription factor signal transducer and activator of transcription (STAT) 3, whereas at E11.5 (mid-gestation) NPCs do not differentiate into astrocytes even when stimulated by STAT3-activating cytokines such as leukemia inhibitory factor (LIF). This can be explained in part by the fact that astrocyte-specific gene promoters are highly methylated in NPCs at E11.5, but other mechanisms are also likely to play a role. We therefore sought to identify genes involved in the inhibition of astrocyte differentiation of NPCs at midgestation. We first examined gene expression profiles in E11.5 and E14.5 NPCs, using Affymetrix GeneChip analysis, applying the Percellome method to normalize gene expression level. We then conducted in situ hybridization analysis for selected genes found to be highly expressed in NPCs at midgestation. Among these genes, we found that N-myc and high mobility group AT-hook 2 (Hmga2) were highly expressed in the E11.5 but not the E14.5 ventricular zone of mouse brain, where NPCs reside. Transduction of N-myc and Hmga2 by retroviruses into E14.5 NPCs, which normally differentiate into astrocytes in response to LIF, resulted in suppression of astrocyte differentiation. However, sustained expression of N-myc and Hmga2 in E11.5 NPCs failed to maintain the hypermethylated status of an astrocyte-specific gene promoter. Taken together, our data suggest that astrocyte differentiation of NPCs is regulated not only by DNA methylation but also by genes whose expression is controlled spatio-temporally during brain development.

Keywords: N-myc and Hmga2, Percellome method, astrocyte differentiation

* Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology

Tsuda, H*., Tokunaga, H., Hirose, A., Kanno, J. : **Hazard identification of nanomaterials**

Yakugaku Zasshi, **128**(12), 1727-1732 (2008)

It is considered that the materials with new properties may lead to novel biological effects or unknown adverse health effects. To gather proper hazard information, it is important to develop both experimental protocols and detection/measurement methods for nanomaterials in the body, in parallel. Since 2005, we are running research projects to develop methods to monitor health risk effects for the assessment of manufactured nanomaterials funded by the Ministry of Health, Labour and Welfare. For the experimental protocols, these projects focus on the development of 1) in vitro experimental systems, 2) in vivo experimental systems (mainly focusing on long-term health implication, especially carcinogenesis), and 3) proper inhalation system. Firstly, fullerene (C60), titanium dioxide and multi-walled carbon nanotube were chosen to be tested because of their high production volume. Safety issues for new materials such as nanoparticles is a new paradigm. The key is that the full scale exposure to the public has not been started yet. Therefore, there is a good chance that information from hazard identification studies can be directly fed back to the product development plan. Manufacturers can produce safer products without risking themselves waiting for the toxicology studies to be finished after their products are widely marketed.

Keywords: toxicity, nanomaterials, carcinogenesis

* Department of Molecular Toxicology, Graduate School of Medical Sciences, Nagoya City University

Myers, J.P.[*1], vom Saal, F.S.[*2], Akingbemi, B.T.[*3], Arizono, K.[*4], Belcher, S.[*5], Colborn, T.[*6], Chahoud, I.[*7], Crain, D.A.[*8], Farabollini, F.[*9], Guillette, Jr, L. J.[*10]., Hassold, T.[*11], Ho, S.[*12], Hunt, P.A.[*11], Iguchi, T.[*13], Jobling, S.[*14], Kanno, J.[*15], Laufer, H.[*15], Marcus, M.[*16], McLachlan, J.A.[*17], Nadal, A.[*18], Oehlmann, J.[*19], Olea, N.[*20], Palanza, P.[*21], Parmigiani, S.[*21], Rubin, B.S.[*22], Schoenfelder, G.[*23], Sonnenschein, C.[*22], Soto, A.M.[*22], Talsness, C.E.[*24], Taylor, J.A.[*2], Vandenberg, L.N.[*22], Vandenbergh, J.G.[*25], Vogel, S.[*26], Watson, C.S.[*27], Welshons, W.V.[*28], and Zoeller, R.T.[*29] : **Why Public Health Agencies Cannot Depend upon 'Good Laboratory Practices' as a Criterion for Selecting Data: The Case of Bisphenol A**

Environmental Health Perspectives, **117**, 309-315 (2009)

In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world. OBJECTIVES: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes. DISCUSSION: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research. CONCLUSIONS: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

Keywords: bisphenol A, endocrine disruptors, GLP

[*1] Environmental Health Sciences, Charlottesville, Virginia, USA

[*2] Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA

[*3] Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA

[*4] Faculty of Environmental and Symbiotic Science, Prefectural University of Kumamoto, Tsukide, Kumamoto, Japan

[*5] Department of Pharmacology and Cell Biophysics, Center for Environmental Genetics, University of Cincinnati, Cincinnati, Ohio, USA

[*6] The Endocrine Disruption Exchange, Paonia, Colorado, USA

[*7] Institut für Klinische Pharmakologie und Toxikologie Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

[*8] Department of Biology, Maryville College, Maryville, Tennessee, USA

[*9] Dipartimento di Fisiologia, Università di Siena, Siena, Italy

[*10] Department of Zoology, University of Florida, Gainesville, Florida, USA

[*11] School of Molecular Biosciences, Washington State University, Pullman, Washington, USA

[*12] Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio, USA

[*13] National Institutes of Natural Science, Okazaki Institute for Integrative Bioscience, Bioenvironmental Science, Okazaki, Japan

[*14] Department of Biological Sciences, Brunel University, Uxbridge, United Kingdom

[*15] Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, USA
[*16] Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA
[*17] Center for Bioenvironmental Research, Tulane and Xavier Universities, New Orleans, Louisiana, USA
[*18] Instituto de Bioingeniería and CIBERDEM, Universidad Miguel Hernández de Elche, Alicante, Spain
[*19] Goethe University Frankfurt am Main, Department Aquatic Ecotoxicology, Frankfurt, Germany
[*20] Hospital Clínico, CIBERESP, University of Granada, Granada, Spain
[*21] Dipartimento di Biologia Evolutiva e Funzionale, Universita'di Parma, Parma, Italy
[*22] Tufts Medical School, Boston, Massachusetts, USA
[*23] Institute of Pharmacology and Toxicology, University of Wuerzburg, Wuerzburg Germany
[*24] Charité University Medical School Berlin, Berlin, Germany
[*25] Department of Biology, North Carolina State University, Raleigh, North Carolina, USA
[*26] Chemical Heritage Foundation, Philadelphia, Pennsylvania, USA
[*27] Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA
[*28] Department of Biomedical Sciences, University of Missouri, Columbia, Missouri, USA
[*29] Biology Department, University of Massachusetts, Amherst, Massachusetts, USA

Matsunaga, N.[*1], Kanno, J., Hamada, C.[*2], Yoshimura, I.[*2] : **An experimental design for judging synergism on consideration to endocrine disruptor animal experiments**

Environmetrics, **20**, 1-13 (2009)

This paper investigates an appropriate statistical design for an animal experiment to evaluate synergism of two test chemicals. It assumes a certain number of animals are divided into group size for each combination of doses, including the case where the dose of either one chemical is zero. The power of *t*-test to detect synergism by positive surplus of response on a simultaneous administration group from the additivity plane composed of the responses on single administration group is adopted as the criterion for the appropriate design. The applicable design is investigated for the application to real cases of endocrine disrupter study conducted at the National Institute of Health Sciences of Japan. It revealed that the dose level of the simultaneous administration group should be located inside or on the boundary of a triangular region and that the total number of animals should be the same as those for single administration groups.

Keywords: experimental design, endocrine disrupter, synergism

[*1] Kyowa Pharmaceutical Inc
[*2] Tokyo University of Science

Yasuhiko, Y., Kitajima, S., Takahashi, Y., Oginuma, M.[*1], Kagiwada, H.[*2], Kanno, J., Saga, Y.[*1] : **Functional importance of evolutionally conserved Tbx6 binding sites in the presomitic mesoderm-specific enhancer of Mesp2**

Development, **135**, 3511-3519 (2008)

The T-box transcription factor Tbx6 controls the expression of *Mesp2*, encoding a basic Helix-Loop-Helix-type transcription factor that has crucial roles in somitogenesis. In cultured cells, Tbx6 binding to the *Mesp2* enhancer region is essential for activation of *Mesp2* by Notch signaling. However, the requirement for this binding in vivo has not been established. Here we report that a *Mesp2* enhancer knockout mouse bearing mutations in two critical Tbx6 binding sites does not express *Mesp2* in the presomitic mesoderm (PSM). This absence leads to impaired skeletal segmentation which is identical to that reported for *Mesp2*-null mice, indicating that these Tbx6 binding sites are indispensable for *Mesp2* expression. The T-box binding to the consensus sequences in the *Mesp2* upstream region were confirmed by chromatin immunoprecipitation assays. Further enhancer analyses indicated that the number and the spatial organization of T-box binding sites were critically important for initiating *Mesp2* transcription via Notch signaling. We also generated a knock-in mouse in which the endogenous enhancer of mouse *Mesp2* was replaced by the core enhancer of medaka *mespb*, an ortholog of mouse *Mesp2*. The homozygous enhancer knock-in mouse was viable and showed normal skeletal segmentation, indicating that the medaka *mespb* enhancer functionally replaced the mouse *Mesp2* enhancer. These results demonstrate that there is significant evolutionary conservation of *Mesp* regulatory mechanisms between fish and mice.

Key words: Mesp2, Tbx6, enhancer

[*1] National Institute of Genetics
[*2] AIST

Sato, K., Saito, Y.[*1], Oka, J-I.[*1], Ohwada, T.[*2], Nakazawa, K., : **Effects of tamoxifen on L-glutamate transporters of astrocytes**

J. Pharmacol. Sci., **107**, 226-230 (2008)

Tamoxifen (Tam) decreased the clearance of L-glutamate (L-glu) by cultured astrocytes at 1 pM, 1 nM and 1 μM, and became toxic at 10 μM. When L-glu transporters were mostly inhibited by threo-β-benzyloxyaspartate (TBOA) (1 mM) or D,L-threo-beta-hydroxyaspartate (THA) (1 mM), Tam (1 nM) did not change extracellular L-glu concentration, confirming that Tam attenuates L-glu transport through L-glu transporters. ICI182,780 (ICI), LY294002 (LY), and U0126 inhibited the effect of Tam dose-dependently, suggesting the involvement of estrogen receptors (ERs), the phosphatidylinositol 3-kinase (PI3K) cascade, and the mitogen-activated protein kinase (MAPK) cascade in the effect of Tam.

