E. Fahy[*1], S. Subramaniam[*1], R. C. Murphy[*2], Nishijima, M., C. R. H.Raetz[*3], Shimizu T.[*4], F. Spener[*5], G. van Meer[*6], M. J. Wakelam[*7], and E. A. Dennis[*1]: **Update of the LIPID MAPS comprehensive classification system for lipids**

J. Lipid. Res., **50**, S9-S14（2009）

In 2005, the International Lipid Classification and Nomenclature Committee under the sponsorship of the LIPID MAPS Consortium developed and established a "Comprehensive Classification System for Lipids" based on well-defined chemical and biochemical principles and using an ontology that is extensible, flexible, and scalable. This classification system, which is compatible with contemporary databasing and informatics needs, has now been accepted internationally and widely adopted. In response to considerable attention and requests from lipid researchers from around the globe and in a variety of fields, the comprehensive classification system has undergone significant revisions over the last few years to more fully represent lipid structures from a wider variety of sources and to provide additional levels of detail as necessary. The details of this classification system are reviewed and updated and are presented here, along with revisions to its suggested nomenclature and structure-drawing recommendations for lipids.

Keywords: lipidomics, nomenclature, databases

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Tomishige, N.[*1], Kumagai, K.[*2], Kusuda, J.[*1], Nishijima, M., and Hanada, K.[*1]: **Casein kinase Iγ2 down-regulates trafficking of ceramide in the synthesis of sphingomyelin**

Mol. Biol. Cell., **20**, 348-357（2009）

Intracellular trafficking of lipids is fundamental to membrane biogenesis. For the synthesis of sphingomyelin, ceramide is transported from the endoplasmic reticulum to the Golgi apparatus by the ceramide transfer protein CERT. CERT is phosphorylated by protein kinase D at S132 and subsequently multiple times in a serine-repeat motif, resulting in its inactivation. However, the kinase involved in the multiple phosphorylation remains unclear. Here, we identify the gamma2 isoform of casein kinase I（CKIgamma2）as a kinase whose overexpression confers sphingomyelin-directed toxin-resistance to Chinese hamster ovary cells. In a transformant stably expressing CKIgamma2, CERT was hyperphosphorylated, and the intracellular trafficking of ceramide was retarded, thereby reducing de novo sphingomyelin synthesis. The reduction in the synthesis of sphingomyelin caused by CKIgamma2 was reversed by the expression of CERT mutants that are not hyperphosphorylated. Furthermore, CKIgamma2 directly phosphorylated CERT in vitro. Among three gamma isoforms, only knockdown of gamma2 isoform caused drastic changes in the ratio of hypo- to hyper-phosphorylated form of CERT in HeLa cells. These results indicate that CKIgamma2 hyperphosphorylates the serine-repeat motif of CERT, thereby inactivating CERT and down-regulating the synthesis of sphingomyelin.

Keywords: ceramide, casein kinase I, CERT

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Nitahara-Kasahara, Y.[*], Fukasawa, M.[*], Shinkai-Ouchi, F.[*], Sato, S.[*], Suzuki, T.[*], Murakami, K.[*], Wakita, T.[*], Hanada, K.[*], Miyamura, T.[*], and Nishijima, M.: **Cellular vimentin content regulates the protein level of hepatitis C virus core protein and the hepatitis C virus production in cultured cells**

Virology., **383**, 319-327（2009）

Hepatitis C virus（HCV）core protein is essential for virus particle formation. Using HCV core-expressing and non-expressing Huh7 cell lines, Uc39-6 and Uc321, respectively, we performed comparative proteomic studies of proteins in the 0.5% Triton X-100-insoluble fractions of cells, and found that core-expressing Uc39-6 cells had much lower vimentin content than Uc321 cells. In experiments using vimentin-overexpressing and vimentin-knocked-down cells, we demonstrated that core protein levels were affected by cellular vimentin content. When vimentin expression was knocked-down, there was no difference in mRNA level of core protein; but proteasome-dependent degradation of the core

protein was strongly reduced. These findings suggest that the turnover rate of core protein is regulated by cellular vimentin content. HCV production was also affected by cellular vimentin content. Our findings together suggest that modulation of hepatic vimentin expression might enable the control of HCV production.

Keywords: hepatitis C virus, core protein, vimentin

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Brown, C. K.[*1], Buhse, L.[*2], Friedel, H.[*3], Keitel, S.[*4], Kraemer, J.[*5], Morris, M.[*6], Stickelmeyer, M.[*7], Yomota, C. and Shah, V. P.[*7]: **FIP Position Paper on Qualification of Paddle and Basket Dissolution Apparatus**

AAPS Pharm. Sci. Tech., **10**, 924-927（2009）

The qualification process for ensuring that a paddle or basket apparatus is suitable for its intended use is a highly debated and controversial topic. Different instrument qualification and suitability methods have been proposed by the pharmacopeias and regulatory bodies. In an effort to internationally harmonize dissolution apparatus suitability requirements, the International Pharmaceutical Federation's（FIP）Dissolution/Drug Release Special Interest Group（SIG）reviewed current instrument suitability requirements listed in the US, European, and Japanese pharmacopeias and the International Conference on Harmonization（ICH）Topic Q4B on harmonization of pharmacopoeial methods, in its Annex 7, Dissolution Test General.

Keywords: basket apparatus, paddle apparatus, performance verification test

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[*5] PHAST
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柘植秀哉[*1]，大内　正[*1]，中島辰巳[*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３報）**

医薬品研究，**40**，505-519（2009）

凝固点降下を用いる浸透圧計に機種間差が存在し，装置校正用オスモル濃度標準液の調製が煩雑であることから，装置メーカーの校正用標準液を使用しているメーカーが多く，測定データに影響が認められないことから，二点校正法やより簡便な校正方法を取り込むことが期待された．また，1000mOsmを越える試料の測定では，現在希釈測定し，希釈倍率を乗じることとなっているが，希釈倍率を記載して，測定値をその表示する方向で，一般試験法の修正が望ましい．

Keywords: 浸透圧測定，機種間差，校正用標準液

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田邊豊重[*1]，高居邦弘[*1]，青木光夫[*1]，大久保恒夫[*1]，大内　正[*2]，寺田三郎[*2]，柘植秀哉[*2]，四方田千佳子：**輸液用ゴム栓試験法の見直し研究（第１報）**

医薬品研究，**41**，221-239（2010）

USPのゴム栓試験法は，2008年に改正されてEP，ISOのゴム栓試験法とほぼ整合するようになった．三薬局方の中でいち早くゴム栓試験法を収載した日局は長らく見直しが行われていない．日局の輸液用ゴム栓試験法を科学的に見直し，注射用ゴム栓試験法として改訂する必要がある．

Keywords: 浸透圧測定，機種間差，校正用標準液

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飯田芳男[*1]，中村利廣[*2]，川瀬　晃[*3]，山崎慎一[*4]，四方田千佳子，松田りえ子，小野昭紘[*5]，柿田和俊[*5]，瀧本憲一[*5]，坂田　衛[*5]：**日本分析化学会標準物質 Jsac0302，Jsac0311，Jsac0401，Jsac0501，Jsac0602-2，JsacPT0711，JsacPT0721の安定性試験結果**

分析化学，**58**，951-962（2009）

標準物質において認証値のトレーサビリティーと安定性が重要である．認証値に関しては，ISOGuide43-1に準拠した共同実験で対応しているが，安定性について，１，３，５，７，10年後に試験を実施することと規定し，認証書には，その結果を公表することとしている．ここでは，7種の標準物質について得られたデータと評価を公表した．

Keywords: 認証標準物質，認証値，安定性

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Izutsu, K., Kadoya, S., Yomota, C., Kawanishi, T., Yonemochi, E.*, and Terada, K.*: **Stabilization of protein structure in freeze-dried amorphous organic acid buffer salts**

Chem. Pharm. Bull., **57**, 1231-1236 (2009)

The purpose of this study was to elucidate the physical properties and protein-stabilizing effects of some pH-adjusting excipients (carboxylic acids and their sodium salts) in frozen solutions and in freeze-dried solids. Thermal and powder X-ray diffraction (XRD) analysis indicated a high propensity of sodium citrates to form glass-state amorphous solids upon freeze-drying. Some salts (e.g., sodium succinate) crystallized in the single-solute frozen solutions. FT-IR analysis of bovine serum albumin (BSA) and bovine immunoglobulin G (IgG) in the aqueous solutions and the freeze-dried solids showed that some glass-forming salts (e.g., monosodium citrate) protected the secondary structure from lyophilization-induced perturbation. Freeze-drying of BSA at different concentrations indicated retention of the secondary structure at similar monosodium citrate/protein concentration ratios, suggesting stabilization through direct interaction that substitute water molecules inevitable for the conformation integrity. The carboxylic acid salts should provide rigid hydrogen bonds and electrostatic interactions that raise the glass transition temperature of the amorphous solids and stabilize protein structure. The relevance of the structural stabilization to the protein formulation design was discussed.

Keywords: freeze-drying, protein formulation, stabilization

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Kadoya, S.*, Fujii, K.*, Izutsu, K., Yonemochi, E.*, Terada, K.*, Yomota, C., Kawanishi, T.: **Freeze-drying of proteins with glass-forming oligosaccharide-derived sugar alcohols**

Int. J. Pharm., **389**, 107-113 (2010)

Physical properties and protein-stabilizing effects of sugar alcohols in frozen aqueous solutions and freeze-dried solids were studied. Various frozen sugar alcohol solutions showed a glass transition of the maximally freeze-concentrated phase at temperatures (T_g's) that depended largely on the solute molecular weights. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure freeze-dried solids. Microscopic observation of frozen maltitol and lactitol solutions under vacuum (FDM) indicated onset of physical collapse at temperatures (T_c) several degrees higher than their T_g's. Freeze-drying of pentitols (e.g., xylitol) and hexitols (e.g., sorbitol, mannitol) resulted in collapsed or crystallized solids. The glass-forming sugar alcohols prevented activity loss of a model protein (LDH: lactate dehydrogenase) during freeze-drying and subsequent storage at 50 degrees C. They also protected bovine serum albumin (BSA) from lyophilization-induced secondary structure perturbation. The glass-forming sugar alcohols showed lower susceptibility to Maillard reaction with co-lyophilized L-lysine compared to reducing and non-reducing disaccharides during storage at elevated temperature. Application of the oligosaccharide-derived sugar alcohols as alternative stabilizers in lyophilized protein formulations was discussed.

Keywords: freeze-drying, amorphous, protein formulation

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Tokunaga, H.*1, Izutsu, K., Arai S.*2, Yonezawa, Y.*2, Kuroki, R.*2, Arakawa, T.*3, Tokunaga, M.*1: **Dimer-tetramer assembly of nucleoside diphosphate kinase from moderately halophilic bacterium Chromohalobacter salexigens DSM3043: Both residues 134 and 136 are critical for the tetramer assembly**

Enz. Microb. Tech., **46**, 129-135 (2010)

The subunit structure of Halomonas nucleoside diphosphate kinase (HaNDK) is a dimer, different from NDKs of other species. We have shown before that it is due to Glu134 in HaNDK, which results in steric hindrance and charge repulsion between two dimeric units and prevents further assembly into the tetramer and that changing the Glu134 to neutral Ala results in formation of a stable tetramer. To our surprise, both wild-type NDK from moderately halophilc Chromohalobacter salexigens (CsNDK/GNE: GNE represents Gly134-Asn135-Glu136) and mutant CsNDK/ANE, both of which have a neutral amino acid at resi-

due 134, were found to form a dimer. These constructs contain Glu136, which may also cause steric barrier and charge repulsion. A double mutant, CsNDK/ANT, having Thr at 136 resulted in stable tetrameric assembly, supporting the above notion. A mutant CsNDK/GNT reverted, however, to a dimmer again, indicating that the introduced Ala residue at 134th in the double mutant generated a hydrophobic cluster consisting of the Ala residues and thereby stabilized dimer-dimer association of CsNDK assembly, while Gly destabilized it due to the loss of this cluster. Based on these observations, it is evident that both residues 134 and 136 contribute to the subunit assembly of CsNDK.

Keywords: nucleoside diphosphate kinase, halophilic, subunit

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Shibata, H., Saito, H., Yomota, C., Kawanishi, T.:
Pharmaceutical quality evaluation of lipid emulsions containing PGE1: alteration in the number of large particles in infusion solutions
Int. J. Pharm., **378**, 167-76 (2009)

There are two generics of a parenteral lipid emulsion of prostaglandin E1 (PGE(1)) (Lipo-PGE(1)) in addition to two innovators. It was reported the change from innovator to generic in clinical practice caused the slowing of drip rate and formation of aggregates in the infusion line. Thus, we investigated the difference of pharmaceutical quality in these Lipo-PGE(1) formulations. After mixing with some infusion solutions, the mean diameter and number of large particles were determined. Although the mean diameter did not change in any infusion solutions, the number of large particles (diameter >1.0 microm) dramatically increased in generics with Hartmann's solution pH 8 or Lactec injection with 7% sodium bicarbonate. Next, we investigated the effect of these infusion solutions on the retention rate of PGE(1) in lipid particles. The retention rate of PGE(1) in these two infusion solutions decreased more quickly than that in normal saline. Nevertheless, there were no significant differences among the formulations tested. Our results suggest that there is no difference between innovators and generics except in mixing with these infusion solutions. Furthermore, that monitoring the number of large particles can be an effective means of evaluating pharmaceutical interactions and/or the stability of lipid emulsions.

Keywords: emulsion, prostaglandin E1, lipid particle

Shibata, H., Saito, H., Yomota, C., Kawanishi, T.:
Ammonium ion level in serum affects doxorubicin release from liposomes
Pharmazie, **65**(4), 251-3 (2010)

In this study, we measured the release of drug from liposome-encapsulated doxorubicin (DXR) in human and mouse serum. While human serum did not induce DXR-release, mouse serum significantly induced DXR-release in a temperature- and time-dependent manner. Release of DXR was clearly observed in ultrafiltrated mouse serum, indicating that low-molecular substances affect DXR-release. Therefore, the level of Na+, Cl(-), NH_4+, and urea nitrogen in each type of serum was measured. Only the concentration of NH_4+ in mouse serum was significantly higher than that in human serum. Furthermore, addition of ammonium acetate to human serum induced DXR release at the same level observed in mouse serum. These results indicate that the NH_4+ concentration in serum might greatly affect the release of DXR from liposomes.

Keywords: liposome, doxorubicin, release

Shibata, H., Abe, Y.[*1], Yoshioka, Y.[*1, 2], Nomura, T.[*1], Sato, M.[*1], Kayamuro, H.[*1, 3], Kawara, T.[*1, 3], Arita, S.[*1, 3], Furuya, T.[*1, 3], Nagano, K.[*1], Yoshikawa, T.[*1, 3], Kamada, H.[*1, 2], Tsunoda, S.[*1, 2, 3], Tsutsumi, Y.[*1, 2, 3]:
Generation of mouse macrophages expressing membrane-bound TNF variants with selectivity for TNFR1 or TNFR2
Cytokine, **50**(1), 75-83 (2010)

Tumor necrosis factor-alpha (TNF) is expressed on the cell surface as a transmembrane form (tmTNF), that can be released as a soluble form (solTNF) via proteolytic cleavage. These two types of TNF exert their biological functions by binding to one of two TNF receptors, TNFR1 or TNFR2. However, the biological function of tmTNF through these two receptors remains to be determined. Here, we generated macrophages that expressed tmTNF mutants with selectivity for either TNFR1 or TNRF2 as a tool to evaluate signaling through these receptors. Wild-type TNF

（wtTNF），TNFR1-selective mutant TNF（mutTNF-R1）or TNFR2-selective mutant TNF（mutTNF-R2）were individually expressed on the TNFR1−/−R2−/− mouse macrophages（Mϕ）as the tmTNF forms. tm-mutTNF-R1-expressing Mϕ exhibited significant selectivity for binding to TNFR1, whereas tm-mutTNF-R2-expressing Mϕ only showed a slight selectivity for binding to TNFR2. Signaling by tm-mutTNF-R1-expressing Mϕ through the hTNFR2 was weaker than that of tm-wtTNF-expressing Mϕ, suggesting that the binding selectivity correlated with functional selectivity. These results indicate tmTNF variants might prove useful for the functional analysis of signaling through TNF receptors.