Keywords: Tamoxifen, L-Glu transporter, Astrocytes

[*1] 東京理科大学

[*2] 東京大学

Kikuchi, A.[*], Shimizu, K.[*], Nibuya, M.[*], Hiramoto T.[*], Kanda, Y., Tanaka, T.[*], Watanabe Y.[*], Takahashi, Y [*], and Nomura, S.[*] : **Relationship between PTSD-like behavior and reduction of hippocampal 5-bromo-2-deoxyuridine positive cells after inescapable shock in rats**

Psychiatry Clin. Neurosci., **62**, 713-720 (2008)

Inescapable shocks (ISs) have been reported to reduce the number of 5-bromo-2-deoxyuridine (BrdU)-positive cells in hippocampus. Antidepressants prevent this reduction, and the role of neurogenesis in depression is now suggested. It is reported, however, that the number of BrdU-positive cells was not different between the rats developed learned helplessness and those did not. This suggests that reduction of neurogenesis does not constitute a primary etiology of depression. We previously showed that ISs can cause post-traumatic stress disorder (PTSD)-like various behavioral changes in rats. In this study, we examined whether the reduction of BrdU-positive cells relates to any PTSD-like behavioral changes in our paradigm. In accordance with previously reported results, IS loading resulted in fewer BrdU-positive cells in the hippocampal subgranular zone (SGZ). Furthermore, in the IS-treated group, the number of BrdU-positive cells in the hippocampal SGZ was negatively correlated at a significant level with several hyperactive behavioral parameters but not with hypoactive behavioral parameters. Our earlier findings had indicated that chronic selective serotonin reuptake inhibitor administration, which is known to increase hippocampal neurogenesis, restored the increase in hypervigilant/hyperarousal behavior but did not attenuate the increase in numbing/avoidance behavior. Taken together, these results suggest that the regulatory mechanism responsible for the decreased proliferation and survival of cells in the hippocampus may be related to the pathogenic processes of hypervigilance/hyperarousal behaviors.

Keywords: stress, neurogenesis, hippocampus

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[*] National Defense Medical College

Horiuchi, S., Ishida, S., Hongo, T.[*1], Ishikawa, Y.[*1], Miyajima, A., Sawada, J., Ohno, Y., Nakazawa, K., Ozawa, S. [*2] : **Global gene expression changes including drug metabolism and disposition induced by three-dimensional culture of HepG2 cells-Involvement of microtubules**

Biochem Biophys Res Commun., **378**, 558-562 (2009)

Constitutive upregulation and a higher degree of induction of drug metabolism and disposition-related genes were found in a three-dimensional HepG2 culture. The upregulated genes are believed to be regulated by different regulatory factors. Global gene expression analysis using the Affymetrix GeneChip indicated that altered expression of microtubule-related genes may change the expressed levels of drug metabolizing and disposition genes. Stabilization of microtubule molecules with docetaxel, a tubulin-stabilizing agent, in the two-dimensional culture showed gene expression patterns similar to those found in the three-dimensional culture, indicating that the culture environment affects drug metabolism functions in HepG2 cells.

Keywords: three-dimensional culture, tubulin satabilization, drug metabolism and disposition-related genes

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[*1] ABLE Corporation

[*2] Iwate Medical University

Nakajima, M.[*1], Mitsunaga, K.[*2], Nakazawa, K. and Usami, M. : **In vivo/in vitro study in rat embryos on indium-caused tail malformations**

Reprod Toxicol, **25**, 426-432 (2008)

Pathogenesis of indium-caused tail malformations was investigated by in vivo and in vitro experiments. In the

in vivo experiment, pregnant Wistar rats received single intravenous administration of indium trichloride at 0.4 mg/kg on day 10 of gestation, and their embryos were examined on days 11, 12 and 13. Embryos in the indium group showed caudal hypoplasia from day 11. Increased apoptosis was observed in their tailbud on day 11. Similar effects were observed in the in vitro experiment, when day 10 rat embryos were cultured in the presence of indium trichloride at 50 μM for 24h and for further 24h in the absence of indium. It was considered from these results that caudal hypoplasia probably due to excessive cell loss by increased apoptosis in the tailbud accounted for indium-caused tail malformations in rat fetuses, and that indium-caused embryotoxic effects were direct effects on the conceptus.

Keywords: indium trichloride, teratogenicity, tail malformation, embryo culture, apoptosis, nile blue sulfate, whole-mount TUNEL

[*1] 旭化成ファーマ（株）医薬研究センター
[*2] 東邦大学薬学部

Omori, T.[*1], Idehara, K.[*2], Kojima, H., Sozu, T.[*3], Arima, K.[*4], Goto, H.[*5], Hanada, T.[*6], Ikarashi, Y., Inoda, T.[*7], Kanazawa, Y.[*8], Kosaka, T.[*9], Maki, E.[*10], Morimoto, T.[*11], Shinoda, S.[*12], Shinoda, N.[*13], Takeyoshi, M.[*14], Tanaka, M.[*15], Uratani, M.[*16], Usami, M.*[17], Yamanaka, A.[*18], Yoneda, T.[*19], Yoshimura I[*20], and Yuasa, A.[*21] : **Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement**

J. Pharmacol. Toxicol. Methods, **58**, 11-26(2008)

Introduction: The murine local lymph node assay (LLNA) is a well-established alternative to the guinea pig maximization test (GPMT) or Buehler test (BT) for the assessment of the skin sensitizing ability of drugs and chemicals. Daicel Chemical Industries Ltd. has developed a modified LLNA based on the adenosine triphosphate (ATP) content (LLNA-DA). We conducted 2 interlaboratory validation studies to evaluate the reliability and relevance of LLNA-DA. Methods: The experiment involved 17 laboratories, wherein 14 chemicals were examined under blinded conditions. In the first study, 3 chemicals were examined in 10 laboratories and the remaining 9 were examined in 3 laboratories. In the second study,1 chemicalwas examined in 7 laboratories and the remaining 4 chemicals were examined in 4 laboratories. The data were expressed as the ATP content for each chemical-treated group, and the stimulation index (SI) for each chemical-treated group was determined as the increase in the ATP content relative to the concurrent vehicle control group. An SI of 3was set as the cut-off value for exhibiting skin sensitization activity. Results: The results of the first study obtained in the experiments conducted for the 3 chemicals that were examined in all the 10 laboratories and for 5 of the remaining 9 chemicals were sufficiently consistent with small variations in their SI values. The sensitivity, specificity, and accuracy of LLNA-DA against those of GPMT/BTwere 7/8 (87.5%), 3/3 (100%), and 10/11 (90.9%), respectively. In the second study, all the 5 chemicals studied demonstrated acceptably small interlaboratory variations. Discussion: In the first study, a large variation was observed for 2 chemicals; in the second study, this variation was small. It was attributed to the application of dimethylsulfoxide as the solvent for the metallic salts. In conclusion, these 2 studies provide good evidence for the reliability of the LLNA-DA.

Keywords: Interlaboratory validation, Local lymph node assay, Skin sensitization

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[*1] Kyoto Univ. SPH.
[*2] Daicel Chemical Industries, Ltd.
[*3] Osaka Univ.
[*4] Taisho Pharmaceutical CO., LTD.
[*5] Otsuka Pharmaceutical Co., Ltd.
[*6] Nippon Shinyaku Co., Ltd.
[*7] Nakano Seiyaku Co., Ltd.
[*8] Food and Drug Safety Center.
[*9] Institute of Environmental Toxicology.
[*10] Biosafety research center, food drugs and pesticides.
[*11] Sumitomo chemical Co., Ltd.
[*12] Drug Safety Testing Center Co., LTD.
[*13] Santen Pharmaceutical Co., Ltd.
[*14] Chemicals Evaluation and Research Institute.
[*15] MEIJI SEIKA KAISHA, LTD.
[*16] Ishihara Sangyo Kaisha, Ltd.
[*17] Hoyu Co., Ltd.
[*18] Pias Corporation.
[*19] TOAEIYO LTD.
[*20] Tokyo University of Science.
[*21] Fuji Film Co., Ltd.

Arai, S., Yamamoto, N.[*1], Kato M.[*2], and Kojima, H. : **An *in vitro* evaluation methods to test ocular irriation using a human corneal epithelium model**

Altern. Animal Test. EXperiment, **13**, 83-90 (2008)

Recently, safety evaluation tests that do not involve animal experiments have been prosperously developing. However, the optimal evaluation materials and methods for assessing ocular irritancy have not been well investigated. In this study, we determined the optimal evaluation method for testing ocular irritation using a human cultured corneal epithelium model (corneal model). In order to assess adequate treatment conditions for the corneal model, we used cetylpyridinium chloride (CPC), which has been recognized as an irritant chemical by the Draize eye test. The irritancy elicited by multiple concentrations of CPC was evaluated by a cytotoxicity assay under nine treatment conditions and compared to the Draize score. The treatment conditions that included a 5-second exposure period followed by a 24-hour post-incubation period (hereafter called protocol "5-sec+-4-h") showed a significant correlation between cytotoxicity and the Draize score. Furthermore, the dose-dependent cytotoxicity of six test chemicals was assessed by protocol "5-sec+24-h" and found to correlate with the Draize eye test results.

Keywords: ocular irritation, corneal model, Draize eye test

[*1] Fujita Health University.

[*2] Japan Tissue Engineering Co., Ltd.

Nakai K[*1], Tanaka H[*1], Hanada K[*1], Ogata H[*1], Suzuki F[*2], Kumada H[*2], Miyajima A, Ishida S, Sunouchi M, Habano W[*3], Kamikawa Y[*4], Kubota K[*4], Kita J[*4], Ozawa S[*3], Ohno Y. : **Decreased expression of cytochromes P450 1A2, 2E1, and 3A4 and drug transporters Na+-taurocholate-cotransporting polypeptide, organic cation transporter 1, and organic anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic hepatitis C patients**

Drug Metab Dispos., **36**, 1786-93 (2008)

Changes in status of drug metabolism and disposition may vary with chronic hepatitis C stage and should be assessed. Total RNA was extracted from liver biopsy specimens (n = 63) and reverse transcribed to yield cDNA. Relative mRNA levels of drug-metabolizing enzymes, transporters, nuclear receptors, and proinflammatory cytokines were analyzed. mRNAs encoding cytochromes P450 1A2, 2E1, and 3A4, and drug transporters, Na(+)-taurocholate-cotransporting polypeptide, organic anion-transporting peptide-C, and organic cation transporter 1 showed remarkable decreases, and tumor necrosis factor-alpha showed an increase according to fibrosis stage progression. CYP1A2 and Na(+)-taurocholate-cotransporting polypeptide mRNA levels significantly decreased in HepG2 cells with interleukin 1beta and interleukin 6 treatments. CYP2E1 and organic cation transporter 1 mRNA levels significantly decreased with tumor necrosis factor-alpha treatment only in HepG2. These results suggested that down-regulation of the above genes manifested in livers of patients with chronic hepatitis C viral infection, was associated, at least in part, with the elevated production of proinflammatory cytokines, including tumor necrosis factor-alpha.

Keywords: drug metabolism related gene expression, hepatitis C patients, liver fibrosis

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[*1] Meiji Pharmaceutical University

[*2] Toranomon Hospital

[*3] Iwate Medical University

[*4] Dokkyo Medical University School of Medicine

Nishikawa, A., Umemura, T., Ishii, Y., Tasaki, M., Okamura, T., Inoue, T., Masumura, K., Nohmi, T. : ***In vivo* approaches to study mechanism of action of genotoxic carcinogens**

Genes Environ., **30**, 120-124 (2008)

Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. However, it is actually difficult to discriminate genotoxic and non-genotoxic carcinogens because both assays are basically independent each other, which raises a simple query as to how much the detected genotoxic potential can consequently contribute to carcinogenicity. To clarify this critical issue, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected as powerful tools for the simultaneous evaluation of both genotoxicity and carcinogenicity at the same organ level. A number of studies of genotoxic carcinogens using these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action. On the other hand, a known non-genotoxic carcinogen dicyclanil increased *in vivo* genotoxicity as well as oxidative DNA damage in female mice, consistently with the sex specificity of its carcinogenicity, albeit without clear evidence of direct DNA reactivity. In contrast, a genotoxic chlorinated water by-product MX failed to exert

in vivo genotoxicity and carcinogenicity in mice. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity as compared with intact counterparts. These results thus indicate that understanding of the detailed mechanism of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose.
Keywords: *in vivo* study, mechanism of action, genotoxic carcinogen

Matsuzaki, Y .[*1], Koyama, M.[*1], Hitomi, T.[*1], Yokota, T. [1], Kawanaka, M.[*1], Nishikawa, A., Germain, D[*2]., Sakai, T.[*1] : **Arctiin induces cell growth inhibition through the down-regulation of cyclin D1 expression**
Oncol. Rep., **19**, 721-7 (2008)

Arctiin is a major lignan constituent of Arctium lappa and has anti-cancer properties in animal models. It was recently reported that arctiin induces growth inhibition in human prostate cancer PC-3 cells. However, the growth inhibitory mechanism of arctiin remains unknown. Herein we report that arctiin induces growth inhibition and dephosphorylates the tumor-suppressor retinoblastoma protein in human immortalized keratinocyte HaCaT cells. We also show that the growth inhibition caused by arctiin is associated with the down-regulation of cyclin D1 protein expression. Furthermore, the arctiin-induced suppression of cyclin D1 protein expression occurs in various types of human tumor cells, including osteosarcoma, lung, colorectal, cervical and breast cancer, melanoma, transformed renal cells and prostate cancer. Depletion of the cyclin D1 protein using small interfering RNA-rendered human breast cancer MCF-7 cells insensitive to the growth inhibitory effects of arctiin, implicates cyclin D1 as an important target of arctiin. Taken together, these results suggest that arctiin down-regulates cyclin D1 protein expression and that this at least partially contributes to the anti-proliferative effect of arctiin.
Keywords: arctiin, cyclin D1, growth inhibition

[*1] Kyoto Prefectural University of Medicine
[*2] Mount Sainai School of Medicine

Nakamura, Y.[*1], Nakamura, K.[*1], Asai, Y.[*1], Wada, T.[*1], Tanaka, K.[*1], Matsuo, T.[*1], Okamoto, S.[*2], Meijer, J.[*1], Kitamura, Y.[*1], Nishikawa, A., Park, E.Y.[*1], Sato, K.[*1], Ohtsuki, K.[*1] : **Comparison of the glucosinolate-myrosinase systems among daikon (Raphanus sativus, Japanese white radish) varieties**
J. Agric. Food Chem., **23**, 2702-2707 (2008)

Myrosinase is a cytosolic plant enzyme present in daikon (Raphanus sativus, Japanese white radish) roots that hydrolyzes 4-methylthio-3-butenyl glucosinolate (MTBGLS) into the natural pungent agent 4-methylthio-3-butenyl isothiocyanate (MTBITC), which possesses antimicrobial, antimutagenic, and anticarcinogenic properties. The concentration of MTBGLS, myrosinase activity, and production of MTBITC in seven daikon varieties (one conventional and six heirlooms) were determined to rank the activity of the glucosinolate-myrosinase system and identify critical factors influencing the production of MTBITC. The six heirloom varieties produced 2.0-11.5 times higher levels of MTBITC as compared to the conventional variety, Aokubi, which is consumed by the present Japanese population. The myrosinase was located exclusively in the outer epidermal layer in Aokubi, and MTBGLS was widely distributed throughout the root tissue. Although the skin is a potentially rich source of myrosinase in Aokubi, the skin is usually peeled off in the current practice of preparing daikon for cooking. New practices are therefore proposed for the preparation of daikon tubers that eliminate the peeling of the skin to avoid removing the enzyme needed to convert MTBGLS to the health-beneficial MTBITC. It is also concluded that the consuconsumption of heirloom daikon varieties in addition to changes in food preparation will optimize the health benefits of daikon.
keywords: daikon, myrosinase activity, 4-methylthio-3-butenyl glucosinolate, 4-methylthio-3-butenyl isothiocyanate

[*1] Kyoto Prefectural University of Medicine
[*2] Kagoshima University
[*3] Swedish University of Agricultural Sciences

Nakamura, Y.[*1], Matsuo, T.[*2], Okamoto, S.[*2], Nishikawa, A., Imai, T., Park, E.Y.[*1], Sato, K.[*1] : **Antimutagenic and anticarcinogenic properties of *Kyo-yasai,* heirloom vesitables in Kyoto**
Genes and Environment., **30**, 41-47 (2008)

Heirloom vegetables in Kyoto, termed Kyo-yasai, have had their seeds preserved by traditional cultivation methods. These heirloom vegetables offer a more distinctive flavor htan conventional vegetables, and extracts from some Kyo-yasai are known to decrease ultraviolet light induced mutations in *E. coli* B/r WP2 (trpE65) significantly

more than extracts from their counterpart of conventional vegetables. 4-Methylthio-3-butenyl isothiocyanate which causes the pungency in daikon, and 3-methylthiopropionic acid ethyl ester, which causes melon-like odor, were identified from heirloom vegetables in Kyoto to be antimutagens in *E*. coli mutagenicity assays. These two chemicals also demonstrated in vivo animal cancer prevention, and induced differentiation, a chemotherapeutic strategy in an in vitro human colon-cancer cell system. The heirloom daikon varieties in Kyoto predued 2.0-11.5 times higher levelsof 4-methylthio-3-butenyl isothiocyanate as compared to the conventional Aokubi variety. The heirloom pickling melon in Kyoto, Katsura-uri began to produce 3-methylthiopropionic acid ethyl ester between the midripening to fully ripening stage of fruit development. Shiro-uri did not contain 3-methylthiopropionic acid ethyl ester. Results also indicate that antimutagenic and anticarinogenic properties change over the ripening stage quantitatively. In this review, we discuss the value of retaining the original phenotypes of vegetables, including the flavors, to maximize the anticarcinogenic properties of these food products.

keywords: heirloom vegetables, 3-methylthiopropionic acid ethyl ester, *E. coli* mutagenicity assay

[*1] Kyoto Prefectural University of Medicine
[*2] Kagoshima University

Kanki, K., Umemura, T., Kitamura, Y., Ishii, Y., Kuroiwa, Y., Kodama, Y., Itoh, K.[*1], Yamamoto, M.[*1], Nishikawa, A., Hirose, M.[*2] : **A possible role of nrf2 in prevention of renal oxidative damage by ferric nitriltriacetate**

Toxicol. Pathol., **36**, 353-361 (2008)

To ascertain the possible roles of nuclear erythroid 2 p45-related factor 2 (Nrf2), a key transcription factor of phase 2 drug-metabolizing enzymes, in renal cellular defense against oxidative stress, wild-type and Nrf2-knockout$^{-/-}$ mice were treated with ferric nitrilotriacetate (Fe-NTA) at doses of 3 or 6 mg iron/kg body weight. After Fe-NTA treatment, Nrf2$^{-/-}$ mice consistently showed lower levels of glutathione (GSH) in the kidney at the low dose and the liver at the high dose than the wild-type mice. Gamma-glutamylcysteine ligase (GCL) activity in the kidney and liver of Nrf2$^{-/-}$ mice was also consistently lower than in wild-type mice after the Fe-NTA treatment. Histopathological examination revealed that nephrotoxicity of Fe-NTA, reflected in necrosis of renal tubule epithelial cells following nuclear damage, was more severe in the Nrf2$^{-/-}$ mice than in their wild-type counterparts. Overall, the data suggest that Nrf2$^{-/-}$ mice are unable to compensate for depletion of renal GSH because of oxidative stress, being more susceptible to Fe-NTA-induced nephrotoxicity. In conclusion, the present study showed that Nrf2 might play an important role in protecting cells from oxidative stress in the kidney through its regulation of antioxidant enzymes.

Key words: nrf2, ferric nitriltriacetate, oxidative damage

[*1] University of Tsukuba
[*2] Food Safety Commission

Umemura, T., Maeda, M., Kijima, A., Ishii, Y., Tasaki, M., Okamura, T., Inoue, T., Hirose, M.[*], Nishikawa, A. : **Lack of promotion activity of deacylglycerol oil on 4-nitroquinoline 1-oxide induced carcinogenesis in the oral cavity of SD rats**

Food Chem. Toxicol., **46**, 3206-3212 (2008)

A recent study using c-Ha-ras proto-oncogene transgenic (rasTg) rats demonstrated possible enhancing effects of diacylglycerol (DAG) on 4-nitroquinoline-1-oxide (4NQO) induced carcinogenesis of the tongue. To assess effects in their Sprague-Dawley back strain, a two-stage carcinogenesis study using 4NQO as an initiator was performed. Groups of 30 male rats were initially treated with 4NQO at a dose of 10 ppm in the drinking water for the first 10 weeks followed after a 1 week recovery interval by 11% DAG, 5.5% DAG+5.5% triacylglycerol (TAG), 2.75% DAG+8.25% TAG, 1.38 DAG+9.62% TAG, 11% TAG, 11% high linoleic acid TAG (HLTG), 5.5% DAG or 2.75% DAG in the diet for 35 weeks. Further groups of animals were treated with distilled water instead of 4NQO followed by 11% DAG, 11% TAG or 11% HLTG in the same manner. The final survival rates in 4NQO-treated groups were from 50 to 77%. However, incidences and multiplicities of squamous cell papillomas and carcinomas in the oral cavity induced by 4NQO were not affected by any of the dietary treatments. Thus, in contrast to the positive data using rasTg rats, DAG had no potential for enhancing 4NQO-induced tumorigenesis in their back strain.