Keywords: Transmembrane TNF, Mutant TNF, Lentiviral vector

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Yoshida, H., Nishikawa, M.[*1], Yasuda, S.[*1], Mizuno, Y.[*1], Toyota, H.[*1], Takahashi, R.[*2], Takakura, Y.[*1]: **TLR9-dependent systemic interferon-β production by intravenous injection of plasmid DNA/cationic liposome complex in mice**

J. Gene. Med., **11**, 708-717（2009）

After an intravenous injection into mice, CpG lipoplex induced a large increase in the levels of IFN-β and IL-6 in the serum, liver, spleen, lung and kidney, whereas non-CpG lipoplex scarcely had any effect. Neither formulation led to significant cytokine production in TLR9$^{-/-}$ mice. Clodronate liposome-treated mice showed a large reduction in both IFN-β and IL-6 levels. Splenectomized mice receiving CpG lipoplex also showed a significantly low production of IL-6 but a similar level of IFN-β production to that of unsplenectomized mice. A large number of monocytes were found in the capillary vessels around the alveoli of mice receiving lipoplex. Thus, unlike the production of IL-6 from splenic Mϕ, IFN-β is produced from phagocytic cells other than splenic Mϕ after the injection of CpG lipoplex TLR9-dependently.

Keywords: IFN-β, lipoplex, TLR9

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Yasuda, S.[*], Yoshida, H., Nishikawa, M.[*], Takakura, Y.[*]: **Comparison of the type of liposome involving cytokine production induced by non-CpG Lipoplex in macrophages**

Mol. Pharm., **7**, 533-542（2010）

The production of IFN-β, TNF-α and IL-6 by lipoplex was confirmed to be induced independently of the interaction between CpG DNA and TLR9 in macrophages from TLR9$^{-/-}$ mice. The level of cytokine production and the increase in the *Z-DNA binding protein-1*（*Zbp1*）, a cytosolic doublestranded DNA sensor, mRNA varied depending on the type of cationic liposome in a macrophage-like cell line, RAW264.7. A good correlation was observed between the cytokine level and the *Zbp1* mRNA. A confocal microscopic study using fluorescently labeled pDNA complexes showed that the complexes that released a lot of cytokines showed an enhanced distribution of pDNA-derived fluorescence into the cytosol. These results suggest that different intracellular trafficking derived from the type of liposomes determines the recognition of pDNA by ZBP1 after uptake of lipoplexes by the macrophages, followed by the release of type I IFNs and inflammatory cytokines.

Keywords: non-CpG pDNA, inflammatory response, ZBP1

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Wang, B.[*], Cicerone, M.T.[*], Aso, Y., Pikal, M. J.[*]: **The impact of thermal treatment on the stability of freeze-dried amorphous pharmaceuticals: II. aggregation in an IgG1 fusion protein**

J. Pharm. Sci. **99**, 683-700（2010）

IgG1の凍結乾燥製剤の保存安定性に及ぼす熱処理の影響を明らかにした．熱処理により，タンパク質の２次構造や局所的な運動性に大きな変化は見られず，熱処理によるIgG1の安定性の向上はグローバルな運動性の低下によって説明できることが示唆された．

Keywords: annealing, stability, mobility

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Sakamoto, T., Portieri, A.[*1], Taday, P.F.[*1], Takada, Y.[*2], Sasakura, D.[*3], Aida, K.[*2], Matsubara, T.[*3], Miura, T.[*3], Terahara T.[*2], Arnone, D.D.[*1], Kawanishi, T., Hiyama, Y.: **Detection of tulobuterol crystal in**

transdermal patches using terahertz pulsed spectroscopy and imaging

Pharmazie, **64**, 361-365（2009）

Applicability of a Terahertz Pulsed Spectroscopy（TPS）and a Terahertz Pulsed Imaging（TPI）for detection of tulobuterol（TBR）crystals in transdermal patches was investigated. Because TBR has high permeability in dermis, crystalline TBR in patch matrices contributes to controlling the release rate of TBR from a matrix. Therefore, crystalline TBR is one of the important factors for quality control of TBR transdermal tapes. A model tape that includes 5w/w%, 10w/w%, 20w/w% or 30w/w% of TBR was measured by TPS/TPI. TBR crystals in the matrices were successfully detected by TPI. Identification of TBR in an image of a crystal-like mass was done by comparison between the spectra of tapes and a TBR standard substance. These results indicate that TPS and TPI are applicable to identifying crystalline lumps of an active drug in tapes for quality control.

Keywords: terahertz pulsed imaging, TDDS, tulobuterol

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Chikuma, T.[*1], Yoshimoto, T.[*1], Ohba, M.[*1], Sawada, M.[*2], Kato, T.[*3], Sakamoto, T., Hiyama Y., Hojo. H.[*1]: **Interleukin-6 induces Prostaglandin E(2) Synthesis in Mouse Astrocytes**

J. Mol. Neurosci., **39**, 175-184（2009）

The physiological function of interleukin-6 within the central nervous system（CNS）is complex; interleukin-6 exerts neurotrophic and neuroprotective effects and yet can also function as a mediator of inflammation, demyelination, and astrogliosis depending on the cellular context. However, the roles of interleukin-6 in astrocytes are poorly understood. In the present study, we investigated the effect of the pro-inflammatory cytokine interleukin-6 on the production of the inflammatory mediator prostaglandin E(2) in mouse astrocytes. Interleukin-6 stimulated prostaglandin E(2) production in a time-dependent fashion via a rapid and transient induction of cyclooxygenase-2 messneger RNA, followed by cyclooxygenase-2 protein synthesis. Interleukin-6 may act on the nervous system by interacting with its specific soluble interleukin-6 receptor and the signal transducer 130-kDa glycoprotein. Simultaneous treatment of astrocytes with interleukin-6 and soluble interleukin-6 receptor caused marked induction of prostaglandin E(2) synthesis, and this effect was suppressed by adding a neutralizing antibody against soluble interleukin-6 receptor. These results indicate that interleukin-6/soluble interleukin-6 receptor complexes and the signal transducer 130-kDa glycoprotein play an important role in the regulation of cyclooxygenase-2 expression and subsequent prostaglandin E(2) formation in mouse astrocytes and that interleukin-6 is an important regulator of immune and inflammatory processes in the CNS.

Keywords: Interleukin-6, Prostaglandin E2, Soluble interleukin-6 receptor

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Sakamoto, T., Mizukai, K., Kawanishi, T., Hiyama, Y.: **Real-Time Analysis for Quality Control of a Reaction Process using Ultra-high performance Liquid Chromatography. Reduction of Phenyl Ketone to Phenyl Alcohol**

J. Pharm. Innov., **4**, 115-120（2009）

The applicability of ultra-high performance liquid chromatography（UHPLC）for real-time analysis of a synthetic process was examined. A reduction reaction was selected as a model process. Quantities of acetophenone（AcPh）and phenyl ethanol（PhEt）in reaction solutions were analyzed at 5- and 15-min intervals. Reaction solutions were diluted using acetonitrile and water（6:4）was used as the mobile phase. PhEt and AcPh were detected at 0.64 and 0.75 min, respectively. Additionally, as many as nine different impurities were detected in the reaction solution. Given the ability to detect impurities in a reaction solution within few minutes, UHPLC technology is deemed applicable for real-time synthetic process control.

Keywords: UHPLC, Real-time analysis, Synthesis process

Sakamoto, T., Tanabe, T.[*1], Sasaki, T.[*2], Oyama, Y.[*1],

Nishizawa, J.[*2], Kawanishi, T., Hiyama, Y.: **Chiral analysis of re-crystallized mixtures of D-, L-amino acid using terahertz spectroscopy**

Malaysian J. Chem., **11**, 88-93 (2009)

Distinguishability by terahertz absorption between D- and L-amino acids and a change in terahertz absorption based on the re-crystallization condition was examined. Terahertz spectra which were obtained from the re-crystallized each enantiomer or mixtures of D- and L-leucine or alanine were compared with those of the purchased reagents. The peak tops of the re-crystallized L- and D- leucine mixtures were shifted toward low frequency side and the half width of the peak at 3.7 THz became narrow to approximately half of that of the reagent. Difference of spectral features from 2.1 THz to 2.8 THz in the THz spectra between D- and L-alanines was observed. According to the result of peak separation against these spectra, distinction between the purchased D- and L-alanine was possible to compare the intensities of the sub-peaks. Moreover, changes of the integrated values of the peaks obtained from L- or D-form-rich mixture of leucine were observed. These results suggested that feasibility of chiral analysis of enantiomers using terahertz spectroscopy would be shown.

Keywords: terahertz spectroscopy, polymorphs, enantiomers

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Sakai-Kato, K., Saito, E., Ishikura, K., Kawanishi, T.: **Analysis of intracellular doxorubicin and its metabolites by ultra-high-performance liquid chromatography**

J. Chromatogr. B., **878**, 1466-1470 (2010)

Doxorubicin, a highly effective anticancer drug, produces severe side effect such as cardiotoxicity, which is mainly caused by its metabolite, doxorubicinol. While in vitro studies by measuring cellular concentration of doxorubicin have been reported, there have been no reports on measuring cellular concentration of the metabolites. In this report, we developed a sensitive and high-throughput method for measuring cellular concentrations of doxorubicin and its metabolites by ultra-high-performance liquid chromatography. The method achieved more than 96% recovery of doxorubicin and its metabolites from cell homogenates. Using simple separation conditions, doxorubicin and its three main metabolites, and the internal standard, were separated within 3 min. The method has a limit of quantification of 17.4 pg (32.0 fmol) injected doxorubicin. This high sensitivity enables the detection and intracellular quantification of doxorubicin and its metabolite, doxorubicinol, in cell homogenates, and its use will facilitate studies of the relationship between doxorubicin pharmacokinetics and therapeutic outcome.

Keywords: Ultra-high-performance liquid chromatography, Doxorubicin, Doxorubicinol

Sakai-Kato, K., Umezawa, Y.[*1], Mikoshiba K.[*2], Aruga, J.[*2], Utsunomiya-Tate, N.[*1]: **Stability of folding structure of Zic zinc finger proteins**

Biochem. Biophys. Res. Commun., **384**, 362-365 (2009)

Zic family proteins have five C2H2-type zinc finger (ZF) motifs. We physicochemically characterized the folding properties of Zic ZFs. Alteration of chelation with zinc ions and of hydrophobic interactions changed circular dichroism spectra, suggesting that they caused structural changes. The motifs were heat stable, but electrostatic interactions had little effect on structural stability. These results highlight the importance of chelating interactions and hydrophobic interactions for the stability of the folding structure of Zic ZF proteins.

Keywords: transcription factor, zinc finger, protein-DNA interaction

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Sano, K.[*1], Miyamoto, Y.[*1], Kawasaki, N., Hashii, N., Itoh, S., Murase, M.[*1], Date, K.[*1], Yokoyama, M.[*2], Sato, C.[*3], Kitajima, K.[*3], Ogawa, H.[*1]: **Survival signals of hepatic stellate cells in liver regeneration are regulated by glycosylation changes in rat vitronectin, especially decreased sialylation**

J. Biol. Chem., **285**, 17301-17309 (2010)

The extracellular matrix (ECM) molecules play important roles in many biological and pathological processes. During tissue remodeling, the ECM molecules that are glycosylated are different from those of normal tissue owing to changes in the expression of many proteins that are responsible for glycan

synthesis. Vitronectin (VN) is a major ECM molecule that recognizes integrin on hepatic stellate cells (HSCs). The present study attempted to elucidate how changes in VN glycans modulate the survival of HSCs, which play a critical role in liver regeneration. Plasma VN was purified from partially hepatectomized (PH) and sham-operated (SH) rats at 24 h after operation and non-operated (NO) rats. Adhesion of rat HSCs (rHSCs), together with phosphorylation of focal adhesion kinase, in PH-VN was decreased to one-half of that in NO- or SH-VN. Spreading of rHSCs on desialylated NO-VN was decreased to one-half of that of control VN, indicating the importance of sialylation of VN for activation of HSCs. Liquid chromatography/multiple-stage mass spectrometry analysis of Glu-C glycopeptides of each VN determined the site-specific glycosylation. In addition to the major biantennary complex-type N-glycans, hybrid-type N-glycans were site-specifically present at Asn (167). Highly sialylated O-glycans were found to be present in the Thr(110)-Thr(124) region. In PH-VN, the disialyl O-glycans and complex-type N-glycans were decreased while core-fucosylated N-glycans were increased. In addition, immunodetection after two-dimensional PAGE indicated the presence of hyper- and hyposialylated molecules in each VN and showed that hypersialylation was markedly attenuated in PH-VN. This study proposes that the alteration of VN glycosylation modulates the substrate adhesion to rat HSCs, which is responsible for matrix restructuring.

Keywords: Apotosis, Extracellular Matrix, Glycoproteins/Carbohydrates

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Wada, Y.*1, Dell, A.*2, Haslam, S. M.*2, Tissot, B.*2, Canis, K.*2, Azadi, P.*3, Backstrom, M.*4, Costello, C. E.*5, Hansson, G. C.*4, Hiki, Y.*6, Ishihara, M.*3, Ito, H.*7, Kakehi, K.*8, Karlsson, N.*9, Kato, K.*10, 11, Kawasaki, N., Khoo, K. H.*12, Kobayashi, K.*13, Kolarich, D.*14, Kondo, A.*15, Lebrilla, C.*16, Nakano, M.*15, Narimatsu, H.*7, Novak, J.*17, Novotny, M. V.*18, Ohno, E.*11, Packer, N. H.*14, Renfrow, M. B.*17, Tajiri, M.*1, Thomsson, K. A.*4, Yagi, H.*11, Yu, S. Y.*12, and Taniguchi, N.*14, 19:

Comparison of methods for profiling O-glycosylation: Human Proteome Organisation Human Disease Glycomics/Proteome Initiative multi-institutional study of IgA1

Mol . Cell. Proteomics., **9**, 719-727 (2010)

The Human Proteome Organisation Human Disease Glycomics/Proteome Initiative recently coordinated a multi-institutional study that evaluated methodologies that are widely used for defining the N-glycan content in glycoproteins. The study convincingly endorsed mass spectrometry as the technique of choice for glycomic profiling in the discovery phase of diagnostic research. The present study reports the extension of the Human Disease Glycomics/Proteome Initiative's activities to an assessment of the methodologies currently used for O-glycan analysis. Three samples of IgA1 isolated from the serum of patients with multiple myeloma were distributed to 15 laboratories worldwide for O-glycomics analysis. A variety of mass spectrometric and chromatographic procedures representative of current methodologies were used. Similar to the previous N-glycan study, the results convincingly confirmed the pre-eminent performance of MS for O-glycan profiling. Two general strategies were found to give the most reliable data, namely direct MS analysis of mixtures of permethylated reduced glycans in the positive ion mode and analysis of native reduced glycans in the negative ion mode using LC-MS approaches. In addition, mass spectrometric methodologies to analyze O-glycopeptides were also successful.

Keywords: O-glycosylation, Human Proteome Organisation Human Disease Glycomics/Proteome Initiative, multi-institutional study

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Hashii, N., Kawasaki, N., Itoh, S., Nakajima, Y., Harazono, A., Kawanishi, T., Yamaguchi, T.: **Identification of glycoproteins carrying a target glycan-motif by liquid chromatography/multiple-stage mass spectrometry: identification of Lewis x-conjugated glycoproteins in mouse kidney**

J. Proteome Res., **8**, 3415-3429 (2009)

Certain glycan motifs in glycoproteins are involved in several biological events and diseases. To understand the roles of these motifs, a method is needed to identify the glycoproteins that carry them. We previously demonstrated that liquid chromatography-multiple-stage mass spectrometry (LC-MS^n) allowed for differentiation of oligosaccharides attached to Lewis-motifs, such as Lewis x (Le^x, Galβ1-4(Fucα1-3)GlcNAc) from other glycans. We successfully discriminated Le^x-conjugated oligosaccharides from other *N*-linked oligosaccharides derived from mouse kidney proteins by using Lewis-motif-distinctive ions, a deoxyhexose (dHex)+hexose (Hex)+*N*-acetylhexsosamine (HexNAc) fragment (*m/z* 512), and a Hex+HexNAc fragment (*m/z* 366). In the present study, we demonstrated that this method could be used to identify the Le^x-conjugated glycoproteins. All proteins in the mouse kidney were digested into peptides, and the fucosylated glycopeptides were enriched by lectin-affinity chromatography. The resulting fucosylated glycopeptides were subjected to two different runs of LC-MS^n using a Fourier- transform ion cyclotron resonance mass spectrometer (FTICR-MS) and an ion trap-type mass spectrometer. After the first run, we picked out product ion spectra of the expected Le^x-conjugated glycopeptides based on the presence of Lewis-motif-distinctive ions and assigned a peptide+HexNAc or peptide+(dHex)HexNAc fragment in each spectrum. Then the fucosylated glycopeptides were subjected to a second run in which the peptide-related fragments were set as precursor ions. We successfully identified γ-glutamyl transpeptidase 1 (γ-GTP1), low-density lipoprotein receptor-related protein 2 (LRP2), and a cubilin precursor as Le^x-conjugated glycoproteins by sequencing of 2-5 glycopeptides. In addition, it was deduced that cadherin 16, dipeptidase I, H-2 class I histocompatibility antigen, K-K α precursor (H2-Kk), and alanyl (membrane) aminopeptidase could be Le^x-conjugated glycoproteins from the good agreement between the experimental and theoretical masses and fragment patterns. The results indicated that our method could be applicable for the identification and screening of glycoproteins carrying target glycan-motifs, such as Lewis epitopes.