Keywords: diacylglycerol, 4-nitroquinoline 1-oxide, rasTg rats

* Food Safety Commission

Tasaki, M., Umemura, T., Inoue, T., Okamura, T., Kuroiwa, Y., Ishii, Y., Maeda, M., Hirose, M.*, Nishikawa, A. : **Induction of characteristic hepatocyte proliferative lesion with dietary exposure of Wister Hannover rats to tocotrienol for 1 year**

Toxicology, **250**, 143-150 (2008)

Tocotrienol is an antioxidant which has found commercial application as a food additive and health supplement. Since there have been no reports regarding toxicological effects of long-term exposure, we performed a 52-week chronic study using Wistar Hannover rats of both sexes given the compound at doses of 0, 0.08, 0.4 or 2% in basal diet. On histopathological examination, hepatocellular nodules were evident with distortion of hepatic cords and compression of the surrounding tissue, almost all including areas of spongiosis hepatis. The constituent hepatocytes were immunohistochemically stained with proliferation cell nuclear antigen at high rates. They were consistently negative for GST-P. Accordingly, we propose the newly categorized but previously used name 'nodular hepatocellular hyperplasia', which may not necessarily have a neoplastic or regenerative nature. However, quantitative GST-P analysis of the liver sections overall showed numbers of GST-P foci in the high dose females to be significantly elevated as compared to the control value. We conclude that the NOAEL is 0.4% (303 mg/kg/day for males, and 472 mg/kg/day for females).

Keywords: tocotrienol, hepatocyte proliferative lesion, GST-P, chronic exposure

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* Food Safety Commission

Inoue, T., Umemura, T., Maeda, M., Ishii, Y., Okamura, T., Tasaki, M., Nishikawa, A. : **Safety assessment of dietary administered paprika color in combined chronic toxicity and carcinogenicity studies using F344 rats**

Food. Chem. Toxicol., **46**, 2689-2693 (2008)

Combined chronic toxicity and carcinogenicity studies of paprika color were performed in male and female F344 rats. Dietary concentrations of 0%, 0.62%, 1.25%, 2.5% and 5% were applied in a 52-week toxicity study and 0%, 2.5% and 5% in a 104-week carcinogenicity study. Treatment with paprika color caused a significant increase in incidence of hepatocellular vacuolation in 5% males, but no toxicological effects were found with any other toxicological parameters at any dose level in either sex in the chronic toxicity study. Also, paprika color did not have carcinogenicity In conclusion, based on slight histopathological changes observed in 5% male livers, NOEL was estimated to be 1,253 mg/kg bw/day and NOAEL was determined to be 2,388 mg/kg bw/day for male rats, and for females, the NOEL was concluded to be 2,826 mg/kg bw/day. Additionally, paprika color was not carcinogenic to male and female F344 rats under the present experimental conditions.

Keywords: paprika color, chronic toxicity and carcinogenicity studies, food additive

Kuroiwa, Y., Yamada, M., Matsui, K., Okamura, T., Ishii, Y., Masumura, K., Tasaki, M., Umemura, T., Mitsumori, K*1, Nohmi, T., Hirose, M.*2, Nishikawa, A. : **Combined ascorbic acid and sodium nitrite treatment induces oxidative DNA damage-associated mutagenicity *in vitro*, but lacks initiation activity in rat forestomach epithelium**

Toxicol. Sci., **104**, 274-283 (2008)

Combination treatment with $NaNO_2$ and ascorbic acid (AsA) is well known to promote forestomach carcinogenesis in rats and weakly enhance esophageal carcinogenesis under acid reflux conditions. The purpose of the present study was to investigate whether oxidative DNA damage-associated genotoxicity and tumor initiating potential are involved in the carcinogenesis. In the bacterial reverse mutation assay using E. coli deficient in the *mutM* gene encoding 8-OHdG DNA glycosylase, the combination with $NaNO_2$ and AsA increased the mutation frequency dramatically, slight increase being evident in the parental strain. *In vivo*, a significant increase in 8-OHdG levels in the rat forestomach epithelium occurred at 24 h after combined treatment. Six-week-old F344 male rats were given drinking water containing 0.2% $NaNO_2$ and a diet supplemented with 1% AsA in combination. After an interval of 2 weeks, they received 1% butylated hydroxyanisole in the diet for promotion until the end of weeks 52 and 78. There was no significant variation in tumor development among the groups.

Keywords: ascorbic acid, sodium nitrite, oxidative DNA damage, forestomach

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*1 Tokyo University of Agriculture and Technology

*2 Food Safety Commission

Tasaki, M., Umemura, T., Maeda, M., Ishii, Y., Okamura, T., Inoue, T., Kuroiwa, Y., Hirose, M.*, Nishikawa, A.
: **Safety assessment of ellagic acid, a food additive, in a subchronic toxicity study using F344 rats**
Food. Chem. Toxicol., **46**, 1119-1124 (2008)

The present 90-day subchronic toxicity study was performed in F344 rats at dose levels of 0, 1.25, 2.5 and 5% of ellagic acid in powdered basal diet, with actual doses of 9.4, 19.1, 39.1 g/kg b.w., respectively, in males, and 10.1, 20.1, 42.3 g/kg b.w. in females. No mortality or treatment-related clinical signs were observed throughout the experimental period. Persistent body weight gain was significantly reduced from early stage to the termination in females of all treated groups, the final body weights being decreased in the 5% (92.5%), 2.5% (94.2%) and 1.25% (94.8%) treated groups as compared to the control. Changes in MCV and serum AST, ALP, Ca, Cl and P were sporadically observed, but these were not considered to be treatment-related alterations. There were no obvious histopathological changes in any of the groups. The no-observed-effect level (NOEL) was estimated to be 5% (3011 mg/kg b.w./day) for males and the NOAEL and NOEL in females were estimated to be 3254 mg/kg b.w./day and <778 mg/kg b.w./day, respectively.
Keywords: Ellagic acid, Subchronic toxicity, F344 rats, Food additive

* Food Safety Commission

Nishimura, J.*, Dewa, Y.*, Okamura, T., Muguruma, M.*, Jin, M.*, Saegusa, Y.*, Umemura, T., Mitsumori, K.,*
: **Possible involvement of oxidative stress in fenofibrate-induced hepatocarcinogenesis in rats**
Arch. Toxicol. **82**, 641-654 (2008)

To clarify whether oxidative stress is involved in the development of hepatocellular preneoplastic foci induced by fenofibrate (FF), a peroxisome proliferator-activated receptor alpha agonist, male F344/N rats were fed a diet containing 6,000, 3,000, or 0 ppm of FF for 13 weeks after N-diethylnitrosamine initiation. Two-third partial hepatectomy was performed 1 week after the FF treatment. Histopathologically, the number of hepatocellular altered foci significantly increased in the FF-treated groups with a concomitant increase in the number of hepatocytes positive for anti-Ki-67 antibody, but the number and area of GST-P-positive foci decreased in these groups, as compared to those in the controls. Microarray analysis or quantitative real-time RT-PCR demonstrated the significant up-regulations of lipid metabolism, metabolic oxidative stress, DNA repair and cell cycle-related genes in the FF-treated groups, and the significant down-regulations of phase I or II metabolism, DNA repair and cell cycle/apoptosis-related genes in these rats. 8-OHdG level in liver DNA significantly increased due to FF treatment. These results suggest that oxidative stress is involved in the development of FF-induced hepatocellular preneoplastic foci in rats.
Keywords: fenofibrate, oxidative stress, 8-OHdG

* Tokyo University of Agriculture and Technology

Nishimura, J.*, Dewa, Y.*, Okamura, T., Jin, M.*, Saegusa, Y.*, Kawai, M.*, Umemura, T., Shibutani, M.*, Mitsumori, K.*
: **Role of Nrf2 and oxidative stress on fenofibrate-induced hepatocarcinogenesis in rats**
Toxicol. Sci., **106**, 339-349 (2008)

Regional specific relationships between oxidative stress and the development of GST-P-positive or negative lesions in rats, induced by fenofibrate (FF) were examined using a two-stage hepatocarcinogenesis model(Ito model) in F344 rats. Animals were sacrificed at week 28. Hepatocellular proliferative lesions attributed to GST-P-negative lesions was increased in the FF-treated groups. GST-P-positive lesions were devoid of intracytoplasmic Nrf2 expression, whereas GST-P-negative lesions expressed higher levels of cytoplasmic Nrf2. Nuclear accumulation of Nrf2 was observed in some cells of GST-P-positive lesions that were negative for Nrf2 in the cytoplasm and in GST-P-negative lesions of the DEN-FF group that were positive for Nrf2 in the cytoplasm. The mRNA of Gpx2 or Gsta2 was increased in GST-P-positive tumors or lesions, respectively. The activation of Nrf2, due to nuclear translocation, might occur in the GST-P-positive lesions. The continuous oxidative stress was identified by mRNA expression, GST activity and 8-OHdG. These results suggest that the relative inhibition of nuclear translocation of Nrf2 in GST-P-negative lesions aggravated the condition of oxidative stress in the liver of rats given FF, resulting in enhanced tumor promotion in FF-induced hepatocarcinogenesis.
Keywords: nrf2, fenofibrate, GST-P

* Tokyo University of Agriculture and Technology

Dewa, Y.*, Nishimura, J.*, Muguruma, M.*, Jin, M.*, Saegusa, Y.*, Okamura, T., Tasaki, M., Umemura,T.,

Mitsumori, K.[*] : **beta-Naphthoflavone enhances oxidative stress responses and the induction of preneoplastic lesions in a diethylnitrosamine-initiated hepatocarcinogenesis model inm partially hepatectomized rats**

Toxicology, **244**, 179-189 (2008)

The tumor-promoting effects of β-naphthoflavone (BNF), a novel AhR agonist, were investigated using a medium-term hepatocarcinogenesis model in rats. Six-week-old male F344 rats received an i.p. injection of N-diethylnitrosamine (DEN) at a dose of 200mg/kg body weight and were fed a diet containing 0, 0.5 or 1% BNF for 6 from 2 weeks after DEN treatment. All animals were subjected to two-thirds partial hepatectomy 1 week after the BNF treatment. The number and area of GST-P positive foci significantly increased in the livers of rats treated with BNF with concomitantly increased cell proliferation compared to those in the livers of the DEN alone group. Global gene expression analysis and quantitative real-time RT-PCR revealed that BNF induced the Nrf2-regulated genes. BNF enhanced oxidative DNA damage and lipid peroxidation, estimated by the levels of 8-OHdG and TBARS. These results suggest that the administration of BNF at a high dose and over a long-term enhance oxidative stress responses which may contribute to its hepatocarcinogenic potential in rats.

Keywords: nrf2, beta-naphthoflavone, 8-OHdG, partially hepatectomized rats

[*] Tokyo University of Agriculture and Technology

Kuroiwa, Y., Okamura, T., Ishii, Y., Umemura, T., Tasaki, M., Kanki, K., Mitsumori, K.[*1], Hirose, M.[*2], Nishikawa, A. : **Enhancement of esophageal carcinogenesis in acid reflux model rats treated with ascorbic acid and sodium nitrite in combination with or without initiation**

Cance Sci., **99**, 7-13 (2008)

Combined treatment with sodium nitrite ($NaNO_2$) and ascorbic acid (AsA) has already been shown to promote rat forestomach carcinogenesis, possibly due to nitric oxide generation under acidic conditions. To clarify a possibility of the similar effect of the luminal pH on acid reflux in the esophagus, reflux esophagitis model rats were coadministered 0.2% $NaNO_2$ in the drinking water and 1% AsA in the diet. After 32 weeks, a significant increase of epithelial hyperplasias of the lower-middle and lowest parts of the esophagus were observed. One squamous cell papilloma was found only in the combined-treatment group. In the enhancing effects of cotreatement with $NaNO_2$ and AsA on rat DHPN-initiated esophageal tumorigenesis model, the incidence of hyperplasia was enhanced in all segments. Thus, the data demonstrate that combined treatment with $NaNO_2$ and AsA exerts promoting effects on rat esophageal carcinogenesis under acid reflux conditions, as in the forestomach. These findings suggest that the risk of excessive intake of a combination of nitrite and antioxidants for esophageal carcinogenesis is appreciable, particularly in patients with reflux esophagitis.

Keywords: sodium nitrite, ascorbic acid, acid reflux model

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commission

Yoshida, M., Sanbuissyo, A.[*1], Hisada, S.[*2], Takahashi, M.[*3], Ohno, Y., Nishikawa, A. : **Morphological characterization of the ovary under normal cycling in rats and its viewpoints of ovarian toxicity detection**

J Toxicol Sci. **34**, SP189-97 (2009)

Identification of ovarian toxicity is very important for safety assessment of drugs and other environmental chemicals. The detection of interference with ovarian function is very hard without a thorough understanding of the normal ovarian morphology based on reproductive physiology. The focus of the present study was therefore a practical analysis in each stage of the estrous cycles using ovaries obtained from 143 rats demonstrating normal cycling. Transversely dissected maximum areas in the ovaries were examined microscopically for the two major features, follicles and corpora lutea (CL). Classification of growing follicles was in reference to Pedersen and Peters (1968), and functionally divided into follicular stimulating hormone (FSH)-independent and dependent categories. The former, small and medium-sized follicles, respectively primordial/primary and preantral follicles, could be readily detected by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). The large antral and Graafian follicles and large sized atretic follicles showed sequential changes depending on the estrous cycle stage. CL could be divided into currently and previously formed examples. Currently formed CL underwent remarkable changes in their appearance with the cycle, reflecting ovulation and progesterone production. Thus morphological analysis

that is synchronized the large antral follicle changes with recently formed CL ones allows the ovary to be classified into the each estrous cycle stage. Morphological deviation from any synchronized combination provides a first pointer of ovarian toxicity. PCNA immunohistochemical staining is also useful to detect small follicles.

Keywords: Ovary, toxicity, morphology, rat

[*1] Daiichi-Saykyo Co.Ltd
[*2] Aska Pharmaceutical Co. Ltd.
[*3] Pathology Peer Review Center

Takahashi, M., Shibutani, M.[*1], Sugita-Konishi, Y., Aihara, M., Inoue, K., Woo, GH., Fujimoto, H. and Hirose, M.[*2] : **A 90-day subchronic toxicity study of nivalenol, a trichothecene mycotoxin, in F344 rats**

Food Chem Toxicol., **46**,125-135 (2008)

A subchronic toxicity study of nivalenol (NIV) was conducted in male and female F344 rats fed diet containing 0, 6.25, 25 or 100 ppm concentration for 90 days. Decrease of body weight and loose stools were observed at 100 ppm in both sexes from the start of the experiment, and body weight reduction was also observed at 25 ppm in males from week 6. Hematologically, decrease of the white blood cell count was found at 100 ppm in males and from 6.25 ppm in females. In addition, decreased platelet counts in both sexes, red blood cell counts in males, and the hemoglobin concentration in females were detected at 100 ppm. Histopathologically, treatment-related changes were observed in the hematopoietic and immune organs and the anterior pituitary in both sexes and female reproductive organs at 100 ppm. Based on the hematological data, the NOAEL of NIV was determined to be less than 0.4 mg/kg body weight/day for both males and females.

Keywords: Subchronic toxicity, nivalenol, mycotoxin

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commission

Inoue, K., Shibutani ,M.[*1], Masutomi, N., Toyoda, K., Takagi, H., Uneyama, C., Nishikawa, A. and Hirose, M.[*2] : **A 13-week subchronic toxicity study of madder color in F344 rats**

Food Chem. Toxicol., **46**, 241-252 (2008)

A 13-week repeated oral dose toxicity study of madder color (MC) was performed using F344 rats. Males and female rats were fed diet containing 0, 0.6, 1.2, 2.5 or 5.0% MC. Lower body weight was evident from the 2.5% dose. Hematological data indicated weak anemia in females. Slight increases of platelet counts and WBC counts were observed at higher doses. Biochemically, slight fluctuations were observed in many parameters. Histopathologically, renal lesions such as microvesicular vacuolar degeneration in the cortex and karyomegaly in the outer medulla involving both sexes changes were observed all treated groups, the lesions being evident even with 0.6%. In the outer medulla, elevation of cell proliferation activity was observed in males from 2.5%. Severity of focal necrosis of hepatocytes was increased only in females at 5.0%, while the increased relative liver weight as with the increased conjugated bilirubin was evident in both sexes from 1.2%. The results thus suggest that MC targeting liver, kidneys, and possibly RBCs and WBCs, some renal changes being evident from 0.6% in diet, that is attributable to be LOAEL (305.8-309.2mg/kg body weight/day).

Keywords: Subchronic toxicity, madder color, Rubia tinctptum L.

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commission

Takahashi, M., Shibutani, M.[*1], Inoue, K., Fujimoto, H., Hirose, M[*2]. and Nishikawa, A. : **Pathological assessment of the nervous and male reproductive systems of rat offspring exposed maternally to acrylamide during the gestation and lactation periods - a preliminary study**

J Toxicol Sci., **33**, 11-24 (2008)

To evaluate the developmental effects of exposure to acrylamide (ACR) on the nervous and male reproductive systems, pregnant Sprague-Dawley rats were given ACR at 0, 50, 100 or 200 ppm in the drinking water from gestational day 10 to postnatal day 21 and offspring was examined at weaning and postnatal week 11. Neurotoxicity was quantitatively assessed in the sciatic nerves, and numbers of aberrant dot-like structures immunoreactive for synaptophysin in the cerebellar molecular layer. Although maternal neurotoxicity was evident from 100 ppm, no changes suggestive of neurotoxicity or testicular toxicity were observed in offspring. However, lowering of body weights was dose-dependently observed from birth at the dose levels of > or =50 ppm in males and > or =100 ppm in females. Maternal malnutrition was apparent at >/=100 ppm during the lactation period. Therefore, poor lactational

ACR-exposure due to maternal toxicity might account for the lack of ACR-induced offspring toxicity other than retarded body growth.

Keywords: Acrylamide, neurotoxicity, testicular toxicity

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commossion

Takahashi, M., Shibutani, M.[*1], Woo, GH., Inoue, K., Fujimoto, H., Igarashi, K., Kanno, J., Hirose, M.[*2] and Nishikawa A. : **Cellular distributions of molecules with altered expression specific to the tumor promotion process from the early stage in a rat two-stage hepatocarcinogenesis model**

Carcinogenesis, **29**, 2218-2226 (2008)

A global gene expression profiling specific to the early process of tumor promotion by fenbendazole (FB) or phenobarbital (PB) in a rat two-stage hepatocarcinogenesis model was investigated using the immunohistochemical distribution of transferrin receptor (Tfrc), nuclear receptor subfamily 0, group B, member 2 (Nr0b2) and minichromosome maintenance deficient 6 (MCM6) in FB- and PB-induced proliferative lesions at both early and late stages of tumor promotion. In the early stage, most hepatocellular foci positive for GST-P showed co-expression of TGFbetaRI and lack of PTEN and pPTEN, some GST-P-positive foci co-expressing Tfrc and Nr0b2. In the late stage, selective expression of TGFbetaRI was also observed in adenomas and carcinomas with consistent expression of GST-P. Nr0b2 was variably expressed in the proliferative lesions, irrespective of the carcinogenic stage. Like the GST-P-positive foci, adenomas and carcinomas consistently lacked PTEN and pPTEN. Expression of Tfrc and MCM6 was increased in parallel with the carcinogenic stage. In conclusion, loss of PTEN and dysregulation of TGFbeta signaling might be involved in rat hepatocarcinogenesis from early stages. Selective expression of Tfrc in proliferative lesions suggests an involvement of changes in iron homeostasis.

Keywords: Hepatocarcinogenesis, fenbendazole, phenobarbital

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commossion

Inoue, K., Shibutani[*1], M., Masutomi, N., Toyoda, K., Takagi, H., Takahashi, M., Fujimoto, H., Hirose, M[*2]. and Nishikawa, A. : **One-year chronic toxicity of madder color in F344 rats--induction of preneoplastic/neoplastic lesions in the kidney and liver**

Food Chem Toxicol., **46**, 3303-3310 (2008)

Chronic toxicity of madder color (MC) was investigated in F344 rats fed in the diet containing 0%, 0.2%, 1.0% or 5.0% MC for 53 weeks. Anemia and serum biochemical parameters indicating hepatotoxicity were demonstrated at 5.0% in both sexes. Liver weights were increased from 1.0% in both sexes, and the kidney weights were increased from 1.0% in males and from 0.2% in females. Atypical renal tubule hyperplasias with increase of cell proliferative activity were increased at 1.0% or higher in both sexe. A renal cell adenoma was observed in a male rat receiving 5.0% MC. In addition, glutathione S-transferase placental form-positive liver cell foci were significantly increased at 5.0% in both sexes. These results indicate that MC has chronic toxicity targeting kidney, liver and blood cells. Moreover, the results strongly suggest that MC may have the carcinogenic potential in the kidney and the liver.

Keywords: Chronic toxicity, madder color, Rubia tinctorum L.