Keywords: LC/MS^n, Lewis-motif, glycopeptide

栫　直孝*，木下充弘*，川崎ナナ，山口照英，早川堯夫*，掛樋一晃*：**日本薬局方医薬品各条へパリンナトリウム純度試験へのキャピラリー電気泳動法の適用について**

薬学雑誌，**129**，1255-1264 (2009)

主に米国で発生した過硫酸化コンドロイチン硫酸（OSCS）混入ヘパリンナトリウムによる有害事象への対応として，日本薬局方医薬品各条へパリンナトリウムの改訂が検討されている．本研究では，キャピラリー電気泳動法がOSCS及びデルマタン硫酸エステル純度試験として適用可能であることを示した．

Keywords: 過硫酸化コンドロイチン硫酸，キャピラリー電気泳動法，日本薬局方

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宮田直樹*，川崎ナナ，内田恵理子，蜂須賀暁子：**「日本薬局方の試験法に関する研究」研究報告－日本薬局方の名称関連項目の科学的整備に関する研究**

医薬品研究，**40**，587-598 (2009)

日本薬局方に既収載の生物薬品及び近々収載が予定されている生物薬品を対象に，医薬品の本質（構造）にかかわる名称関連項目の記載内容及び表記方法について調査研究を行い，今後整備が必要となる事項を明らかにした．

Keywords: INN，JAN，生物薬品

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Morita, I.*1, Kakuda, S.*1, Takeuchi, Y.*1, Itoh, S., Kawasaki, N., Kizuka, Y.*1, Kawasaki, T.*2, Oka, S.*1: **HNK-1 glyco-epitope regulates the stability of the glutamate receptor subunitGluR2 on the neuronal cell surface**

J. Biol. Chem., **284**, 30209-30217 (2009)

HNK-1 (human natural killer-1) glyco-epitope, a

sulfated glucuronic acid attached to N-acetyllactosamine on the nonreducing termini of glycans, is highly expressed in the nervous system. Our previous report showed that mice lacking a glucuronyltransferase (GlcAT-P), a key enzyme for biosynthesis of the HNK-1 epitope, showed reduced long term potentiation at hippocampal CA1 synapses. In this study, we identified an alpha-amino-3-hydroxy-5-methylisoxazole propionate (AMPA) -type glutamate receptor subunit, GluR2, which directly contributes to excitatory synaptic transmission and synaptic plasticity, as a novel HNK-1 carrier molecule. We demonstrated that the HNK-1 epitope is specifically expressed on the N-linked glycan(s) on GluR2 among the glutamate receptors tested, and the glycan structure, including HNK-1 on GluR2, was determined using liquid chromatography-tandem mass spectrometry. As for the function of HNK-1 on GluR2, we found that the GluR2 not carrying HNK-1 was dramatically endocytosed and expressed less on the cell surface compared with GluR2 carrying HNK-1 in both cultured hippocampal neurons and heterologous cells. These results suggest that HNK-1 stabilizes GluR2 on neuronal surface membranes and regulates the number of surface AMPA receptors. Moreover, we showed that the expression of the HNK-1 epitope enhanced the interaction between GluR2 and N-cadherin, which has important roles in AMPA receptor trafficking. Our findings suggest that the HNK-1 epitope on GluR2 regulates cell surface stability of GluR2 by modulating the interaction with N-cadherin.

Keywords: HNK-1, GluR2, AMPA

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Suzuki, T., Ishii-Watabe, A., Tada, M., Kobayashi, T., Kanayasu-Toyoda, T., Kawanishi, T., and Yamaguchi, T.: **Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: A comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR**

J. Immunol., **184**, 1968-1976 (2010)

The neonatal FcR (FcRn) binds to the Fc domain of IgG at acidic pH in the endosome and protects IgG from degradation, thereby contributing to the long serum half-life of IgG. To date, more than 20 mAb products and 5 Fc-fusion protein products have received marketing authorization approval in the United States, the European Union, or Japan. Many of these therapeutic proteins have the Fc domain of human IgG1; however, the serum half-lives differ in each protein. To elucidate the role of FcRn in the pharmacokinetics of Fc domain-containing therapeutic proteins, we evaluated the affinity of the clinically used human, humanized, chimeric, or mouse mAbs and Fc-fusion proteins to recombinant human FcRn by surface plasmon resonance analysis. The affinities of these therapeutic proteins to FcRn were found to be closely correlated with the serum half-lives reported from clinical studies, suggesting the important role of FcRn in regulating their serum half-lives. The relatively short serum half-life of Fc-fusion proteins was thought to arise from the low affinity to FcRn. The existence of some mAbs having high affinity to FcRn and a short serum half-life, however, suggested the involvement of other critical factor(s) in determining the serum half-life of such Abs. We further investigated the reason for the relatively low affinity of Fc-fusion proteins to FcRn and suggested the possibility that the receptor domain of Fc-fusion protein influences the structural environment of the FcRn binding region but not of the FcgammaRI binding region of the Fc domain.

Keywords: FcRn, monoclonal antibody products, Fc-fusion protein products

Kita, T.*, Nishida, H.*, Shibata, H.*, Niimi, S., Higuti, T.* and Arakaki, N.*: **Possible role of mitochondria remodeling on cellular triacylglycerol accumulation**

J. Biochem., **146**, 787-796 (2009)

Mitochondrial fusion and fission processes play a role in a variety of cell functions, including energy metabolism, cell differentiation and programmed cell death. Still, it is not clear how these processes contribute to the cell functions. Here, we investigated the role of mitochondrial remodeling on lipid metabolism in adipocytes. In 3T3-L1 pre-adipocytes, the morphology of mitochondria is organized as a continuous reticulum. Upon differentiation of adipocytes manifested by cellular triacylglycerol (TG) accumulation, mitochondrial morphology altered from filamentous to fragmented and/or punctate structures. When the mito-

chondrial fusion was induced in adipocytes by silencing of mitochondrial fission proteins including Fis1 and Drp1, the cellular TG content was decreased. In contrast, the silencing of mitochondrial fusion proteins including mitofusion 2 and Opa1 increased the cellular TG content followed by fragmentation of mitochondria. It also appears that polyphenolic phytochemicals, negative regulators of lipid accumulation, have mitochondrial fusion activity and that there is a good correlation between mitochondrial fusion activity and the cellular TG accumulation-reducing activity of the phytochemicals. These results suggest that cellular TG accumulation is regulated, at least in part, via mitochondrial fusion and fussion processes.

Keywords: adipocytes, fission, fusion, mitochondria, phytochemicals, triacylglycerol

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Nugroho, A. E.[*1], Hirasawa, Y.[*1], Kawahara, N.[*2], Goda, Y., Awang, K.[*3], Hadi, A. H. A.[*3], Morita, H.[*1]:
Bisnicalaterine A, a Vobasine-Vobasine bisindole alkaloid from *Hunteria zeylanica*
J. Nat. Prod., **72**, 1502－1506 (2009)

A new bisindole alkaloid, bisnicalaterine A, consisting of two vobasine-type skeletons, and 3-epivobasinol and 3-*O*-methylepivobasinol, with vobasine-type skeletons, were isolated from the leaves of *Hunteria zeylanica*, and their structures were elucidated on the basis of spectroscopic data and chemical correlation. Bisnicalaterine A showed moderate cytotoxicity against various human cancer cell lines.

Keywords: Bisnicalaterine A, Vobasine-Vobasine bisindole alkaloid, *Hunteria zeylanica*

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Sato, M.[*], Anetai, M.[*], Kamakura, H., Goda, Y.:
Analysis of Organophosphorus Pesticide Residues in Crude Drugs (Part 3)
Pharmaceutical and Medical Device Regulatory Science, **41**, 324-337 (2010)

A method was developed for simultaneous determination of 28 organophosphorus pesticides in Angelica Dahurica Root (Angelicae Dahuricae Radix), Astragalus Root (Astragali Radix), Cnidium Rhizome (Cnidii Rhizoma), Gardenia Fruit (Gardeniae Fructus), Glehnia Root (Glehniae Radix Cum Rhizoma), Magnolia Bark (Magnoliae Cortex), Pinellia Tuber (Pinelliae Tuber), Poria Sclerotium (Poria), Rehmannia Root (Rehmanniae Radix), Rhubarb (Rhei Rhizoma) and Senega (Senegae Radix). The determination was performed by gas chromatography with FPD detection. The recoveries of organophosphorus pesticides added at the concentration of 0.4μg/g to the crude drugs, except for Angelica Dahurica Root, Cnidium Rhizome, Glehnia Root and Magnolia Bark were mostly in the range of 70～120% (peak area method). The recoveries of methidathion, phosmet, edifenphos and phosalone added to Angelica Dahurica Root, Cnidium Rhizome and Glehnia Root were greater than 120%. The recoveries of quinalphos and iprobenfos added to Magnolia Bark were 42% and 33%, respectively. These lower recoveries may be due to reactions with components of the crude drug during extraction procedures. The established method was applied to 111 samples of 16 kinds of crude drugs. Seven kinds of organophosphorus pesticides were detected in 6 samples of 3 kinds of crude drugs produced in Japan and 12 samples of 4 kinds of crude drugs produced in the People's Republic of China in the range of trace to 0.54 ppm. The Agricultural Chemicals Regulation Law was revised on March 10, 2003, and states that a person who uses agricultural chemicals shall not use them contrary to the regulation determined by ministerial ordinances (Article 12). Four kinds of organophosphorus pesticides regulated by this law were detected in 4 samples of 2 kinds of crude drugs.

Keywords: crude drugs, organophosphorus pesticide residue, GC-FPD

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Maruyama, T., Abbaskhan, A.[*1], Choudhary, M. I.[*1], Tsuda, Y.[*1], Goda, Y., Farille M.[*2], Reduron J. P.[*3]:
Botanical origin of Indian celery seed (fruit)
J. Nat. Med., **63**, 248-253 (2009)

In the course of our study on the traditional medicines and foodstuffs used in Pakistan, we investigated the

origin of Indian celery using the analysis of internal transcribed spacer (ITS) sequence of nuclear rDNA and a phytochemical approach. As the findings of this study, the source plant of the Indian celery containing coumarin derivatives such as seselin (**1**), bergapten (**2**), isopimpinellin (**3**), etc., was not a common celery, *Apium graveolens*, but it was suggested to be *Seseli diffusum*, although Indian workers reported that *A. graveolens* seeds contain these compounds. In addition, a market survey of the Indian celery in Pakistan and related countries revealed that the Indian celery seeds in Pakistani markets are mainly composed of 3 species which have been confused in rural markets.

Keywords: Indian celery, *Seseli diffusum*, rDNA internal transcribed spacer sequence

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Maruyama, T., Kawamura, M., Kikura-Hanajiri, R., Takayama, H.[*], Goda, Y.: **The botanical origin of kratom (*Mitragyna speciosa*; Rubiaceae) available as abused drugs in the Japanese markets**

J. Nat. Med., **63**, 340-344 (2009)

Kratom is the leaves of *Mitragyna speciosa* (Rubiaceae). Recently, kratom has been sold in street shops or on the Internet in Japan for the purpose of abuse due to its opium-like effects. In this study, we investigated the botanical origin of the commercial kratom products using the internal transcribed spacer (ITS) sequence analysis of rDNA in preparation for future regulation of this product. In addition, a previously reported method to authenticate the plant, utilizing PCR-restriction fragment length polymorphism (RFLP) was applied to the same products in order to estimate the method's accuracy and utility. The ITS sequence analysis of the commercial kratoms revealed that most of them were derived from *M. speciosa* or closely related plants, while the others were made from the same tribe plant as *M. speciosa*. The reported PCR-RFLP method could clearly distinguish kratoms from the other psychoactive plants available in the Japanese markets and also from related plants. The authentication method is considered to be useful for the practical regulation of the plant due to its wide range of application, high accuracy and simplicity.

Keywords: *Mitragyna speciosa*, rDNA internal transcribed spacer, PCR-restriction fragment length polymorphism (RFLP)

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Kondo, K.[*1], Shiba, M.[*1], Yotsuyanagi, Y.[*2], Nishimura, N.[*2], Maruyama, T., Goda, Y.: **Discrimination between *Atractylodes* Rhizome (Byaku-jutsu) and *Atractylodes lancea* Rhizome (So-jutsu) by the PCR-RFLP analysis of ITS region on nrDNA**

J. Jpn. Bot., **84**, 356-359 (2009)

The purity test for *Atractylodes* rhizome by the molecular biological method based on ARMS was established in the general information on the first supplement of the Japanese Pharmacopoeia 15th ed., to stop commingling of *Atractylodes lancea* rhizome with *Atractylodes* rhizome. The ARMS requires rigorous experimental conditions. Therefore, we established a new, simple, quick and stable method based on PCR-RFLP for discrimination between *Atractylodes* rhizome and *Atractylodes lancea* rhizome.

Keywords: *Atractylodes* rhizome, *Atractylodes* lancea rhizome, PCR-restriction fragment length polymorphism (RFLP)

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丸山卓郎，宮井美穂，鎌倉浩之，中島育美[*1]，川崎武志[*1]，小松かつ子[*2]，藤田正雄[*1]，山本 豊[*3]，柴田敏郎[*4]，合田幸広：**遺伝子情報を利用したシゴカの基原種鑑別と純度試験法の検討**

生薬学雑誌，**64**，15-20 (2010)

In our continuous study on the quality assurance of Eleutherococcus Senticosus Rhizome (ESR), we investigated the botanical origin of the commercial ESR obtained in Heilongjian, China using ITS sequence analysis of nuclear rDNA. Furthermore, we established a simple and rapid authentication method of ESR based on the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and estimated the detection limit of the method in preparation for its application as a purity test. As a result, two ITS genotypes were observed in the commercial ESR and they were supposed to be the inherent origin,

Eleutherococcus senticosus and the related plants such as *E. sessiliflorus*. These data are in accord with our previous study. The authentication method based on the PCR-RFLP method could clearly discriminate the inherent material from the counterfeits. In addition, the related plants were detected in all 11 samples when artificial 5% adulterant samples, which consisted of 95% *E. senticosus* and 5% related plant in weight, were assayed by the authentication method. Therefore, it was found that the purity test of ESR utilizing the PCR-RFLP method can detect contaminants at the 5% level.

Keywords: Eleutherococcus Senticosus Rhizome, internal transcribed spacer, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

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Hosoe, T.[*1], Moriyama, H.[*1], Wakana, D., Itabashi, T.[*1], Kawai, K.[*1], Yaguchi, T.[*2], Iizuka, T.[*3], Hoshi, K.[*4], Fukuyama, Y.[*4], Kouda, Y.[*4], Lau, F. C.[*5]: **Inhibitory effects of dihydroterrein and terrein isolated from *Aspergillus novofumigatus* on platelet aggregation**
Mycotoxins, **59**, 75-82 (2009)

Dihydroterrein (**1**) and terrein (**2**) were isolated from methanol extracts of the solid culture of *Asperillus novofumigatus* IFM 55215. Both **1** and **2** inhibited human platelet aggregation induced by collagen at a concentration of 5.0×10^2 μmol/L in vitro and they displayed slight inhibitory effects when platelet aggregation was induced by adenosine diphosphate (ADP). Inhibitory activity of dihydroterrein diacetate (**3**) and terrein diacetate (**4**) on platelet aggregation was also examined. Compound **4** was found to possess the highest inhibitory effect on both collagen- and ADP-induced platelet aggregation among **1**-**4**.

Keywords: *Aspergillus novofumigatus*, platelet aggregation inhibition, terrein

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Ishikawa, K.[*1], Hosoe, T.[*1], Itabashi, T.[*1], Wakana, D., Takizawa, K.[*2], Yaguchi, T.[*2], Kawai, K.[*1]: **Novoamauromine and *ent*-Cycloechinulin: Two new diketopiperazine derivatives from *Aspergillus novofumigatus***
Chem. Pharm. Bull., **58**, 717-719 (2010)

The fungus *Aspergillus fumigatus* is known as an important human pathogen and a strain that produces many secondary metabolites. The fungus *Aspergillus novofumigatus* CBS117520 was isolated originally as A. fumigatus from Equadorian soil in 1965. In 2005, Hong *et al.* re-identified it as the new *Aspergillus* sp., closely related to *A. fumigatus*. We have isolated two new diketopiperazines, novoamauromine (**1**) and *ent*-cycloechinulin (**2**), along with *epi*aszonalenins A and C, and helivolic acid, from the methanolic extract of this fungus cultivated on rice using a thin layer chromatography (TLC) analysis-guided fractionation. This report describes the isolation, structure, and antifungal and cytotoxic activities of 1 and 2.