[*1] Tokyo University of Agriculture and Technology
[*2] Food Safety Commossion

Yahia, D.[*1,2], Tsukuba, C.[*2], Yoshida, M., Sato, I.[*2] and Tsuda, S.[*2]. **Neonatal death of mice treated with perfluorooctane sulfonate (PFOS)**

J Toxicol Sci., **33**, 219-26 (2008)

Pregnant mice exposure to PFOS causes neonatal death. Ten pregnant ICR mice per group were given 1, 10 or 20 mg/kg PFOS daily by gavage from gestational day (GD) 0 to the end of the study. Five dams per group were sacrificed on GD 18 for prenatal evaluation, the others were left to give birth. PFOS treatment (20 mg/kg) reduced the maternal weight gain and feed intake but increased the water intake. The liver weight increased in a dose-dependent manner accompanied by hepatic hypertrophy at 20 mg/kg. PFOS reduced the fetal body weight in a dose-dependent manner and caused a bilateral enlargement in the neck region in all fetuses at 20 mg/kg and mild enlargement in some fetuses at 10 mg/kg, in addition to skeletal malformations. Almost all fetuses at 20 mg/kg were alive on GD18 and showed normal lung structure; but at parturition, all neonates were inactive and weak, showed severe lung atelectasis and severe dilatation of intracranial blood vessel, and died within a few hours. At 10 mg/kg, all neonates were born alive, 27% showed slight

lung atelectasis, all of them had mild to severe dilatation of the intracranial blood vessel, and 45% of neonates died within 24 hr. The cause of neonatal death in mice exposed to PFOS may be attributed either to the intracranial blood vessel dilatation or to respiratory dysfunction. The former might be a cause of the latter.

Keywords: PFOS, Neonate, mouse

[*1,2] Assiut University, Egypt

[*2] Iwate University.

Cho, Y. M., Imai, T., Ota, Y., Hasumura, M., Takami, S., Hirose, M.[*] and Nishikawa, A. : **A new medium-term rat colorectal bioassay applying neoplastic lesions as end points for detection of carcinogenesis modifiers effects with weak or controversial modifiers**

Toxicol. Pathol., **36**, 459-464 (2008)

We investigated the ability of a two-stage, medium-term rat colorectal carcinogenesis model to detect weak modifiers. F344 male rats were given three subcutaneous injections of DMH, 40 mg/kg b.w. in one week followed by drinking water containing 1% dextran sodium sulfate (DSS) for a second week. One week after this regimen, basal diet alone, or diets containing 10% perilla oil, 10% corn oil, 10% dextrin, or 0.1% indole-3-carbinol (I3C) were supplied. The perilla oil and corn oil groups did not show significant differences in the numbers of aberrant crypt foci (ACF) and incidences or multiplicity of proliferative lesions at either time point. In the dextrin group, the total number of ACF at week ten was significantly increased. With I3C, the total number of ACF and incidence and multiplicities of adenocarcinomas at week 10 and the incidence of invasive tumors at Week 20 were increased. These data essentially correspond with earlier reported results, except in the vegetable oil cases. Thus, the system is suitable for detection of colorectal carcinogenesis modifiers with advantages over previous models using ACF alone as end points.

Keywords: colorectal cancer, dextran sodium sulfate, mid-term bioassay

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[*1] Food Safety Commission

Hirata, A.[*1], Tsukamoto, T.[*1], Sakai, H.[*1], Takasu, S.[*1], Ban, H.[*1], Imai, T., Totsuka, Y.[*2], Nishigaki, R.[*2], Wakabayashi, K.[*2], Yanai, T.[*3], Masegi, T.[*3] and Tatematsu, M.[*1] : **Carcinogenic risk of heterocyclic amines in combination - assessment with a liver initiation model**

Food Chem. Toxicol., **46**, 2003-2009 (2008)

Carcinogenic potential of heterocyclic amines (HCAs) was investigated using an in vivo 5-week initiation assay with quantitative evaluation of GST-P positive foci in rat liver. GST-P positive foci were increased with individual administration of six different HCAs, indicating utility of the assay. It was therefore applied to investigate risk with multiple HCAs in combination. Concomitant treatment with PhIP and MeIQx did not result in any additive carcinogenicity. In the rats taking MeIQx prior to PhIP the value was almost equal to the sum total of individual data, indicating additive initiation activities. Simultaneous or prior administration of PhIP rather exerted inhibitory effects on the carcinogenic potential of MeIQx. Moreover, microarray and quantitative RT-PCR assessment revealed that PhIP induced cytochrome P450 1A1 more strongly than MeIQx. It is noteworthy that complex exposure to multiple HCAs is not necessarily associated with increased risk of carcinogenesis because they are simultaneously and continuously ingested under normal circumstances.

Keywords:Heterocyclic amines, glutathione S-transferase placental form positive foci, liver initiation model

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[*1] Institute of Aichi Cancer Center

[*2] National Institute of Cancer

[*3] Gifu University

Onose, J.[*1], Imai, T., Hasumura, M., Ueda, M., Ozeki, Y. and Hirose, M.[*2] : **Evaluation of subchronic toxicity of dietary administered Cry1Ab protein from Bacillus thuringiensis var. Kurustaki HD-1 in F344 male rats with chemically induced gastrointestinal impairment**

Food Chem. Toxicol., **46**, 2184-2189 (2008)

We evaluated following four F344 rat groups with a purified *Bacillus thuringiensis* (Bt) protein Cry1Ab from *B. thuringiensis* var. *Kurustaki* HD-1. Gastrointestinal impairment (GI) alone and GI + Bt protein fed (GI + Bt) groups were given i.p. injections of famotidine to reduce gastric acid secretion twice a day at 30 mg/kg body weight in weeks 2 and 4. GI and GI + Bt groups were additionally fed diets containing 80 ppm indomethacin for induction of intestinal damage during weeks 1 and 3. Bt alone and GI + Bt groups were also fed diet containing Bt protein

Cry1Ab at a concentration of 10 ppm in weeks 2 and 4. A no treatment control group was also included. At the end of week 4, all animals were euthanized under ether anesthesia, blood samples were collected for hematology and serum biochemistry and a complete necropsy was performed. No significant changes indicative of toxicity of the Bt protein Cry1Ab used here were noted with any of the parameters investigated. In conclusion, no significant toxicological effects were detected in this subchronic gastrointestinal impairment rat model.

Keywords: subchronic toxicity, Cry1Ab protein from *Bacillus thuringiensis* var. *Kurustaki*, gastrointestinal impairment

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[*1] Tokyo University of Agriculture
[*2] Food Safety Commission

Takami, S., Imai, T., Hasumura, M., Cho, Y.M., Onose, J.[*1] and Hirose, M.[*2] : **Evaluation of toxicity of green tea catechins with 90-day dietary administration to F344 rats**

Food Chem. Toxicol., **46**, 2224-2229 (2008)

As a part of their safety assessment, subchronic toxicity of green tea catechins (GTC), was investigated in male and female F344 rats with dietary administration at concentrations of 0 (control), 0.3%, 1.25% and 5.0% for 90 days. No mortality or obvious clinical signs were observed throughout the experimental period but body weights were reduced from week 1 to the end of the experiment in 5.0% males. In serum biochemistry, alanine transaminase and alkaline phosphatase in 5.0% males and females and aspartate transaminase in 5.0% females were increased, together with the relative liver weights in both sexes receiving 5.0%. Although decreases were evident for total cholesterol in 0.3-5.0% males and triglycerides in 1.25% and 5.0% males and 5.0% females, these changes were not considered to be adverse. Hematology and histopathological observation revealed no GTC-related toxicological changes. Based on above findings, NOAEL of GTC was estimated to be 764mg/kg body weight/day for males and 820mg/kg body weight/day for females.

Keywords: green tea catechins, subchronic toxicity, F344 rats

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[*1] Tokyo University of Agriculture
[*2] Food Safety Commission

Yatagai, F.[*1], Suzuki, M.[*2], Ishioka, N.[*3], Ohmori, H.[*1], and Honma, M. : **Repair of I-SceI induced DSB at a specific site of chromosome in human cells: influence of low-dose, low-dose-rate gamma-rays**

Radiat. Environ. Biophys., **47**, 439-444 (2008)

We investigated the influence of low-dose, low-dose-rate gamma-ray irradiation on DNA double strand break (DSB) repair in human lymphoblastoid TK6 cells. A single DSB was introduced at intron 4 of the TK+ allele by transfection with the I-SceI expression vector. We assessed for DSB repair due to non-homologous end-joining (NHEJ) by determining the generation of TK-deficient mutants in the TK6 derivative TSCE5 (TK+/-) carrying an I-SceI recognition site. We similarly estimated DSB repair via homologous recombination (HR) at the same site in the derived compound heterozygote (TK-/-) cell line TSCER2 that carries an additional point mutation in exon 5. The NHEJ repair of DSB was barely influenced by pre-irradiation of the cells with 30 mGy c-rays at 1.2 mGy h^{-1}. DSB repair by HR, in contrast, was enhanced by 50% after pre-irradiation of the cells under these conditions. Furthermore, when I-SceI digestion was followed by irradiation at a dose of 8.5 mGy, delivered at a dose rate of only 0.125 mGy h^{-1}, HR repair efficiency was enhanced by 80%. This experimental approach can be applied to characterize DSB repair in the low-dose region of ionizing radiation.

Keywords:Double strand break (DSB), Low-dose effect, Homologous recombination (HR)

[*1] 理化学研究所
[*2] 放射線医学総合研究所
[*3] 宇宙航空研究開発機構

Anderson, H.J.[*1], Vonarx, E.J.[*1], Pastushok, L.[*2], Nakagawa, M.[*3], Katafuchi, A., Gruz, P., Di Rubbo, A.[*1], Grice, D.M.[*1], Osmond, M.J.[*1], Sakamoto, A.N.[*3], Nohmi, T., Xiao, W.[*2], and Kunz, B.A.[*1] : **Arabidopsis thaliana Y-family DNA polymerase eta catalyses translesion synthesis and interacts functionally with PCNA2**

Plant J., **55**, 895-908 (2008)

We assessed the roles of *Arabidopsis thaliana POLH*, which encodes a homologue of Y-family polymerase eta (Polη), PCNA1 and PCNA2 in TLS-mediated UV resistance. A T-DNA insertion in *POLH* sensitized the growth of roots and whole plants to UV radiation, indicating that At Polη contributes to UV resistance. *POLH*

alone did not complement the UV sensitivity conferred by deletion of yeast *RAD30*, which encodes Polη, although AtPolη exhibited cyclobutane dimer bypass activity in vitro, and interacted with yeast PCNA, as well as with *Arabidopsis* PCNA1 and PCNA2. Co-expression of POLH and PCNA2, but not PCNA1, restored normal UV resistance and mutation kinetics in the *rad30* mutant. PCNA-interacting protein boxes and an ubiquitin-binding motif in AtPolη were found to be required for the restoration of UV resistance in the *rad30* mutant by POLH and PCNA2. These observations indicate that AtPolη can catalyse TLS past UV-induced DNA damage, and links the biological activity of AtPolη in UV-irradiated cells to PCNA2 and PCNA- and ubiquitin-binding motifs in AtPolη.