Keywords: *Aspergillus novofumigatus*, novoamauromine, *ent*-cycloechinulin

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日向野太郎[*]，岡本　仁[*]，植竹厚裕[*]，明戸孝夫[*]，袴塚高志：**ビルベリー配合食品中のアントシアニン類及びアントシアニジン類の分析**
日本食品化学学会誌，**16**，60-65 (2009)

日本に流通するビルベリー製品の品質評価を行うため，ビルベリー標榜健康食品７検体についてアントシアニン類及びアントシアニジン類を指標にHPLCで分析した．３検体のHPLCプロファイルは欧州薬局方規格のビルベリー乾燥エキス標準品のプロファイルとほぼ同等であったが，３検体ではビルベリー由来のアントシアニンが検出されず，１検体ではビルベリー以外の植物に由来するアントシアニンの含有の可能性が示唆された．市販のビルベリー健康食品には品質上に大きな差があり，また，欧州薬局方規格と成分組成の大きく異なる製品の存在が確認された．

Keywords: bilberry, anthocyanin, health food

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Kakigi, Y.[*], Mochizuki, N.[*], Icho, T.[*], Hakamatsuka, T., Goda, Y.: **Analysis of terpene lactones in Ginkgo leaf extracts by high-performance liquid chromatography using charged aerosol detection**

Biosci. Biotechnol. Biochem., **74**, 590-594（2010）

A new HPLC method using charged aerosol detection（CAD）was developed for the determination of terpene lactones in Ginkgo leaf extracts. The linearity of the standard curves was excellent（$r > 0.999$）. The repeatability of the method was lower than 3%, and its reproducibility was lower than 5% for each analyte. The LODs（limits of detection）were between 0.087 and 0.45 mg/L. Then, the developed method was applied to the analysis of terpene lactones in Ginkgo leaf products distributed in Japanese market. The results suggest that some health food products contain approximately equivalent amounts of terpene lactones to those in the medical product and the abundance of terpene lactones varies in each health product.

Keywords: ginkgo, terpene lactone, charged aerosol detection

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El-Halawany, AM.[*1], Chung MH., Abdallah, HM.[*1], Nishihara T.[*2], Hattori M.[*3]: **Estrogenic activity of a naringinase-treated extract of *Sophora japonica* cultivated in Egypt**

Pharm. Biol., **2**, 177-181（2010）

The naringinase-treated methanol extract of *Sophora japonica* L.（Fabaceae）seeds showed potent estrogen agonist activity. Through bioassay-guided isolation of the main active constituents from the naringinase-treated methanol extract of *S. japonica*, the aglycones genistein and kaempferol were found to be the main phytoestrogens in the naringinase-treated extract. In addition, kaempferol was nearly equipotent to genistein as an estrogen agonist. Concerning the compounds isolated from the untreated methanol extract, sophoricoside showed weak estrogenic activity on ERβ only.

Keywords: Estrogenic activity, ER, *Sophora japonica*

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Ma, H.[*1, 2], Chung, MH., Lu, Y.[*3], Nishihara, T.[*4], Hattori, M.[*2]: **Estrogenic effects of a herbal formula, Menoprogen, in ovariectomized rats**

Biol. Pharm. Bull., **33**, 455-460（2010）

Despite the health risks for postmenopausal women, the indications and ideal candidates for hormone replacement therapy remain unclear. The present study used ovariectomized rats to examine the safety and effects of the Chinese herbal formula Menoprogen（MPG）, which is prescribed for menopausal syndrome. Daily oral MPG（1000 mg/kg body weight）for 2 weeks significantly recovered uterine and adrenal gland atrophy and restored serum estradiol, estrone and progesterone levels that were decreased in rats by bilateral ovariectomy. However, yeast two-hybrid and nuclear receptor cofactor assays showed that MPG did not bind estrogen receptors α（ERα）and β, and immunohistochemical staining revealed that unlike 17 β-estradiol, MPG did not stimulate the protein expression of ERα, progesterone receptor, c-jun and c-fos in the uterus. No side effects of MPG were confirmed in vivo. These findings suggest that MPG would be useful for treating women with premenopausal and postmenopausal syndromes.

Keywords: Menoprogen, estrogen, menopausal syndrome

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Wang, J.[*1], Chung, MH., Xue, B.[*1], Ma, H.[*2, 3], Ma, C.[*2], Hattori, M.[*2]: **Estrogenic and antiestrogenic activities of Phloridzin**

Biol. Pharm. Bull., **33**, 592-597（2010）

Phloridzin, a phloretin 2'-β-D-glucoside, belongs to dihydrochalcones and mainly exists in the fruits of *Malus pumila* Mill., *Lithocarpus polystachyus* REHD and the root skins, stems, tender leaves and fruits of *Malus hupehensis*. It has many pharmacological activi-

ties, such as regulating blood sugar level and blood pressure, protecting heart, scavenging of oxygen free radicals and antioxidant injuries. Thus, market demand of products containing phloridzin is increasing year by year. Our research results demonstrated that phloridzin is provided with a double directional adjusting function of estrogenic and antiestrogenic activities. It showed significant effects on the proliferation of estrogen sensitive estrogen receptor (ER) (+)MCF-7 cells in the absence of estrogen. When added with 17β-estradiol, phloridzin showed antagonism on estradiol-induced MCF-7 cell proliferation, but it did not significantly affect proliferation of estrogen insensitive ER (-)MDA-MB-231 cells. Phloridzin induced β-galactosidase activity in a yeast two-hybrid assay. Light increase of the uterine weight and serum estradiol content of mouse was observed when the glucoside was administered orally for 7 d. After oral administration, phloridzin was found mainly in the blood and a small part was metabolized to phloretin. Our investigation proved that phloridzin was distributed at the target organ and played the role of phytoestrogen.

Keywords: *Malus hupehensis*, phloridzin, phytoestrogen

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Kawamura, M., Kikura-Hanajiri R., Goda, Y.: **Simple and rapid screening for psychotropic natural products using Direct Analysis in Real Time (DART)-TOFMS**

Yakugaku Zasshi, **129**, 719-725 (2009)

Direct Analysis in Real Time (DART) is a novel ionization technique that provides for the rapid ionization of small molecules under ambient conditions. To investigate the trend of non-controlled psychotropic plants of abuse in Japan, a rapid screening method, without sample preparation, was developed using DART-time of flight mass spectrometer (TOFMS) for plant products. The major psychotropic constituents of these products were determined using liquid chromatography-mass spectrometry (LC/MS). As a result of the DART-TOFMS analyses of 36 products, the protonated molecular ions $[M+H]^+$, corresponding to 6 kinds of major hallucinogenic constituents (mescaline, salvinorin A, *N,N*-dimethyltryptamine, harmine, harmaline and lysergamide), were detected in 21 products. It was possible to estimate their accurate elemental compositions through exact mass measurements. These results were consistent with those of the LC/MS analyses and the contents of the 6 psychotropic constituents were in the range from 0.05 to 45 μg/mg. Typical controlled narcotic drugs, tetrahydrocannabinol, opioid alkaloids and psilocin were also directly detected in marijuana cigarette, opium gum and magic mushroom respectively. Although it is difficult to estimate the matrix effects caused by other plant ingredients, the DART-TOFMS could be useful as a simple and rapid screening method for the targeted psychotropic natural products, because it provides the molecular information of the target compounds without time-consuming extraction and pretreatment steps.

Keywords: Direct Analysis in Real Time (DART), TOFMS, psychotropic plants

Kikura-Hanajiri, R., Kawamura, M., Maruyama, T., Kitajima, M.*, Takayama, H.*, Goda, Y.: **Simultaneous analysis of opioid agonists; Mitragynine, 7-Hydroxymitragynine and other alkaloids in a psychotropic plant "Kratom" (*Mitragyna speciosa*) by LC-ESI-MS**

Forensic Toxicol., **27**, 67-74 (2009)

The leaves of a tropical plant, *Mitragyna speciosa* (known as "Kratom") have been being traditionally used as a substitute for opium in Thailand and Malaysia. Mitragynine, a major constituent of *M. speciosa*, has an opioid agonistic activity, and its derivative 7-hydroxymitragynine (a minor constituent) is much more potent than mitragynine and morphine. Recently, many kinds of products containing this plant are distributed as "incense" on the drug market in Japan for their expected narcotic effects. Despite their potency and their wide distribution for abuse, there are no reports on quantitative analysis of mitragynine and 7-hydroxymitragynine in the raw materials or in the commercial products of Kratom. In this study, a method for simultaneous analysis of mitragynine,

7-hydroxymitragynine and other indole alkaloids (speciogynine, speciociliatine and paynantheine), contained in the raw materials and commercial products of Kratom, by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) was developed. By the LC-ESI-MS method, mitragynine, 7-hydroxymitragynine and other alkaloids were detected in 11 of the 13 products. The contents of mitragynine in the products ranged from 1% to 6%, and those of 7-hydroxymitragynine from 0.01% to 0.04%. 7-Hydroxymitragynine has a highly potent narcotic activity; it is even more powerful than morphine. Therefore, *M. speciosa* abuse is a matter of a major concern. This analytical method is considered useful for the screening of *M. speciosa* products in the drug market.

Keywords: Kratom, opioid agonists, LC-MS

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Min, J. Z.*, Hatanaka, S.*, Toyo'oka, T.*, Inagaki, S.*, Kikura-Hanajiri, R., Goda, Y.: **Rapid, sensitive and simultaneous determination of fluorescence-labeled designated substances controlled by the Pharmaceutical Affairs Law in Japan by ultra-performance liquid chromatography coupled with electrospray-ionization time-of-flight mass spectrometry**

Anal. Bioanal. Chem., **395**, 1411-1422 (2009)

A simultaneous determination method based on ultra-performance liquid chromatography (UPLC) with fluorescence (FL) detection and electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) was developed for 16 "designated substances" (Shitei-Yakubutsu) controlled by the Pharmaceutical Affairs Law in Japan. These substances were first labeled with 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole at 60 °C for 2 h in 0.1 M borax (pH 9.3). The resulting fluorophores were well separated by reversed-phase chromatography using an Acquity UPLC BEH C_{18} column (1.7 μm, 100 mm x 2.1 mm i.d.) by isocratic elution with a mixture of water and acetonitrile-methanol (20:80) containing 0.1% formic acid. The separated derivatives were sensitively detected by both FL and TOF-MS. However, the determination of several designated substances by FL detection showed interference from endogenous substances in biological samples. Therefore, the determination in real samples was carried out by a combination of UPLC separation and ESI-TOF-MS detection. The structures of the designated substances were identified from the protonated-molecular ions $[M+H]^+$ obtained from the TOF-MS measurement. The calibration curves obtained from the peak area ratios of the internal standard (I.S.), i.e., 3-phenyl-1-propylamine, and the designated substances versus the injection amounts showed good linearity. The limits of detection and the limits of quantification in 0.1 mL of human plasma and urine for the present method were 0.30-150 pmol and 1.0-500 pmol, respectively. Good accuracy and precision (according to intraday and interday assays) were also obtained with the present procedure. This method was applied to analyses of human plasma, urine and real products.

Keywords: Designated substances, Fluorescence labeling, Time-of-flight mass spectrometry

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Kikura-Hanajiri, R., Maruyama, T., Miyashita, A., Goda, Y.: **Chemical and DNA analyses for the products of a psychoactive plant, *Voacanga Africana***

Yakugaku Zasshi, **129**, 975-982 (2009)

Voacanga africana (Apocynaceae) is a small tropical African tree. The root bark and seeds of this tree contain a number of alkaloids, including ibogaine (a hallucinogenic/aphrodisiac compound in bark), tabersonine (a major constituteent of seeds) and other voacanga alkaloids, traditionally used in Africa for religious purposes. Recently, some kinds of products containing this plant (root bark and seeds) have been distributed in the drug market in expectation of its hallucinogenic/aphrodisiac effects. There has been no report that has discussed quantitative analyses of these alkaloids in the products and their botanical origins. In this study, to investigate the trend of such a non-controlled psychotropic plant of abuse, a simultaneous analytical method was developed using LC/MS for the voacanga alkaloids including ibogaine and tabersonine in the commercial products of *V. africana*. Moreover, the botanical origins of these products were investigated

by DNA analyses. As a result of the LC/MS analyses, the products were classified into two chemical types; an ibogaine-type and a tabersonine-type. The samples of the ibogaine-type contain ibogaine (0.05-0.6%) and other voacanga alkaloids; voacamine, voacamidine and voacangine, while those of the tabersonine-type mainly contain tabersonine (0.6-1.6%). The sequence analyses of chloroplast DNA, *trn*L-F region suggested that most of the products were derived from *V. africana* or closely related plants. They were classified into four genotypes based on nucleotide sequence of the *trn*L-F IGS region. The proposed methods of chemical and DNA analyses would be useful for investigating the trend in the distribution of the products of *V. africana*.
Keywords: *Voacanga africana*, LC/MS, DNA analysis

Matsushima, Y.[*1], Shirota, O.[*2], Kikura-Hanajiri, R., Goda, Y., Eguchi, F.[*1]: **Effects of *Psilocybe argentipes* on marble-burying behavior in mice**
Biosci Biotechnol Biochem., **73**, 1866-1868 (2009)

Psilocybe argentipes is a hallucinogenic mushroom. The present study examined the effects of *P. argentipes* on marble-burying behavior, which is considered an animal model of obsessive-compulsive disorder. *P. argentipes* significantly inhibited marble-burying behavior without affecting locomotor activity as compared with the same dose of authentic psilocybin. These findings suggest that *P. argentipes* would be efficient in clinical obsessive-compulsive disorder therapy.
Keywords: *Psilocybe argentipes*, marble-burying behavior, obsessive-compulsive disorder

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Kikura-Hanajiri, R., Kawamura, M., Miyajima-Tabata, A., Sunouchi, M., Goda, Y.: **Determination of a new designer drug, *N*-hydroxy-3,4-methylenedioxy methamphetamine and its metabolites in rats using ultra-performance liquid chromatography-tandem mass spectrometry**
Forensic Sci Int., **198**, 62-69 (2010)

An *N*-hydroxy analogue of 3,4-methylendioxymetha mphetamine (MDMA), *N*-hydroxy MDMA (*N*-OH MDMA), has recently been distributed as a new designer drug in some drug markets. Very little data is available to the metabolic and pharmacological properties of *N*-OH MDMA, although it has been reported that the *N*-demethyl analogue, *N*-hydroxy-3,4-methylenedioxyamphetamine (*N*-OH MDA), is mainly metabolized to MDA in rats. In this study, an analytical method for the determination of *N*-OH MDMA and its metabolites in biological samples was developed, and the metabolic properties of *N*-OH MDMA in rats were investigated. After the i.p. administration of *N*-OH MDMA to pigmented hairy rats (5 mg/kg/day, 10 days), *N*-OH MDMA and its *N*-dehydroxy and *N*-demethyl metabolites (MDMA, *N*-OH MDA and MDA) in rat plasma, urine and hair samples were determined by ultra-performance LC (UPLC)-MS/MS. The hair sample was extracted by 1-h sonication and overnight soaking in 5 M hydrochloric acid-methanol (1:20). The plasma, urine, and hair extract samples were purified using a solid-phase extraction procedure. *N*-OH MDMA in the samples could be precisely analyzed by avoiding an alkaline environment. The parent compound very rapidly disappeared from the rat plasma (<15 min) and urine (<10 h), and most of the *N*-OH MDMA was excreted in the rat urine as MDMA and MDA in 72 h. In the rat hair samples collected 4 weeks after the first administration, *N*-OH MDMA (0.03 ng/mg) and *N*-OH MDA (0.13 ng/mg) were clearly detected as well as MDMA (149 ng/mg) and MDA (52 ng/mg). This analytical method will be useful for the analysis of *N*-OH MDMA and its metabolites in biological samples.
Keywords: *N*-OH MDMA, biological samples, UPLC-MS/MS

Uchiyama, N., Kikura-Hanajiri, R., Kawahara, N.[*], Goda, Y.: **Identification of a cannabimimetic indole as a designer drug in a herbal product**
Forensic Toxicol., **27**, 61-66 (2009)

A cannabimimetic indole has been identified as a new adulterant in a herbal product being sold illegally in Japan for its expected narcotic effect. LC-MS and GC-MS analyses indicated that the product contained two major compounds. One was identified as a cannabinoid analog (1*RS*, 3*SR*)-3-[4-(1,1-dimethyloctyl) -2-hydroxyphenyl] cyclohexan-1-ol (**1**) by direct comparison with the authentic compound, which we reported previously. Another compound (**2**) showed a

molecular weight of 341 daltons, and accurate mass spectral measurements showed its elemental composition to be $C_{24}H_{23}NO$. Both mass and NMR spectrometric data revealed that **2** was 1-pentyl-3-(1-naphthoyl)indole [or naphthalen-1-yl-(1-pentylindol-3-yl)methanone], which was identical to a compound JWH-018, which had been synthesized by Huffman et al. in 1998. This compound was reported as a potent cannabinoid receptor agonist possessing a pharmacological cannabimimetic activity.