Keywords: *Arabidopsis*, POLH, PCNA

[*1] Deakin University, Australia
[*2] University of Saskatchewan, Canada
[*3] 日本原子力研究開発機構

Uchida, K.[*1], Furukohri, A.[*1], Shinozaki, Y.[*1], Mori, T.[*1], Ogawara, D.[*1], Kanaya, S.[*2], Nohmi, T., Maki, H.[*1], and Akiyama, M.[*1] : **Overproduction of *Escherichia coli* DNA polymerase DinB (Pol IV) inhibits replication fork progression and is lethal**

Mol Microbiol., **70**, 608-622 (2008)

We overexpressed *dinB*, which encodes a TLS DNA polymerase in *E. coli*, under the tightly regulable arabinose promoter and looked for a distinct phenotype. Upon induction of *dinB* expression, progression of the replication fork was immediately inhibited at random genomic positions, and the colony-forming ability of the cells was reduced. Overexpression of mutated dinB alleles revealed that the structural requirements for these two inhibitory effects and for TLS were distinct. We suggest that DinB targets Pol III, thereby acting as a brake on replication fork progression. Because the brake operates when cells have excess DinB, as they do under stress conditions, it may serve as a checkpoint that modulates replication to safeguard genome stability.

Keywords: *dinB*, replication fork, Pol III

[*1] 奈良先端大学・バイオサイエンス研究科
[*2] 奈良先端大学・情報科学研究科

Hashimoto, A.[*], Amanuma, K.[*], Masumura, K., Nohmi, T., and Aoki, Y.[*] : ***In vivo* mutagenesis caused by diesel exhaust in the testis of *gpt* delta transgenic mice**

Genes & Environ., **31**, 1-8 (2009)

Diesel exhaust (DE) is a major airborne pollutant in urban areas. In this study, we estimated the systemic effect of diesel exhaust inhalation by investigating mutations in extraplumonary organs such as the testis and liver. *gpt* delta Transgenic mice were exposed to inhalation of 3 mg m^{-3} diesel exhaust (as suspended particulate matter) for 12 or 24 weeks. Compared to the control mice, DE resulted in a 2.0-fold increase in mutant frequency in the testis of mice that were exposed to DE for 24 weeks, but not in the testis of mice exposed for 12 weeks. The mutant frequency in the lungs was 2.6-fold higher in mice exposed to DE for 24 weeks than the control group, but it was not elevated in the liver. In the testis, the major mutations on the *gpt* gene were G:C→T:A transversions, 1 base deletions and G:C→A:T transitions, while the major mutation in the lung was G:C→A:T transitions. Our results suggest that inhalation of diesel exhaust is genotoxic to the testis as well as respiratory organs.

Keywords: diesel exhaust, testis, *gpt* delta transgenic mouse

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[*] 国立環境研究所

Xu, A.[*1,2], Chai, Y.[*1], Nohmi, T., and Hei, T.K.[*1] : **Genotoxic responses to titanium dioxide nanoparticles and fullerene in *gpt* delta transgenic MEF cells**

Particle and Fibre Toxicology, **6**, 3 (2009)

Titanium dioxide (TiO_2) nanoparticles and fullerene (C60) are two attractive manufactured nanoparticles with great promise in industrial and medical applications. However, little is known about the genotoxic response of TiO_2 nanoparticles and C60 in mammalian cells. In the present study, we determined the mutation fractions induced by either TiO_2 nanoparticles or C60 in *gpt* delta transgenic mouse primary embryo fibroblasts (MEF) and identified peroxynitrite anions ($ONOO^-$) as an essential mediator involved in such process. Our results provided novel information that both TiO_2 nanoparticles and C60 were taken up by cells and induced kilo-base pair deletion mutations in a transgenic mouse mutation system. The induction of $ONOO^-$ may be a critical signaling event for nanoparticle genotoxicity.

Keywords: nanoparticle, titanium dioxide, fullerene

[*1] Columbia University, USA
[*2] Institute of Plasma Physics, Chinese Academy of

Sciences, China

Sakamoto Y[*1], Nakae D[*1*2], Fukumori N[*1], Tayama K[*1], Maekawa A[*2*3], Imai K[*4], Hirose A, Nishimura T, Ohashi N[*1] and Ogata A[*1]. : **Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats**

J. Toxicol Sci., **34**, 65-76 (2008)

The present study assessed a carcinogenic hazard of multi-wall carbon nanotube (MWCNT) in intact (not genetically modified) rodents. MWCNT (1 mg/kg body weight, 7 animals), crocidolite (2 mg/kg body weight, 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) was administered to male Fischer 344 rats (12 weeks old) by a single intrascrotal injection. Rats were autopsied immediately after death, when becoming moribund or at the end of the maximal observation period scheduled to be 52 weeks. After 37-40 weeks, however, 6 MWCNT-treated animals died or became moribund due to intraperitoneally disseminated mesothelioma (6/7, 85.7%) with bloody ascites. Peritoneal mesothelium was generally hypertrophic, and numerous nodular or papillary lesions of mesothelioma and mesothelial hyperplasia were developed. While mesothelioid cells were predominant in relatively early stage tumors, advanced stage mesotheliomas were constituted by 2 portions occupied by mesothelioid cells on the surface and spindle-shaped sarcomatous cells in the depth. In the latter, the histological transition was apparently observed between these 2 portions. Mesotheliomas were invasive to adjacent organs and tissues, and frequently metastasized into the pleura. Only 1 rat survived for 52 weeks in the MWCNT-treated group, and similar findings except mesothelioma were observed. All 10 crocidolite-treated and 5 vehicle-treated rats survived for 52 weeks without any particular changes except deposition of asbestos in the former case. It is thus indicated that MWCNT possesses carcinogenicity causing mesothelioma at a high rate in intact male rats under the present experimental conditions. The present data identifies a carcinogenic hazard of MWCNT and will serve as one of the indispensable evidences to be used for the risk assessment crucial for not only protection and improvement of human health and welfare, but also safe and acceptable development and prevalence of this and similar upcoming materials.

Keywords: Multi-wall carbon nanotube, Carcinogenicity, Hazard identification

[*1] National Institute of Advanced Industrial Science and Technology, Tsukuba Central 5, Tsukuba, Ibaraki 305-8565, Japan
[*2] Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health
[*3] Tokyo University of Agriculture
[*4] Safety Assessment Division, Chemical Management Center, National Institute of Technology and Evaluation
[*4] Biosafety Research Center Foods, Drugs and Pesticides

Ema, M.[*1], Fukunishi, K.[*2], Hirose, A., Hirata-Koizumi, M., Matsumoto, M. and Kamata, E. : **Repeated-dose and reproductive toxicity of the ultraviolet absorber 2-(3',5'-di- tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole in rats**

Drug. Chem. Toxicol.,**31**, 399-412 (2008)

2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as an ultraviolet (UV) absorber. In this study, the repeated dose and reproductive toxicity of DBHCB was evaluated in rats. Crj:CD(SD)IGS rats were given DBHCB by gavage at 0, 2.5, 25, or 250 mg/kg/d. Male and female rats were dosed beginning 28 d before mating, and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56-57 d, and females were dosed for a total of 55-69 d up to Day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed on the next day of the last administration, and 10 females were killed on Days 4-6 after parturition. Five rats/sex treated at 0 and 250 mg/kg/d for 56 d were then kept without treatment for 14 d (recovery period). No deaths were found in any group. No effects of DBHCB on general condition, body weight, food consumption, or reproductive/developmental parameters were observed. Significant increases in serum albumin and an albumin/globulin ratio at 25 mg/kg/d and higher and alkaline phosphatase levels at 250 mg/kg/d were noted in males. The absolute and relative weights of the liver were significantly increased in males at 25 mg/kg/d and higher. Significantly increased serum albumin and absolute and relative liver weight were also found in males at 250 mg/kg/d after the recovery period. No changes in these parameters were observed in females of any DBHCB-treated groups. No significant changes in organ histopathology were found in males or females. These findings indicated a sex difference in the toxicity of DBHCB in rats.

Keywords: Ultraviolet absorber, Reproductive toxicity, Triazoles

[*1] National Institute of Advanced Industrial Science and

Technology
*2 Shin Nippon Biomedical Laboratories, Ltd.

Ema, M.*1, Fujii, S.*2, Hirata-Koizumi, M. and Matsumoto, M. : **Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats**

Reprod. Toxicol.,**25**, 335-351 (2008)

Male and female rats were fed a diet containing flame retardant hexabromocyclododecane (HBCD) at 0, 150, 1500 or 15,000 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation and lactation periods for two generations. The mean daily intakes of HBCD during the whole period of administration were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively. The incidence of rats with decreased thyroid follicles size was increased in F0 and F1 males and females at 1500 ppm and higher. Serum TSH levels were increased in F0 and F1 females at 1500 ppm and higher, and serum T4 levels were decreased in F0 males and females at 15,000 ppm. The number of the primordial follicles in the ovary of F1 females was reduced at 1500 ppm and higher. There were increases in the absolute and relative weights of the liver in male adults and male and female weanlings at 1500 ppm and higher, and in female adults at 15,000 ppm, and of the thyroid in male and female adults at 15,000 ppm. Decreased body weight and body weight gain associated with reduced food consumption were found in F1 males and females at 15,000 ppm. Decreases were found in the viability index of F2 pups and the body weight of male F1 and F2 pups and female F2 pups at 15,000 ppm. In F2 pups, there were low incidences of the completion of eye opening in males at 15,000 ppm and in females at 1500 ppm and higher, and of completed mid-air righting in females at 15,000 ppm. The data indicate that the NOAEL of HBCD in this study was 150 ppm (10.2mg/kg bw/day). The estimated human intake of HBCD is well below the NOAEL in the present study.

Keywords: Hexabromocyclododecane, Reproductive toxicity, Spermatozoa

*1 National Institute of Advanced Industrial Science and Technology
*2 Safety Research Institute for Chemical Compounds Co., Ltd.

Harada, T., Kimura, E.*, Hirata-Koizumi, M., Hirose, A., Kamata, E. and Ema, M. : **Reproductive and developmental toxicity screening study of 4-aminophenol in rats**

Drug. Chem. Toxicol.,**31**, 473-486 (2008)

Twelve male and female rats per group were given 4-aminophenol (PAP) by gavage at 0, 20, 100, or 500 mg/kg/day. Males were dosed for a total of 49 days, beginning 14 days before mating. Females were dosed for a total of 40-60 days, from 14 days before mating to Day 3 of lactation throughout the mating and gestation periods. Four males and 2 females died at 500 mg/kg/day, and all surviving males and females showed brown urine at 100 mg/kg/day and above. Body-weight gain was lower in males and females at 500 mg/kg/day, and food consumption was decreased in males at 500 mg/kg/day and in females at 100 and 500 mg/kg/day. Absolute and relative weights of the testes and epididymides were decreased at 500 mg/kg/day. Histopathological examinations revealed decreased spermatocyte and spermatid levels in the testis, debris of germ cell in the epididymis lumen, basophilic tubules in the kidney, and deposits of hemosiderin in the red pulp and extramedullary hematopoiesis in the spleen in males at 500 mg/kg/day. Longer gestation period, decreased delivery index, and lower body weight of pups on postnatal day (PND) 0 and increased number of stillborns at 500 mg/kg/day were also observed. At this dose, the viability of pups on PND 4 was decreased markedly. No adverse effects on reproduction or development were detected at 20 and 100 mg/kg/day. These findings indicate that PAP is general and reproductive/developmental toxic, but is unlikely to be teratogenic, in rats.

Keywords: 4-Aminophenol, Testicular toxicity, Neonatal death

* Mitsubishi Chemical Safety Institute Ltd.

Hirata-Koizumi, M., Matsuyama, T.*1, Imai, T., Hirose, A., Kamata, E. and Ema, M.*2 : **Gender-related difference in the toxicity of ultraviolet absorber 2-(3',5'-di-tert-butyl-2'-hydroxyphenyl)-5-chlorobenzotriazole in rats**

Drug. Chem. Toxicol.,**31**, 383-398 (2008)

2-(3',5'-Di-tert-butyl-2'-hydroxyphenyl)-5-

chlorobenzotriazole (DBHCB) is widely used as an ultraviolet absorber. Previously, we showed that male rats had more than a 100 times higher susceptibility to the toxic effects of DBHCB than females. In order to investigate the role of sex steroids in the mediation of this gender-related difference, DBHCB (0 or 250 mg/kg/day) was given to male and female young intact and castrated rats by gavage for 28 days in the current study. In intact rats, relative liver weight increased to more than two times that of the control in males, while the rate of change was less than 10% in females. On histopathology, hypertrophy of hepatocytes was observed in males but not in females. In castrated rats, an approximately 40% increase in the relative liver weight was found only in males, and no histopathological changes in the liver were detected in either sex. The gender-related difference was also determined in preweaning rats administered DBHCB at 0, 250, or 500 mg/kg/day by gavage from postnatal days 4 to 21. Blood biochemical changes, including increases in the levels of AST, ALT, and ALP, 80-95% increase in the relative liver weight and histopathological changes in the liver, such as hypertrophy and single cell necrosis of hepatocytes, were observed at both doses in both sexes. In conclusion, the gender-related difference in the toxicity of DBHCB, which was observed in young rats, was markedly reduced by castration and abolished in preweaning rats.

Keywords: Ultraviolet absorber, Gender-related difference, Preweaning rats

[*1] Shin Nippon Biomedical Laboratories, Ltd.

[*2] National Institute of Advanced Industrial Science and Technology

Takahashi, M., Sunaga, M.[*], Hirata-Koizumi, M., Hirose, A., Kamata, E. and Ema, M. : **Reproductive and developmental toxicity screening study of 2,4-dinitrophenol in rats**

Environ. Toxicol.,**24**, 74-81 (2009)

Rats were treated by gavage once daily with 2,4-dinitrophenol (DNP) at 0 (control), 3, 10, or 30 mg/kg bw. Males were dosed for 46 days, beginning 14 days before mating, and females were dosed for 40-47 days, from 14 days before mating to day 3 of lactation. No deaths were observed in males and females of any group. A significant decrease in body weight gain and significant increase in liver weight were found in males and females at 30 mg/kg bw/day. The number of live pups on postnatal days (PNDs) 0 and 4, live birth index, and body weight of live male and female pups on PNDs 0 and 1 were significantly lowered at 30 mg/kg bw/day. External and internal examinations of pups revealed no increased incidence of malformations in DNP-treated groups. On the basis of these findings, we concluded that DNP has general and reproductive/developmental toxicity, but not teratogenicity, under the present conditions. The NOAEL of DNP is considered to be 10 mg/kg bw/day in rats.

Keywords: 2,4-Dinitrophenol, Reproduction, Development

[*] Safety Research Institute for Chemical Compounds Co., Ltd.

Hirode, M.[*1], Ono, A., Miyagishima, T.[*2], Nagao, T.[*3], Ohno, Y. and Urushidani, T.[*4] : **Gene expression profiling in rat liver treated with compounds inducing phospholipidosis**

Toxicol. Appl. Pharmacol.,**229**, 290-299 (2008)

We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for diagnosis of hepatic phospholipidosis, we extracted 78 probe sets of rat hepatic genes from data of 5 drugs, amiodarone, amitriptyline, clomipramine, imipramine, and ketoconazole, which actually induced this phenotype. Principal component analysis (PCA) using these probes clearly separated dose- and time-dependent clusters of treated groups from their controls. Moreover, 6 drugs (chloramphenicol, chlorpromazine, gentamicin, perhexiline, promethazine, and tamoxifen), which were reported to cause phospholipidosis but judged as negative by histopathological examination, were designated as positive by PCA using these probe sets. Eight drugs (carbon tetrachloride, coumarin, tetracycline, metformin, hydroxyzine, diltiazem, 2-bromoethylamine, and ethionamide), which showed phospholipidosis-like vacuolar formation in the histopathology, could be distinguished from the typical drugs causing phospholipidosis. Moreover, the possible induction of phospholipidosis was predictable by the expression of these genes 24 h after single administration in some of the drugs. We conclude that these identified 78 probe sets could be useful for diagnosis of phospholipidosis, and that toxicogenomics would be a promising approach for prediction of this type of toxicity.

Keywords: Toxicogenomics, Phospholipids, Principal Component Analysis

[*1] Takeda Pharmaceutical Company Limited
[*2] National Institute of Biomedical Innovation
[*3] Food Safety Commission of Japan
[*4] Doshisha Women's College of Liberal Arts

Uehara, T.[*1], Kiyosawa, N.[*1], Shimizu, T.[*1], Omura, K.[*1], Hirode, M.[*1], Imazawa, T.[*1], Mizukawa, Y.[*2], Ono, A., Miyagishima, T.[*1], Nagao, T.[*3] and Urushidani, T.[*2] : **Species-specific differences in coumarin-induced hepatotoxicity as an example toxicogenomics-based approach to assessing risk of toxicity to humans**

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One expected result from toxicogenomics technology is to overcome the barrier because of species-specific differences in prediction of clinical toxicity using animals. The present study serves as a model case to test if the well-known species-specific difference in the toxicity of coumarin could be elucidated using comprehensive gene expression data from rat in-vivo, rat in-vitro, and human in-vitro systems. Coumarin 150 mg/kg produced obvious pathological changes in the liver of rats after repeated administration for 7 days or more. Moreover, 24 h after a single dose, we observed minor and transient morphological changes, suggesting that some early events leading to hepatic injury occur soon after coumarin is administered to rats. Comprehensive gene expression changes were analyzed using an Affymetrix GeneChip approach, and differentially expressed probe sets were statistically extracted. The changes in expression of the selected probe sets were further examined in primary cultured rat hepatocytes exposed to coumarin, and differentially expressed probe sets common to the in-vivo and in-vitro datasets were selected for further study. These contained many genes related to glutathione metabolism and the oxidative stress response. To incorporate human data, human hepatocyte cultured cells were exposed to coumarin and changes in expression of the bridging gene set were examined. In total, we identified 14 up-regulated and 11 down-regulated probe sets representing rat-human bridging genes. The overall responsiveness of these genes to coumarin was much higher in rats than humans, consistent with the reported species difference in coumarin toxicity. Next, we examined changes in expression of the rat-human bridging genes in cultured rat and human hepatocytes treated with another hepatotoxicant, diclofenac sodium, for which hepatotoxicity does not differ between the species. Both rat and human hepatocytes responded to the marker genes to the same extent when the same concentrations of diclofenac sodium were exposed. We conclude that toxicogenomics-based approaches show promise for overcoming species-specific differences that create a bottleneck in analysis of the toxicity of potential therapeutic treatments.

Keywords: Toxicogenomics, Coumarins, Species Specificity

[*1] National Institute of Biomedical Innovation
[*2] Doshisha Women's College of Liberal Arts
[*3] Food Safety Commission of Japan

Uehara, T.[*1], Hirode, M. [*1], Ono, A., Kiyosawa, N. [*1], Omura, K. [*1], Shimizu, T. [*1], Mizukawa, Y. [*2], Miyagishima, T. [*1], Nagao, T. and Urushidani, T. [*2] : **A toxicogenomics approach for early assessment of potential non-genotoxic hepatocarcinogenicity of chemicals in rats**

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For assessing carcinogenicity in animals, it is difficult and costly, an alternative strategy has been desired. We explored the possibility of applying a toxicogenomics approach by using comprehensive gene expression data in rat liver treated with various compounds. As prototypic non-genotoxic hepatocarcinogens, thioacetamide (TAA) and methapyrilene (MP) were selected and 349 commonly changed genes were extracted by statistical analysis. Taking both compounds as positive with six compounds, acetaminophen, aspirin, phenylbutazone, rifampicin, alpha-naphthylisothiocyanate, and amiodarone as negative, prediction analysis of microarray (PAM) was performed. By training and 10-fold cross validation, a classifier containing 112 probe sets that gave an overall success rate of 95% was obtained. The validity of the present discriminator was checked for 30 chemicals. The PAM score showed characteristic time-dependent increases by treatment with several non-genotoxic hepatocarcinogens, including TAA, MP, coumarin, ethionine and WY-14643, while almost all of the non-carcinogenic samples were correctly predicted. Measurement of hepatic glutathione content suggested that MP and TAA cause glutathione depletion followed by a protective increase, but the protective response is exhausted during repeated administration. Therefore, the presently obtained PAM classifier could predict potential non-genotoxic hepatocarcinogenesis within 24 h after single dose and the inevitable pseudo-positives could be eliminated by checking data of repeated administrations up to 28

days. Tests for carcinogenicity using rats takes at least 2 years, while the present work suggests the possibility of lowering the time to 28 days with high precision, at least for a category of non-genotoxic hepatocarcinogens causing oxidative stress.

Keywords: Toxicogenomics, Hepatocarcinogenesis, Non-genotoxic

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[*1] National Institute of Biomedical Innovation

[*2] Doshisha Women's College of Liberal Arts