Keywords: 1-Pentyl-3-(1-naphthoyl) indole, Naphthalen-1-yl-(1-pentylindol-3-yl)methanone, JWH-018

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Uchiyama, N., Miyazawa, N.*, Kawamura, M., Kikura-Hanajiri, R., Goda, Y.: **Analysis of newly distributed designer drugs detected in the products purchased in fiscal year 2008**

Yakugaku Zasshi, **130**, 263-270 (2010)

Thirty-two psychotropic substances were listed as designated substances (Shitei-Yakubutsu, 31 compounds and 1 plant) in Japan by the Pharmaceutical Affairs Law in April 2007 for preventing the abuse of these substances. Subsequently, other psychoactive compounds were also added to this category, 40 substances (classified as 12 tryptamines, 17 phenethylamines, 3 piperazines, 6 alkyl nitrites, 1 diterpene and 1 plant) are controlled as designated substances as of July 2009. However, new designer drugs are still distributed in illegal drug market according to the results of our annual survey. This study presents the analysis of newly distributed four designer drugs detected from two products, which purchased from October 2008 to February 2009 in Japan. As the results of NMR, GC-MS and LC-MS analyses, three phenethylamine derivertives, 1-(2-fluorophenyl)-*N*-methylpropan-2-amine (*N*-Me-2-FMP), 1-(2,5-dimethoxy-4-isopropylsulfanylphenyl) propan-2-amine (ALEPH-4) and 1-(2,5-dimethoxy-4-nitrophenyl) propan-2-amine (DON) and a tryptamine derivative, *N*-ethyl-5-methoxy-*N*-propyltryptamine (5-MeO-EPT), were detected. *N*-Me-2-FMP and 5-MeO-EPT were newly identified in this study. Additionally, ALEPH-4 and DON were found as novel illegal drugs distributed in Japan.

Keywords: psychotropic substances, NMR, GC-MS

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Hirasawa, Y.*1, Shoji, T.*1, Arai, T.*1, Nugroho, AE.*1, Deguchi, J.*1, Hosoya, T.*1, Uchiyama, N., Goda, Y., Awang, K.*2, Hadi, AH.*2, Shiro, M.*3, Morita, H.*1: **Bisleuconothine A, an eburnane-aspidosperma bisindole alkaloid from *Leuconotis griffithii***

Bioorg Med Chem Lett., **20**, 2021-2024 (2010)

A new bisindole alkaloid, bisleuconothine A (**1**) consisting of an eburnane－aspidosperma type skeleton, was isolated from the bark of *Leuconotis griffithii*. The structure including absolute stereochemistry was elucidated on the basis of 2D NMR data and X-ray analysis. Bisleuconothine A (**1**) showed cell growth inhibitory activity against various human cancer cell lines.

Keywords: Bisindole alkaloid, Bisleuconothine A, *Leuconotis griffithii*

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Uchiyama, N., Kikura-Hanajiri, R., Ogata, J., Goda, Y.: **Chemical analysis of synthetic cannabinoids as designer drugs in herbal products**

Forensic Sci. Int., **198**, 31-38 (2010)

Several synthetic cannabinoids were found in 44 of 46 different kinds of herbal products that are currently distributed on the illegal drug market in Japan due to their expected narcotic effects. Gas chromatography－mass spectrometry (GC-MS) and liquid chromatography－mass spectrometry (LC-MS) analyses indicated that most of the products contained two major synthetic cannabinoids: (1*RS*, 3*SR*)-3-[2-hydroxy-4-(2-methylnonan-2-yl)phenyl]cyclohexan-1-ol, renamed cannabicyclohexanol with the agreement of Pfizer Inc., and/or 1-naphthalenyl (1-pentyl-1*H*-indol-3-yl)methanone, named JWH-018. Oleamide (*cis*-9,10-octadecenoamide), which is an endogenous cannabinoid, was also detected in 7 products. Additionally, two synthetic cannabinoids were identified as minor components in some products. One was (1*RS*, 3*SR*)-3-[2-hydroxy-4-(2-methyloctan-2-yl)phenyl] cyclohexan-1-ol, which is named CP-47,497 and is a homolog of cannabicyclohexanol. The other was 1-naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone, which is named JWH-073 and is a homolog of JWH-018. These compounds were reported as synthetic cannabinoids

possessing pharmacological cannabimimetic activity. The concentrations of cannabicyclohexanol, JWH-018 and oleamide in the products ranged from 1.1 to 16.9 mg/g, 2.0 to 35.9 mg/g and 7.6 to 210.9 mg/g, respectively, and showed considerable variation. In this study, details of the analysis and identification of these synthetic cannabinoids in herbal products being sold on the Japanese drug market are described.

Keywords: cannabicyclohexanol, JWH-018, oleamide

Sakamoto, K.*, Hiraiwa, M.*, Saito, M.*, Nakahara, T.*, Sato, Y., Nagao, T., Ishii, K.*: **Protective effect of all-trans retinoic acid on NMDA-induced neuronal cell death in rat retina**

Eur. J. Pharmacol., **635**, 56-61 (2010)

We histologically examined the effects of all-trans retinoic acid (ATRA) on neuronal injury induced by intravitreous injection of N-methyl-D-aspartic acid (NMDA) (200 nmol/eye). Treatment with ATRA for 7 days (15 mg/kg for the first two days and 10 mg/kg for the following five days, p.o.) reduced the decrease of cell number in the ganglion cell layer and the inner nuclear layer 7 days after NMDA injection. TUNEL staining 6 h after NMDA injection showed that treatment with ATRA (15 mg/kg, p.o.) 1 h prior to NMDA injection reduced the number of apoptotic cells in the ganglion cell layer and inner nuclear layer. The anti-apoptotic effect of ATRA was vanished by intravitreous injection of U0126, an extracellular signal-regulated kinase/ mitogen-activated protein kinase kinase inhibitor (1 nmol/eye). These results suggest that ATRA has a protective effect, which is medicated by extracellular signal-regulated kinase pathway, on NMDA-induced apoptosis in the rat retina. ATRA may be useful as a therapeutic drug against retinal diseases that cause glutamate neurotoxicity.

Keywords: retinoic acid, retina, apoptosis

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Nishida, M.*1, Suda, R.*1, Nagamatsu, Y.*1, Tanabe, S., Onohara, N.*1, Nakaya, M.*1, Kanaho, Y.*2, Shibata, T.*3, Uchida, K.*3, Sumimoto, H.*1, Sato, Y., Kurose, H.*1: **Pertussis toxin up-regulates angiotensin type 1 receptors through Toll-like receptor 4-mediated Rac activation**

J. Biol. Chem., **285**, 15268-15277 (2010)

Pertussis toxin (PTX) is recognized as a specific tool that uncouples receptors from G_i and G_o through ADP-ribosylation. During the study analyzing the effects of PTX on angiotensin II type 1 receptor (AT1R) function in cardiac fibroblasts, we found that PTX increases the number of AT1Rs and enhances AT1R-mediated response. Microarray analysis revealed that PTX increases the induction of interleukin (IL)-1β among cytokines. Inhibition of IL-1β suppressed the enhancement of AT1R-mediated response by PTX. PTX increased the expression of IL-1β and AT1R through NF-κB, and a small GTP-binding protein, Rac, mediated PTX-induced NF-κB activation through NADPH oxidase-dependent production of reactive oxygen species. PTX induced biphasic increases in Rac activity, and the Rac activation in a late but not an early phase was suppressed by IL-1β siRNA, suggesting that IL-1β-induced Rac activation contributes to the amplification of Rac-dependent signaling induced by PTX. Furthermore, inhibition of Toll-like receptor 4 (TLR4) abolished PTX-induced Rac activation and enhancement of AT1R function. However, ADP-ribosylation of G_i/G_o by PTX was not affected by inhibition of TLR4. Thus, PTX binds to two receptors: one is TLR4, which activates Rac, and another is the binding site that is required for ADP-ribosylation of G_i/G_o.

Keywords: PTX, AT1R, Rac

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Nishida, M.*1, Watanabe, K.*1, Sato, Y., Nakaya, M.*1, Kitajima, N.*1, Ide, T.*2, Inoue, R.*3, Kurose, H.*1: **Phosphorylation of TRPC6 channels at Thr69 is required for anti-hypertrophic effects of phosphodiesterase 5 inhibition**

J. Biol. Chem., **285**, 13244-13253 (2009)

Activation of Ca^{2+} signaling induced by receptor stimulation and mechanical stress plays a critical role in the development of cardiac hypertrophy. A canonical transient receptor potential protein subfamily member, TRPC6, which is activated by diacylglycerol and mechanical stretch, works as an upstream regulator of the Ca^{2+} signaling pathway. Although activation of protein kinase G (PKG) inhibits TRPC6 channel activity

and cardiac hypertrophy, respectively, it is unclear whether PKG suppresses cardiac hypertrophy through inhibition of TRPC6. Here, we show that inhibition of cGMP-selective PDE5（phosphodiesterase 5）suppresses endothelin-1-, diacylglycerol analog-, and mechanical stretch-induced hypertrophy through inhibition of Ca^{2+} influx in rat neonatal cardiomyocytes. Inhibition of PDE5 suppressed the increase in frequency of Ca^{2+} spikes induced by agonists or mechanical stretch. However, PDE5 inhibition did not suppress the hypertrophic responses induced by high KCl or the activation of protein kinase C, suggesting that PDE5 inhibition suppresses Ca^{2+} influx itself or molecule(s) upstream of Ca^{2+} influx. PKG activated by PDE5 inhibition phosphorylated TRPC6 proteins at Thr(69) and prevented TRPC6-mediated Ca^{2+} influx. Substitution of Ala for Thr(69) in TRPC6 abolished the anti-hypertrophic effects of PDE5 inhibition. In addition, chronic PDE5 inhibition by oral sildenafil treatment actually induced TRPC6 phosphorylation in mouse hearts. Knockdown of RGS2（regulator of G protein signaling 2）and RGS4, both of which are activated by PKG to reduce G alpha(q)-mediated signaling, did not affect the suppression of receptor-activated Ca^{2+} influx by PDE5 inhibition. These results suggest that phosphorylation and functional suppression of TRPC6 underlie prevention of pathological hypertrophy by PDE5 inhibition.

Keywords: phosphodiesterase, cardiac hypertrophy, TRP channel

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早川堯夫[*1]，梅澤明弘[*2]，山中伸弥[*3]，小澤敬也[*4]，大和雅之[*5]，澤　芳樹[*6]，山口照英，松山晃文[*7]，佐藤陽治，中内啓光[*8]：**細胞・組織加工医薬品等の品質及び安全性確保に関する研究（その１）ヒト（自己）体性幹細胞加工医薬品等の品質及び安全性の確保に関する指針案（中間報告）**

再生医療，**9**，116-127（2010）

ヒト（自己）体性幹細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件に関する指針案（中間報告）について紹介する。多分化能を有し、かつ自己複製能力を維持している体性幹細胞から加工した製品は、加工内容や適用部位によっては、たとえ自己に由来するものであっても、元来の細胞そのものではなく、また、存在していた、あるいは存在すべきであった細胞環境とは異なる状態のものとして臨床上適用される可能性がある。これらの点に関する留意事項がベースとなった薬食発第0208003号に付加された部分である。

Keywords: somatic stem cells, quality, safety

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再生医療，**9**，128-138（2010）

ヒト（同種）体性幹細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件に関する指針案（中間報告）について紹介する。多分化能を有し、かつ自己複製能力を維持している体性幹細胞から加工した製品は、加工内容や適用部位に応じて、元来の細胞とは異なり、また、存在していた、あるいは存在すべきであった細胞環境とは異なる状態のものとして臨床上適用される可能性がある。これらの点に関する留意事項がベースとなった薬食発第0912006号に付加された部分である。

Keywords: somatic stem cells, quality, safety

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早川堯夫[*1]，梅澤明弘[*2]，山中伸弥[*3]，小澤敬也[*4]，大和雅之[*5]，澤　芳樹[*6]，山口照英，松山晃文[*7]，佐藤陽治，中内啓光[*8]：**細胞・組織加工医薬品等の品質及び安全性確保に関する研究（その３）ヒト（自己）iPS（様）細胞加工医薬品等の品質及び安全性の確保に関する指針案（中間報告）**

再生医療，**9**，139-151（2010）

ヒト（自己）iPS（様）細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件に関する指針案（中間報告）について紹介する。山中らによるiPS細胞の作製は、分化した細胞を人為的にリプログラミング（初期化）できることを示した。これは細胞の分化・脱分化が人為的に自在に操作できる可能性を示唆する金字塔である。その活用により、生命現象解明のための基礎研究、病因や発症機構解明などの医学研究、毒性・薬効評価系確立などを通した創薬研究、さらに再生医療の実用化にも無限の可能性が拓かれた。ところで再生医療の究極の目的は治療である。したがって、常に治療（目的）から発想する考え方、アプローチが肝要であり、どのような疾患を対象に、どのような製品を開発するかが第一義的課題である。iPS細胞の作製による細胞の分化・脱分化に関するパラダイムシフトは、再生医療への応用に無限の可能性（手段）を提供するが、このことは、初期化の程度や特定iPS細胞の標準化が全ての再生医療への応用の前提であるということを必ずしも意味する訳ではない。初期化の程度を一定にすることができ、iPS細胞の標準化ができることは、再生医療に利用される細胞・組織加工医薬品等の創製のための特性が明らかな原材料、すなわち重要な素材（手段）の１つの提供という大きな意義を持つ。しかし、全ての製品のもとが、特定のiPS細胞でなければならないという必然性はない。ある個別の製品に対して、素材として適切な細胞があれば、それはそれで良い。重要なことは、細胞の分化・脱分化が人為的に操作できるというパラダイムの中で、ある特定の治療（目的）に叶う品質・有効性・安全性を有する最終製品を製造するのに適切な素材として人工的に誘導された多能性の細胞が適切に位置づけられることである。どの細胞から、どの手段で、どの程度初期化（多能性化）した細胞を得て、どのような分化誘導で、どのような細胞を経て、目的細胞に至るかが、各開発研究関係者の挑戦課題であると思われる。

Keywords: iPS cells, quality, safety

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早川堯夫[*1]，梅澤明弘[*2]，山中伸弥[*3]，小澤敬也[*4]，大和雅之[*5]，澤　芳樹[*6]，山口照英，松山晃文[*7]，佐藤陽治，中内啓光[*8]：**組織加工医薬品等の品質及び安全性確保に関する研究（その４）ヒト（同種）iPS（様）細胞加工医薬品等の品質及び安全性の確保に関する指針案（中間報告）**

再生医療，**9**，152-165（2010）

ヒト（同種）iPS（様）細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件に関する指針案（中間報告）について紹介する。山中らによるiPS細胞の作製は、分化した細胞を人為的にリプログラミング（初期化）できることを示した。これは細胞の分化・脱分化が人為的に自在に操作できる可能性を示唆する金字塔である。その活用により、生命現象解明のための基礎研究、病因や発症機構解明などの医学研究、毒性・薬効評価系確立などを通した創薬研究、さらに再生医療の実用化にも無限の可能性が拓かれた。ところで再生医療の究極の目的は治療である。したがって、常に治療（目的）から発想する考え方、アプローチが肝要であり、どのような疾患を対象に、どのような製品を開発するかが第一義的課題である。iPS細胞の作製による細胞の分化・脱分化に関するパラダイムシフトは、再生医療への応用に無限の可能性（手段）を提供するが、このことは、初期化の程度や特定iPS細胞の標準化が全ての再生医療への応用の前提であるということを必ずしも意味する訳ではない。初期化の程度を一定にすることができ、iPS細胞の標準化ができることは、再生医療に利用される細胞・組織加工医薬品等の創製のための特性が明らかな原材料、すなわち重要な素材（手段）の1つの提供という大きな意義を持つ。しかし、全ての製品のもとが、特定のiPS細胞でなければならないという必然性はない。ある個別の製品に対して、素材として適切な細胞があれば、それはそれで良い。重要なことは、細胞の分化・脱分化が人為的に操作できるというパラダイムの中で、ある特定の治療（目的）に叶う品質・有効性・安全性を有する最終製品を製造するのに適切な素材として人工的に誘導された多能性の細胞が適切に位置づけられることである。どの細胞から、どの手段で、どの程度初期化（多能性化）した細胞を得て、どのような分化誘導で、どのような細胞を経て、目的細胞に至るかが、各開発研究関係者の挑戦課題であると思われる。

Keywords: iPS cells, quality, safety

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再生医療，**9**，166-180（2010）

ヒトES細胞を加工した医薬品又は医療機器の品質及び安全性の確保のための基本的な技術要件に関する指針案（中間報告）について紹介する．分化能及び自己複製能が有限である体性幹細胞と比較した場合，ヒトES細胞はその幅広い多能性ゆえに，いままで入手が困難であった各種細胞を作製することのできる素材となることが期待され，またその無限の自己複製能ゆえに，ひとたび目的細胞への効率的分化誘導方法が確立すれば，再生医療に利用できる細胞を大量に，安定に供給することが可能となることが期待されている．最近米国では，再生医療におけるヒトES細胞の活用について，治験開始の試みが具体的になされるまでに至っている．しかし，ヒトES細胞が人の生命の萌芽であるヒト胚を滅失させて樹立されたものであること，また，すべての細胞に分化する可能性があること等の生命倫理上の問題が存在することから，ヒトES細胞の樹立・使用には慎重な配慮が必要とされる．

Keywords: ES cells, quality, safety

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Fujishita, K.[*], Ozawa, T.[*], Shibata, K.[*], Tanabe, S., Sato, Y., Okuda, T.[*], Maeda, S.[*], Koizumi, S.[*]: **Grape seed extract（GSE）acting on astrocytes reveals neuronal protection against oxidative stress via interleukin-6-mediated mechanisms**
Cell Mol Neurobiol., **29**, 1121-1129（2009）

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, oxidative stress, neuron

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Yasuda, S., Kai, M.[*1], Imai, S.[*1], Takeishi, K.[*2], Taketomi, A.[*2], Toyota, M.[*1], Kanoh, H.[*1], Sakane, F.[*3]: **Diacylglycerol kinase eta augments C-Raf activity and B-Raf/C-Raf heterodimerization**
J. Biol. Chem., **284**, 29559-29570（2009）

The Ras/B-Raf/C-Raf/MEK/ERK signaling cascade is critical for the control of many fundamental cellular processes, including proliferation, survival, and differentiation. This study demonstrated that small interfering RNA-dependent knockdown of diacylglycerol kinase eta（DGKeta）impaired the Ras/B-Raf/C-Raf/MEK/ERK pathway activated by epidermal growth factor（EGF）in HeLa cells. Conversely, the overexpression of DGKeta1 could activate the Ras/B-Raf/C-Raf/MEK/

ERK pathway in a DGK activity-independent manner, suggesting that DGKeta serves as a scaffold/adaptor protein. By determining the activity of all the components of the pathway in DGKeta-silenced HeLa cells, this study revealed that DGKeta activated C-Raf but not B-Raf. Moreover, this study demonstrated that DGKeta enhanced EGF-induced heterodimerization of C-Raf with B-Raf, which transmits the signal to C-Raf. DGKeta physically interacted with B-Raf and C-Raf, regulating EGF-induced recruitment of B-Raf and C-Raf from the cytosol to membranes. The DGKeta-dependent activation of C-Raf occurred downstream or independently of the already known C-Raf modifications, such as dephosphorylation at Ser-259, phosphorylation at Ser-338, and interaction with 14-3-3 protein. Taken together, the results obtained strongly support that DGKeta acts as a novel critical regulatory component of the Ras/B-Raf/C-Raf/MEK/ERK signaling cascade via a previously unidentified mechanism.

Keywords: diacylglycerol kinase, Raf kinase, cell growth

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Kai, M.[*1], Yasuda, S., Imai, S.[*1], Toyota, M.[*1], Kanoh, H.[*1], Sakane, F.[*2]: **Diacylglycerol kinase alpha enhances protein kinase Czeta-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-kappaB**

FEBS Lett., **583**, 3265-3268 (2009)

We recently reported that diacylglycerol kinase (DGK) alpha enhanced tumor necrosis factor-alpha (TNF-alpha)-induced activation of nuclear factor-kappaB (NF-kappaB). However, the signaling pathway between DGKalpha and NF-kappaB remains unclear. Here, we found that small interfering RNA-mediated knockdown of DGKalpha strongly attenuated protein kinase C (PKC) zeta-dependent phosphorylation of a large subunit of NF-kappaB, p65/RelA, at Ser311 but not PKCzeta-independent phosphorylation at Ser468 or Ser536. Moreover, knockdown and overexpression of PKCzeta suppressed and synergistically enhanced DGKalpha-mediated NF-kappaB activation, respectively. These results strongly suggest that DGKalpha positively regulates TNF-alpha-dependent NF-kappaB activation via the PKCzeta-mediated Ser311 phosphorylation of p65/RelA.

Keywords: diacylglycerol kinase, protein kinase C, nuclear factor-kappaB

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Yamaguchi, T.[*], Suzuki, T., Arai, H.[*], Tanabe, S., and Atomi, Y.[*]: **Continuous mild heat stress induces differentiation of mammalian myoblasts, shifting fiber type from fast to slow**

Am. J. Physiol. Cell Physiol., **298**, C140-C148 (2010)

Local hyperthermia has been widely used as physical therapy for a number of diseases such as inflammatory osteoarticular disorders, tendinitis, and muscle injury. Local hyperthermia is clinically applied to improve blood and lymphatic flow to decrease swelling of tissues (e.g., skeletal muscle). As for muscle repair following injury, the mechanisms underlying the beneficial effects of hyperthermia-induced muscle repair are unknown. In this study, we investigated the direct effects of continuous heat stress on the differentiation of cultured mammalian myoblasts. Compared with control cultures grown at 37°C, incubation at 39°C (continuous mild heat stress; CMHS) enhanced myotube diameter, whereas myotubes were poorly formed at 41 °C by primary human skeletal muscle culture cells, human skeletal muscle myoblasts (HSMMs), and C2C12 mouse myoblasts. In HSMMs and C2C12 cells exposed to CMHS, mRNA and protein levels of myosin heavy chain (MyHC) type I were increased compared with the control cultures. The mRNA level of MyHC IIx was unaltered in HSMMs and decreased in C2C12 cells, compared with cells that were not exposed to heat stress. These results indicated a fast-to-slow fiber-type shift in myoblasts. We also examined upstream signals that might be responsible for the fast-to-slow shift of fiber types. CMHS enhanced the mRNA and protein levels of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α in HSMMs and C2C12 cells but not the activities of MAPKs (ERK1/2 and p38 MAPK) in HSMMs and C2C12 cells. These data suggest that CMHS induces a fast-to-slow fiber-type shift of mammalian myoblasts through PGC-1α.

Keywords: mild heat stress, human skeletal muscle myoblasts, differentiation

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Matsuoka, A., Öfelt, A.[*1], Matsuda, Y., Nakaoka, R., Haishima, Y., Yudasaka, M.[*2], Iijima, S.[*3] and Tsuchiya, T.: **Development of an in vitro screening method for safety evaluation of nanomaterials**

Bio-Med. Mater. Engineering., **19**, 19-27（2009）

To evaluate the role of particle size in cytotoxicity tests of nanomaterials（NMs）, we exposed Chinese hamster cells to polystyrene（PS）spheres with defined diameters ranging from 0.1 to 9.2μm. We found that the 4.45-μm PS particles were most cytotoxic while sizes 0.1 and 0.2μm showed no cytotoxicity up to 1000μg/ml. In the chromosome aberration test, the 4.45-μm PS particles induced polyploidy in a mass concentration-dependent manner in 24- and 48-h treatments. The 5.26-μm PS particles induced polyploidy only at 1000μg/ml for 48 h. Next, we performed the cytotoxicity test with as-grown single walled carbon nanohorns（NHas）. These were suspended in DMSO and then transferred into the culture medium followed by sonication. Six suspensions differently sonicated showed the same apparent toxicity, although the total particle size distributions differed. However, the sizes of NHas particles predicted to be most toxic from the experiments with PS particles, i.e. 1.01-4.47μm constituted 40-60% of all particles in all six suspensions. The results suggest that the cytotoxicity of NMs in suspension depends on specific sizes of aggregates and therefore suspensions should be checked with regard to particle size distributions in assays of toxic effects. The uptake of particles into cells was confirmed by confocal microscopy.

Keywords: polystyrene particles, single-walled carbon nanohorns, cytotoxicity, polyploidy

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Nakagawa, K.[*], Nakamura, K.[*], Haishima, Y., Yamagami, M.[*], Saito, K.[*], Sakagumi, H.[*] and Ogawa, H.[*]: **Pseudoproteoglycan（pseudoPG）probes that simulate PG macromolecular structure for screening and isolation of PG-binding proteins**

Glycoconj. J., **26**, 1007-1017（2009）

To elucidate the functions of higher-order proteoglycan（PG）structures, pseudoPGs that imitate the PG structure were prepared to develop probes and affinity adsorbents. Poly-L-lysine（PLL）or polyacrylamide（PAA）was coupled with various glycosaminoglycans（GAGs）, then biotinylated, and the remaining amino groups were blocked to obtain the pseudoPG probes, biotinyl PLL（BPL）- or PAA（BPA）-GAGs. Lactoferrin exhibited 30-single-strand probe, biotin-hydrazide-heparin. Heparin-PLL was immobilized on a formyl-Sepharose and compared with the Hep-Sepharose. Screening for ligands in normal rat brain revealed several proteins that specifically bound to either of the two adsorbents, indicating that the heparin-binding proteins exhibit specific recognition depending on the higher-order structure of the PG.

Keywords: proteoglycan, glycosaminoglycan, pseudoPG probe

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伊佐間和郎，河上強志，土屋利江，松岡厚子：**キャピラリー電気泳動法による家庭用品塗膜の鉛溶出量調査**

生活衛生，**54**，27-32（2010）

乳幼児用玩具の塗膜の鉛溶出量は，食品衛生法の「食品，添加物等の規格基準」において，90μg/g以下でなければならないと規制されている．しかし，乳幼児が触れやすい状態で使用される製品であっても，食品衛生法の対象外である製品については，塗膜の鉛溶出量が規制されていない．そこで，家庭内の生活空間に乳幼児が触れやすい状態で置かれる家庭用品として，文具及び髪留め等について，食品衛生法に基づく溶出試験を行い，塗膜の鉛溶出量の実態調査を実施した．また，試験溶液の鉛濃度の測定におけるキャピラリー電気泳動法の妥当性を確認した．調査した文具及び髪留め等の計105製品（107検体）中，塗膜から鉛の溶出が認められたのは髪留め（パッチンピン）の１製品のみで，食品衛生法の規格基準を超える量ではなかった．なお，この製品の梱包には，鉛の含有に対する注意表示があった．しかし，国際的な鉛フリーの情勢を鑑みれば，家庭内の生活空間に乳幼児が触れやすい状態で置かれる家庭用品の鉛含有量をより低減する努力がさらに必要であろう．

Keywords: capillary electrophoresis, lead, paint film

伊佐間和郎，河上強志，土屋利江，松岡厚子：**鉛含有**

金属製品の酸溶出試験法の比較

薬学雑誌，**130**，763-768（2010）

The international standard ISO 8124-3:1997 “Safety of toys － Part 3: Migration of certain elements” and “Interim Enforcement Policy for Children’s Metal Jewelry Containing Lead－2/3/2005” by the U.S. Consumer Product Safety Commission（CPSC）to control the amount of eluted lead from metal accessories cannot be simply compared, because the acid extraction methods and the limit values are different from each other. Therefore, the acid extraction tests based on the ISO standard and the CPSC policy were conducted for the small metal products, and the amounts of eluted lead were compared between both tests. There was less amount of eluted lead in the ISO method than in the CPSC method. Moreover, the amount of eluted lead in the ISO method did not even reach that of the first elution in the CPSC method. It became clear that the acid extraction test of the ISO standard was not as good in the ability of lead elution as that of the CPSC policy. In 16 products, seven products were unsuitable for the ISO standard and 14 products were unsuitable for the CPSC policy, but all these products were originally inapplicable to the ISO standard and the CPSC policy. The calculation grounds of the limit values were also different between the ISO standard and the CPSC policy. The standardization of acid extraction test that simulates the lead elution to gastric juice is expected, in order to prevent the adverse health effects in children due to the accidental ingestion of small metal products containing lead.

Keywords: lead, acid extraction test, metal product

大嶋智子[*1]，尾崎麻子[*1]，中島晴信[*2]，伊佐間和郎，土屋利江：**ポリ乳酸プラスチック中の有機スズ化合物の分析**

大阪市立環境科学研究所報告，**71**，21-26（2009）

Tin octylate（tin 2-ethylhexanoate）is widely used as a catalyst in the polymerization of polylactide plastics. Moreover, organotin compounds such as dibutyltin（DBT）and dioctyltin（DOT）are used as stabilizers of polyvinyl chloride. Therefore, residual organotin（butyltin, phenyltin and octyltin）compounds in polylactide plastics were simultaneously determined by gas chromatography－mass spectrometry（GC-MS）after ethyl derivatization with sodium tetraethylborate（$NaBEt_4$）. Tin octylate was detected as tetraethyltin by this method. Tin octylate level was 192μg/g in 1 out of 4 samples of polylactide plastics. The other organotin compounds were not detected as contaminants of tin octylate in the 4 tested samples.

Keywords: tin 2-ethylhexanoate, polylactide plastics, GC-MS

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河上強志，伊佐間和郎，中島晴信[*1]，大嶋智子[*2]，土屋利江，松岡厚子：**ガスクロマトグラフィー質量分析法による水性塗料及び水性接着剤中の有機スズ化合物の分析**

薬学雑誌，**130**，223-235（2010）

The use of tributyltin（TBT）and triphenyltin（TPT）in some household products are prohibited by “Act on the Control of Household Products Containing Harmful Substances” in Japan. In this study, methods for determination of TBT and TPT in water soluble paints and adhesives were developed by GC-MS. These compounds in paints and adhesives, which were mainly composed of vinyl acetate, urethane and acryl resins, and chloroprene rubber, were firstly extracted with HCl-acetone, and then extracted with hexane. On the other hand, the adhesive composed of natural rubber was firstly dispersed in water before acidification. The organotins were extracted with hexane from this solution and then these compounds were extracted with acetonitrile from hexane extract. These extracts were purified by a florisil cartridge column after ethylderivation with sodium tetraethylborate, and analyzed by GC-MS. The quantifications using deuterated compound of both organotins as surrogate standard were conducted, and good results were obtained. The recoveries were 81 to 118 % and the coefficients of variation were 0.83 to 4.3 %（TBT and TPT added; 5μg/g）. The method quantification limits were 0.0090 to 0.025μg/g, which were lower than those of an official method. These methods were applied to monobutyltin（MBT）, dibutyltin（DBT）, monophenyltin（MPT）, and diphenyltin（DPT）. DBT and DPT in paints and adhesives were quantified, except for DPT in natural rubber. These methods were applied to commercial products. DBT was detected at low concentrations（t.r.～0.19μg/g）in some paint samples, while TBT and TPT were not detected in all samples.

Keywords: organotin compounds, household goods, GC/MS

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Kishi, T.*, Shinkura, T.*, Suzuki, S.*, Kawakami, T., Takeda, K.*, Onodera, S.*: **Suppression of PCDD/Fs formation because of the presence of DEHP during the model slow combustion of 2,4,6-trichlorophenol**
Chemosphere, **78**, 1207-1212（2010）

The thermal reactions of 2,4,6-T_3CP in the presence and absence of DEHP in a dry air stream was investigated using a silica flow reactor at a residence time of 10 s and a temperature range from 450℃ to 850℃. Two isomers of T_4CDDs（1,3,6,8- and 1,3,7,9-T_4CDDs）were the most abundant products during the combustion of 2,4,6-T_3CP alone and were observed at temperatures ranging from 550℃ to 800℃. In the presence of DEHP, we observed a remarkable decrease in the yields of T_4CDDs during the combustion of 2,4,6-T_3CP. The suppression ratio of the T4CDDs formation was more than 90% in the case of the co-combustion with 10% DEHP in molar ratio. Other PCDD/Fs except for 2,7-/2,8-DCDD and 2,8-DCDF also decreased upon the combustion of 2,4,6-T_3CP in the presence of DEHP. During the co-combustion of 2,4,6-T_3CP and DEHP, the residual ratio of 2,4,6-T_3CP increased slightly and formations of lower chlorinated phenols were observed. The suppression of the T_4CDDs was strongly dependent on the DEHP ratio in the starting material. The prospective pathways of the suppressions of the T_4CDDs formations during the combustion of 2,4,6-T_3CP in the presence of DEHP were proposed.

Keywords: PCDD/Fs, suppression, DEHP

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Nishi, I.*, Komuro, T.*, Kawakami, T., Onodera, S.*: **In vitro Cyclooxygenase Inhibition Assay for Evaluating Ecotoxicity of the Surface Water and Domestic Wastewater in the Tone Canal, Japan**
Arch. Environ. Contam. Tocicol, **58**, 535-542（2010）

Cyclooxygenase（COX）plays an important role in eicosanoid metabolism. Nonsteroidal anti-inflammatory drugs（NSAIDs）function as COX inhibitors and are frequently detected in the aquatic environment. Here, we measured the in vitro COX-inhibiting activity of the surface water and domestic wastewater in the Tone Canal, Japan. The concentrations of several NSAIDs in the some samples were also determined using gas chromatography－tandem mass spectrometry for confirming the validity of the assay. The target compounds were extracted from the samples using a solid-phase extraction cartridge. A dose－response relationship between the inhibiting activity and sample volume were observed in the wastewater sample. The higher COX-inhibiting activities were observed in the wastewater sample, as compared with the samples of the surface water in the canal. These inhibiting activities reflected the trends of NSAIDs distribution in the canal. However, the inhibiting activities of the water samples could not be entirely explained by the NSAIDs that were selected for instrumental analysis in this study. Other compounds that were not measured by instrumental analysis in this study might contribute to the inhibiting activities. Therefore, the COX-inhibiting assay would be effective for evaluating inclusive ecotoxicity in the aquatic environment.

Keywords: cyclooxygenase, NSAIDs, ecotoxicity

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迫田秀行，石川 格，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高密度架橋ポリエチレンの疲労特性評価**
臨床バイオメカニクス，**30**，263-268（2009）

Highly crosslinked polyethylene（HXLPE）was introduced due to its superior wear property compared to conventional ultra-high molecular weight polyethylene（UHMWPE）. 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 the required specimen size. Therefore, there is not sufficient data to relate the fatigue property of UHMWPE to clinical outcome. The authors have developed a new test method using small specimens for evaluation of

fatigue property applicable to retrieved components or final products. This study directly compares the fatigue property of HXLPE and retrieved components using the same test method.

HXLPE that was gamma irradiated at 100kGy followed by melt treatment showed 30% reduction in the stress level of the fatigue property. This reduction in fatigue property was similar to that of a retrieved component which showed oxidation and delamination. This result indicated the possibility of fatigue-related failure of HXLPE components depending on its manufacturing process, condition and design.

Keywords: joint prosthesis, crosslinked polyethylene, fatigue

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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　第二報　人工股関節**

臨床バイオメカニクス，**30**，319-323（2009）

Although joint arthroplasty contributes to recovering the quality of life in patients with osteoarthritis or rheumatoid arthritis, considerable numbers of revision surgeries are performed due to failure of the joint prosthesis. It is necessary to understand factors affecting joint prosthesis failure in order to reduce the number of failures. Failed and retrieved implants provide very valuable information for identification of factors related to failure since non of the tests, including in vitro mechanical tests, animal tests and clinical trials, can fully simulate the complex biological and biomechanical environment over the long term in vivo.

However, most retrieved implants are old and were manufactured by outdated technologies. Therefore, for efficient analysis of retrieved implants, it would be desirable that failures due to well known factors that have already been addressed are screened out of these analyses, preferably without collecting clinical information, since the collection and analysis of these data are costly and time-consuming.

In this study, 16 retrieved hip implants were obtained without clinical information and visually inspected followed by FTIR analysis of UHMWPE components.

Oxidative degradation of the UHMWPE components, which is known as a major factor contributing to implant failure, was considered the reason for failure in most cases. These cases could be eliminated by visual inspection. The remaining three cases were considered to have failed due to factors other than oxidative degradation of UHMWPE components. Identification of factors related to failure of these cases by detailed analysis using clinical information is expected to provide useful information for the development of future implants.

Keywords: joint prosthesis, retrieved implants, implant failure

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Teramura, S.[*1], Sakoda, H., Terao, T.[*1], Fujiwara, K.[*2], Kawai, K.[*1] and Tomita, N.[*1]: **Reduction of wear volume from accelerated aged UHMWPE knee components by the addition of vitamin E**

Journal of Biomechanical Science and Engineering, **4**, 589-596（2010）

Accelerated ageing was conducted on UHMWPE with and without vitamin E（D, L-α tocopherol, VE）. Wear performance was investigated using a knee simulator and wear debris was analysed. Aged UHMWPE with VE showed significantly lower wear volume than that of aged virgin UHMWPE and showed approximately similar wear volume as non-aged virgin UHMWPE. There were no significant differences among the materials as far as shape factor of the debris is concerned.

Keywords: artificial joint, vitamin E, wear

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Ahmed, S., Tsuchiya, T., Nagahata-Ishiguro, M., Sawada, R., Banu, N., Nagira, T.: **Enhancing action by sulfated hyaluronan on connexin-26, -32, and -43 gene expressions during the culture of normal human astrocytes**

J. Biomed. Mater. Res. A, **90**(3), 713-719（2009）

Astrocyte proliferation is strictly controlled during development and in the adult nervous system. In this study, we examined the role of sulfated hyaluronan（SHya）in the proliferation and differentiation of normal human astrocytes（NHAs）. Cells were cultured with different concentrations of SHya for 7 days, and

the number of viable cells and the presence of neural cell-specific genes were determined to assess their proliferation and development, respectively. With SHya, cell proliferation increased nonsignificantly. Furthermore, remarkable enhancing action by SHya on connexin-26, -32, and -43 gene expressions were observed during the culture of NHAs. It has been suggested that a fraction of NHAs have neural precursor activity that gives rise to astrocytes themselves, oligodendrocytes, and neurons. Our results clearly demonstrated that the expression of specific genes for neural precursor cells, astrocytes, neurons, and oligodendrocytes was significantly increased to 50 μg/mL in SHya-treated cultures when compared with that of the control culture. These findings suggest that SHya plays an important role in the proliferation and differentiation of NHAs and in the production of a novel material for tissue engineering.

Keywords: sulfated hyaluronan, normal human astrocytes, connexin

Yamada, T., Nakaoka, R., Sawada, R., Matsuoka, A., Tsuchiya, T.: **Effects of intracerebral microinjection of hydroxylated-[60] fullerene on brain monoamine concentrations and locomotor behavior in rats**

J. Nanosci. Nanotechnol., **10**, 604-611 (2010)

Fullerenes are condensed ring aromatic compounds with extended pi systems; they have unique cage structures. Current studies suggest that several fullerene derivatives have neuroprotective effects, and it is expected that fullerenes will be useful in drug delivery system and novel medical devices targeting the brain. However, little is known about the effects of fullerenes and its derivative on brain function. We examined the effect of fullerene$(OH)_{24}$ on the central nervous system in this study. In a V79 colony assay, the IC_{50} of fullerene$(OH)_{24}$ was 1.74 μg/ml. In an MTT assay, fullerene$(OH)_{24}$ reduced proliferation of normal human astrocytes obviously. In an vivo study, 0.25 mg/kg of fullerene$(OH)_{24}$ was injected into the lateral ventricle of rat brains. The intracerebral injection of fullerene $(OH)_{24}$ remarkably decreased body weight and locomotor behavior of rats on day 1, but drastically increased locomotor behavior on day 7. The intracerebral injection of fullerene$(OH)_{24}$ changed the monoamine concentration greatly on day 1 and slightly on day 30 after the injection. These results suggest that intracerebral injection of fullerene$(OH)_{24}$ had strong and acute effects on the central nervous system, but that the effects we,re not permanent. In conclusion, we suggest that fullerene's derivative, fullerene$(OH)_{24}$ had toxic effects on brain cells and that intracerebral injection of fullerene$(OH)_{24}$ had acute harmful effects on brain monoamines neurotransmission and locomotor activity.

Keywords: Nanomaterials, Fullerene, Neurotoxicity

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電気学会論文誌C（電子・情報・システム部門誌），**130**(3)，414-419 (2010)

To establish safe, precise, and minimally invasive surgery, Computer Aided Surgery (CAS) systems, such as intra-operative imaging and navigation system to detect the location of the target of therapy, and surgical robot system, are very powerful tools. There is strong need to combine these CAS systems for fusion of advanced diagnosis and treatment technologies. In this paper, we introduce our new method to register the intraoperative imaging information, robotic surgery system, and patient using surgical navigation system. Using our Open-MRI navigation system and laser surgery system for neurosurgery, we can make registration between these system and patient precisely. The experimental result shows that the error on the registration between image data and the laser surgery system is low enough to fulfill the requirement of laser surgery system in the use of high-resolution image data. This system realizes the safe, precise and minimally invasive neurosurgery by the combination of intra-operative diagnosis and advanced therapeutic device.

Keywords: surgical navigation system, robotic surgery, registration

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中島晴信[*]，鹿庭正昭：**乳幼児用繊維製品（衣服及び玩具）に使用されている染料成分中の芳香族第一アミン類の分析調査**

大阪府公衆衛生研究所研究報告，**47**，75-80（2009）

発がん性を有するために，欧州ではEN71「玩具の安全性規制」により規制されている，９種の芳香族第一アミン類について，GC/MSによる最終試験分析法を確立し，市販乳幼児用繊維製品（衣服，玩具）における分析調査を行った．

Keywords: textile products for infant and baby, dye, aromatic primary amines

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Sato, K.[*1], Umemura, T.[*1], Tamura, T.[*1], Kusaka, Y.[*1], Aoyama, K.[*2], Ueda, A.[*3], Harada, K.[*3], Minamoto, K.[*3], Otsuki, T.[*4], Yamashita, K.[*5], Takeshita, T.[*6], Shibata, E.[*7], Dobashi, K.[*8], Kameo, S.[*8], Miyagawa, M.[*9], Kaniwa, M., Endo, Y.[*10], Yuda, K.[*11]: **Skin sensitization study by quantitative structure-activity relationship (QSAR)**

AATEX, **14**(3)，940-946（2009）

In silico assessment of skin sensitization is increasingly needed owing to the problems concerning animal welfare, as well as excessive time consumed and cost involved in the development and testing of new chemicals. Skin sensitization positive/negative prediction models with discriminant function were generated and parameter analysis was discussed on the basis of QSAR technology.

This is the first QSAR model for skin sensitization from Japan. Future studies of this QSAR model are needed to improve its efficacy.

Keywords: skin sensitization, QSAR, animal study

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Hanioka, N.[*], Yamamoto, M.[*], Tanaka-Kagawa, T., Jinno, H., Narimatsu, S.[*]: **Functional characterization of human cytochrome P4502E1 allelic variants: *in vitro* metabolism of benzene and toluene by recombinant enzymes expressed in yeast cells**

Arch. Toxicol., **84**, 363-371（2010）

Benzene and toluene are common organic solvents currently in worldwide industrial usage, which are metabolized mainly by hepatic cytochrome P450 2E1 (CYP2E1) in humans. Genetic polymorphism of *CYP2E1* in 5'-flanking and coding regions has been found previously in Caucasian and Chinese populations. In this study, the effects of *CYP2E1* alleles causing amino acid substitutions (*CYP2E1*2*, *CYP2E1*3* and *CYP2E1*4*; wild-type, *CYP2E1.1A*) on benzene hydroxylation and toluene methylhydroxylation were studied using recombinant CYP2E1 enzymes of wild-type (CYP2E1.1) and variants (CYP2E1.2 having Arg76His, CYP2E1.3 having Val389Ile and CYP2E1.4 having Val179Ile) expressed in yeast cells. The *K*m, *V*max and *CL*int values of CYP2E1.1 were 10.1 mM, 9.38 pmol/min/pmol CYP and 0.99 nL/min/pmol CYP for benzene hydroxylation, and 3.97 mM, 19.9 pmol/min/pmol CYP and 5.26 nL/min/pmol CYP for toluene methylhydroxylation, respectively. The *K*m, *V*max and *CL*int values for benzene and toluene metabolism of CYP2E1.2, CYP2E1.3 and CYP2E1.4 were comparable to those of wild-type CYP2E1. These findings may mean that the polymorphic alleles of *CYP2E1* causing amino acid substitutions are not directly associated with the metabolic activation of benzene and toluene. The information gained in this study should help to identify the variations in the toxicity of environmental pollutants.

Keywords: Cytochrome P450 2E1 (CYP2E1), Genetic polymorphism, Benzene and Toluene

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Senzui, M.[*], Tamura, T.[*], Miura, K.[*], Ikarashi, Y., Watanabe, Y.[*], Fujii, M.[*]: **Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin *in vitro***

J. Toxicol. Sci., **35**, 107-113（2010）

It is important for toxicological assessment of nanoparticles to determine the penetration of nanoparticle in skin qualitatively and quantitatively. Skin penetration of four different types of rutile titanium dioxide (TiO_2) (T-35, 35 nm, non-coating; TC-35, 35 nm, with

almina/silica/silicon coating; T-disp, 10 × 100 nm, mixture of almina coated and silicon coated particles, dispersed in cyclopentasiloxan; T-250, 250 nm, non-coating）was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs to study the effect of dispersion and skin conditions. The TiO_2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose 2μL/cm^2 for 24 h, followed by cyanoacrylate stripping. The Ti concentration in skin was determined by ICP-MS. T-35 and T-250 easily aggregated in suspension with a mean diameter greater than 1μm. TC-35 and T-disp showed good dispersion properties with a mean diameter in suspension of approximately 100 nm. No penetration was observed regardless of TiO_2 type in intact and stripped skin. The concentration of Ti in skin was significantly higher when TC-35 was applied on hair-removed skin. SEM-EDS observation showed that Ti penetrated into vacant hair follicles（greater than 1 mm below the skin surface）, however, it did not penetrate into dermis or viable epidermis.

Keywords: nanoparticle, skin penetration, titanium oxide

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内野 正，五十嵐良明，関 泰三[*1]，森岡恒男[*1]，奥村秀信[*2]，高良健作[*3]，和田浩二[*3]，徳永裕司[*4]，西村哲治：**黒糖から抽出した化合物のβ-hexosaminidase放出抑制活性並びに抗酸化活性について**

日本香粧品学会誌，**34**，14-18（2010）

Oxidative stress is known to be connected with allergic dermatitis such as atopy, which was those recently increased. Kokuto condenced sugar cane sap., is reported to contain anti-oxidants. We reported that oxidative stress induces β-hexosaminidase（connected with allergic reaction）release from cultured cells. In this study, we screened kokuto extracts by the detection of β-hexosaminidase released from RBL-2H3 cells. We found out that 5 kinds of phenyl glucoside inhibit β-hexosaminidase release and 4-hydroxy-3-methoxy-phenyl-*O*-β-D-glucoside shows the highest inhibition activity. This result suggested that these compounds have *in vitro* anti-allergic activity.

Keywords: kokuto extracts, β-hexosaminidase, anti-allergic activity

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Amakura, Y.[*], Yoshimura, M.[*], Sugimoto, N., Yamazaki, T., Yoshida, T.[*]: **Marker constituents of the natural antioxidant "Eucalyptus leaf extract" for the evaluation of food additives**

Biosci. Biotech. Biochem., **73**, 1060-1065（2009）

In order to establish the marker constituents or natural antioxidant rood-additive Eucalyptus leaf extract, the UV-absorbing constituents or two eucalyptus leaf extract registered as food additives（eucalyptus A and B）were investigated. Several major peaks on the reversed-phase HPLC chromatogram or eucalyptus were characterized as gallic acid, ellagic acid, 3-*O*-β-D-glucuronides or quercetin and kaempferol, and a hydrolyzable tannin dimer, oenothein B, by direct comparison with authentic specimens isolated from *Eucalyptus globuls* leaves. A new gallotannin was round in *E. globulus* leaf extract, and its structure was found to be 1,2,3,6-tetra-*O*-galloyl-β-D-galactose. Two major peaks on the HPLC chromatogram or eucalyptus were identified as gallic acid and ellagic acid, indicative of degradation products from hydrolyzable tannins in the leaves. Considering the evaluation of antioxidant activity by radical scavenging ability, a standardization of eucalyptus leaf extract, including the antioxidative polyphenol, oenothein B, is proposed.

Keywords: natural antioxidant, eucalyptus, polyphenol

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日本食品化学学会誌，**16**，28-33（2009）

On the quantitative analysis of pesticide residues by LC/MS or GC/MS, the standard samples of pesticides are essential. But most of their purities are not traceable to the International System of Units（SI）and it results in degrading the reliability of analysis data.

Therefore, the SI-traceable quality control of pesticide standard samples will be most important. We are developing quantitative nuclear magnetic resonance (qNMR) as one of simple quality control methods that is able to determine the purities or contents with SI traceability. We demonstrated that qNMR was used for the purity determination of two standard samples of isoxathion oxon (IXO), an organophosphorus pesticide. The purities of the two samples were certificated by the manufacture as 96.9 % and 98.9 % which were calculated from the peak area percentages using GC/FID. On the qNMR spectrum, IXO showed the proton signals in the range of δ 1.0 − 8.0 ppm, and the quantitation was performed by calculating the relative peak area ratios of selected proton signals of the target compound to the known purity and amount of the internal standard, hexadimethylsilane which was calibrated with SI-traceable diethyl phthalate. For this method no reference compound of IXO is needed. The purities of two IXO samples showed 75.4 % and 98.5 % by qNMR. The relative ratio of the two purities was equivalent to the ratio of IXO peak areas in the two samples observed by GC/MS. This result shows that qNMR does not only lead to SI-traceable purity, but it also will be a rapid and simple SI-traceable quality control method of any pesticides with overall analysis time of only 20 min.

Keywords: quantitative NMR, isoxathion oxon, pesticide

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杉本直樹，多田敦子，末松孝子[*1]，有福和紀[*1]，齋藤剛[*2]，井原俊英[*2]，吉田雄一[*3]，久保田領志，田原麻衣子，清水久美子，伊藤澄夫[*4]，山崎　壮，河村葉子，西村哲治：**定量NMRを用いたコチニール色素中のカルミン酸の絶対定量**
食品衛生学雑誌，**51**，19-27 (2010)

Quantitative NMR (qNMR) method was applied for the determination of carminic acid. Carminic acid is the main component in cochineal dye that is widely used as a natural food colorant. Since several manufacturers only provide the reagent grade carminic acids of which purities are not determined exactly, there is no its reference material that is traceable to International System of Units (SI units). Hence if using the reagent as the reference material for quantitation, it will result in degrading the reliability of analysis data. To improve the reliability of analytical data, we are developing quantitative nuclear magnetic resonance (qNMR) as one of simple absolute quantitation methods that is able to determine the contents with SI traceability. qNMR is based on the fact that the signal intensities of a given NMR resonance are directly proportional to the molar amount of that nucleus in the sample. The purities and contents of carminic acid were calculated from the ratio of the signal intensities of an aromatic proton on carminic acid to nine protons of three methyl groups on DSS-d_6 used as the internal standard, after the concentration of DSS-d_6 was made a correction using potassium hydrogen phthalate, which is one of certified reference material (CRM). In the result, the purities of the reagents and the contents in cochineal dye products were determined with SI-traceability to 25.3-92.9% and 4.6-30.5% as the crystalline formula, carminic acid potassium salt trihydrate, which had been confirmed by X-lay analysis. qNMR method needs no its reference compound but also it is rapid and simple with overall analysis time of only 10 min. Our approach thus represents an absolute quantitation method with SI-traceability that will be readily utilized to analysis and quality control of any natural product.

Keywords: quantitative NMR, carminic acid, cochineal dye

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Hasada, K.[*1, 2], Yoshida, T.[*1], Yamazaki, T., Sugimoto, N., Nishimura, T., Nagatsu, A.[*2], Mizukami, H.[*1]: **Quantitative derermination of atractylon in Atractylodis rhizoma and Atractylodis lanceae rhizoma by ^{1}H-NMR spectrometry**
J. Nat. Med., **64**, 161-166 (2010)

^{1}H-NMR spectroscopy was successfully applied to the quantitative determination of atractylon in Atractylodis Rhizoma (dried rhizomes of *Atractylodes ovata* and *A. japonica*) and Atractylodis Lanceae Rhizoma (dried

rhizomes of *Atractylodes lancea* and *A. chinensis*). The analysis was carried out by comparing the integral of the H-12 singlet signal of atractylon, which was well separated in the range of δ6.95－7.05 ppm in the NMR spectrum, with the integral of a hexamethyldisilane (HMD) signal at δ0 ppm. The atractylon contents obtained by the ^{1}H-NMR spectroscopy were consistent with those obtained by the conventional HPLC analysis. The present method requires neither reference compounds for calibration curves nor sample pre-purification. It also allows simultaneous determination of multiple constituents in a crude extract. Thus, it is applicable to chemical evaluation of crude drugs as a powerful alternative to various chromatographic methods.

Keywords: quantitative NMR, qNMR, Atractylon

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鈴木俊成*，矢口久美子*，栗田雅行*，西村哲治，小縣昭夫*：**河川水中の医薬品の分析法**

東京都健康安全研究センター研究年報，**60**，253-258（2009）

河川水中の医薬品を固相抽出法により抽出・濃縮後，GC/MSまたはLC/MSで分離定量する分析法について検討した．分析対象は解熱鎮痛消炎剤，高脂血症薬，抗アレルギー薬，抗てんかん薬，高圧症治療薬，糖尿病治療薬および精神科用薬の105医薬品であった．本分析法ではスルピリン，ヒドラジン，ピンドルールおよびメチルドーパの回収率はいずれも8%以下と低く，これらの分析は不可能であった．一方，その他の101医薬品については，回収率が37～200%と広範囲に及んだが，変動係数は23%以下と比較的良好であり，実態調査に適用可能であった．しかし，定量に際しては回収率が120%を超えるものや80%に満たないものは標準添加法により定量する必要があることが示唆された．

Keywords: 医薬品，分析法，実態調査

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宮原　誠，廣庭隆行*1，増水章季*2，原　英之*3，岡野和史*4，武川哲也*5，須永博美*6：**アラニン線量計を用いた北海道士幌町農業協同組合士幌アイソトープ照射センターの線量分布測定**

Radioisotopes, **58**, 815-825（2009）

北海道士幌町のばれいしょの照射施設はフリッケ線量計で線量管理を行ってきた．これと同等にアラニン線量計システムが適用できることを確かめる目的で，照射コンテナの表面線量分布を測定した．結果，表面の均一性は1.08で，最大線量領域はコンテナの端から80cmにある垂直線の上端から20cm～60cmの範囲にあった．この分布は設計仕様概念にほぼ一致しており，これらは同等の線量分布測定システムと考えられた．

Keywords: food irradiation, alanine dosimeter, Shihoro, dose distribution

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高附　巧，渡邉敬浩，坂井隆敏，松田りえ子，米谷民雄*：**葉菜およびミネラルウォーター中の過塩素酸濃度の実態調査**

食品衛生学雑誌，**50**(5)，184-189（2009）

過塩素酸塩は天然および人工物が存在し，人への健康影響は甲状腺へのヨウ素の取り込み阻害および甲状腺機能を抑制である．アメリカでは様々な食品中から過塩素酸塩が検出されている．我が国における食品中の過塩素酸塩濃度の実態を調査するため，市販の葉菜82検体およびミネラルウォーター20試料中の過塩素酸塩濃度を測定した．過塩素酸塩の試験法はFDAの試験法を参考に$^{18}O_4$-標識過塩素酸塩を内部標準物質としたIC-MS/MSにより行った．葉菜82検体中，3検体がLOQ（0.3 ng/g）未満で，79検体から0.3～29.7 ng/gの過塩素酸塩を検出した．ミネラルウォーター20検体中6検体から0.14～0.35 ng/mLの過塩素酸塩を検出し，14検体はLOQ（0.1 ng/g）未満であった．

Keywords: perchlorate, IC-MS/MS, leafy vegetable, mineral water

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渡邉敬浩，松田りえ子：**TaqMan Chemistryに基づくリアルタイムPCRにより得られるデータの新規解析ソフトウェアー（*GiMlet*）の開発とそれを用いたCt値変動要因の検討**

食品衛生学雑誌，**50**(5)，208-215（2009）

リアルタイムPCRの一義的な測定量である蛍光データを，高い自由度をもって解析するためのアプリケーション（*GiMlet*）を開発した．またこれを用いて，併行条件下でTaqMan Chemistryに基づくリアルタイムPCRによ

り得られるCt値の変動要因について，複数の異なる機種（ABI PRISM7500，7700，7900HT）を対象に検討した．また，ベースラインの補正方法の分析結果への影響について検討した．その結果，1)機種により測定値およびPCR効率のウェルおよびくり返し測定間での変動が異なること，2)ベースラインの補正方法が測定値の変動に影響を及ぼす要因になりうること，の２点が示された．
Keywords: real-time PCR, Ct value, precision, data analysis

Kotani, A.*, Yuan, Y.*, Yang, B.*, Hayashi, Y., Matsuda, R., Kusu, F.*: **Selection of the optimal solvent grade for the mobile phase in HPLC with electrochemical detection based on FUMI theory**
Anal.Sci., **25**, 925-929 (2009)

The optimum conditions of the mobile phase for HPLC with electrochemical detection (HPLC-ECD) were selected from among solvents of different grades with the standard deviation of area measurements based on FUMI theory as criterion. This selection method saves considerable amounts of chemicals and experimental time, and would be a useful exploration technique for the routine check and troubleshooting of HPLC-ECD.
Keywords: HPLC-ECD, optimization, precision, FUMI theory

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久保田浩樹，大槻　崇，原　貴彦*1，平川佳則*1，飯塚太由*1，田中麻紀子*2，岩村真実*2，佐藤恭子，河村葉子：**果実，種実，香辛料およびその加工食品に存在する安息香酸並びにソルビン酸含有量の調査**
日本食品化学会誌，**17**，54-61（2010）

水蒸気蒸留法および溶媒抽出法の２種類の抽出方法を用い，HPLCおよびLC/MS/MS法により，24種の果物を含む39種の試料に存在する安息香酸およびソルビン酸の含有量を調査した．安息香酸の平均含有量は，水蒸気蒸留法では，29種の試料で１～424 mg/kg，溶媒抽出法では，15種の試料で１～126 mg/kgであった．一方，ソルビン酸は，両抽出方法ともに，これらの試料から検出されなかった．検出誤認を避けるため，安息香酸誘導体を多く含む食品に関しては，溶媒抽出法が水蒸気蒸留法と比較して適切と考えられた．またLC/MS/MS法は，夾雑成分を多く含む食品においても安息香酸およびソルビン酸を高い精度で定量できることが確認された．
Keywords: benzoic acid, sorbic acid

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Tatebe C., Kawasaki H., Kubota H., Sato K., Tanamoto K. and Kawamura Y.: **Analysis of residual solvent in thickeners by headspace gas chromatography using a standard addition method**
Nihon Shokuhin Kagaku Gakkaishi, **16**, 78-83 (2009)

Headspace gas chromatography (HS-GC) is an accepted method for analysis of residual solvents in pharmaceuticals, food additives and food. The amounts of residual solvent present in various food thickeners were analysed by HS-GC standard addition method. Conditions for the HS-GC were optimised, and equilibration time was determined to be 40 min at 60℃ for the determination of residual solvent. The results were very similar to those obtained by distillation and gas chromatography (Distillation－GC). We conclude that both methods are equally efficient for the determination of residual solvent in thickeners. In addition capillary column (Aquatic-2 GL Sciences Co.) was used to analyze by headspace or distillation.
Keywords: residual solvent, headspace gas chromatography (HS-GC), standard addition method

大槻 崇，久保田浩樹，佐藤恭子，河村葉子：**アセトニトリル不足に対応した*tert*-ブチルヒドロキノン試験法におけるメタノールの適用性**
日本食品化学学会誌，**16**，66-71（2009）

世界的な景気後退によるアセトニトリルの供給量の減少に対応するため，*tert*-ブチルヒドロキノン通知法におけるアセトニトリルの代替溶媒としてのメタノールの適用性について検討し，通知法にほぼ準じた操作で良好な回収率，定量再現性が得られることを明らかにした．
Keywords: *tert*-butylhydroquinone, alternative method, shortage of acetonitrile

箕川　剛，久保田浩樹，佐藤恭子，河村葉子：**アセトニトリル不足に対応した食品中のサイクラミン酸分析法の改良**
食品化学学会誌，**17**，62-64（2010）

世界的なアセトニトリル不足を受けて，それが移動相溶媒に使われている食品中のサイクラミン酸分析法（通知法）のHPLC条件を改良した．その結果，移動相にメタノール-水（80：20）を使用することにより，サイクラミン酸の良好な分離および改良前の条件と同等の検出限

界が得られた．さらにこのHPLC条件の実試料への適用性を評価するため，7種類の市販食品に標準添加した後，通知法に従って前処理し，HPLC分析に供した．その結果いずれの試料からも妨害ピークのない良好なクロマトグラムと十分な回収率が得られ，本HPLC条件はサイクラミン酸の分離検出条件として通知法に適用しても支障ないことが示唆された．

Keywords: cyclamic acid, high performance liquid chromatography, shortage of acetonitrile

多田敦子，杉本直樹，古庄紀子，佐藤恭子，山崎壮，棚元憲一：**既存添加物オゾケライトの成分調査**
日本食品化学学会誌，**16**，92-96（2009）

オゾケライトは天然由来のガムベースの一つで，既存添加物に関連した通知（1996年，既存添加物名簿収載品目リスト）には，「ワックスシュールの鉱脈に含まれるロウを精製したものである．主成分はC_{29}～C_{53}の炭化水素である．」と記載されている．オゾケライト国内流通製品の品質を明らかにするため，成分分析を行った．GC/MS分析の結果，主にC_{22}～C_{38}の飽和炭化水素群が検出され，微量ながらC_{39}～C_{58}の飽和炭化水素も検出された．したがって，主要炭化水素の炭素数分布は，通知の記載より低炭素数に分布していた．また，GC/FID分析でC_{22}～C_{38}の主要飽和炭化水素を定量したところ，合計81％であった．

Keywords: food additive, ozokerite（ozocerite）, wax

Ito, Y., Sugimoto, N., Akiyama, T., Yamazaki, T., and Tanamoto, K.: **Cepaic acid, a novel yellow xanthylium pigment from the dried outer scales of the yellow onion *Allium* cepa**
Tetrahedron Letters, **50**, 4084-4086（2009）

Cepaic acid was isolated as a novel xanthylium yellow pigment from the dried outer scales of the yellow onion *Allium* cepa Linne. Its structure was elucidated on the basis of ESI-MS and 2D NMR spectroscopy as a 9-carboxy-1,3,6,8-tetrahydroxyxanthylium, which suggests that cepaic acid and other yellow pigments in the dried outer skin of onion was formed by the nucleophilic reaction of phloroglucinol derived from quercetin, a flavonol in onion scales, by autoxidation to glyoxylic acid. To our knowledge, this is the first report of such pigment in yellow onion.

Keywords: onion, cepaic acid, xanthylium

六鹿元雄，李　演捩，河村葉子，棚元憲一：**紙製品中の芳香族第一級アミン類の分析**
食品衛生学雑誌，**50**，160-166（2009）

紙製品中の芳香族第一級アミン類25種類およびそれらを生成するアゾ色素類の高感度分析法を確立した．試料中の遊離アミンと総アミンを分析し，アゾ色素量は総アミンから遊離アミンを差し引くことで求めた．アミン類およびアゾ色素の溶出は，23℃の水に24時間浸漬して行った．遊離アミンは溶出液に水酸化ナトリウムを加えてアルカリ性とし，ジクロロメタンで抽出した．総アミンは溶出液中のアゾ色素を亜ジチオン酸ナトリウムでアミンに還元分解したのち同様に操作した．試験溶液はGC/MSで測定した．試料あたり100μg/kg相当量のアミン類を溶出液に添加した場合の回収率は，4,4'-oxydianilineと4,4'-diaminodiphenylmethaneで40%程度と低かったが，それ以外のアミン類は69～122%とほぼ良好であり，定量限界は4～20μg/kgであった．本法を用いて原紙17試料および食品用紙製品16試料の分析を行ったところ，大部分の再生紙試料からアニリンが4～20μg/kg検出されたが，それ以外のアミン類はいずれの試料からも検出されなかった．

Keywords: primary aromatic amine, azo-dye, paper product

大野浩之，鈴木昌子，六鹿元雄，河村葉子：**合成樹脂製器具・容器包装および玩具における過マンガン酸カリウム消費量および全有機炭素の検討**
食品衛生学雑誌，**50**，230-236（2009）

合成樹脂製器具・容器包装および乳幼児用玩具から溶出する有機物総量の指標として過マンガン酸カリウム消費量と全有機炭素（TOC）を検討した．定量限界はいずれも0.5μg/mLであった．器具・容器包装97検体を測定したところ，過マンガン酸カリウム消費量およびTOCの値はポリ塩化ビニル（PVC）製急須口とナイロン製器具で0.5～10.9μg/mLおよびND～18.9μg/mLであった．また，玩具32検体ではPVC製玩具とエチレン・酢酸ビニル樹脂製ブロック玩具で0.8～45.5μg/mLおよび0.5～8.9μg/mLであった．一方，その他の試料では両者はほとんど検出されなかった．過マンガン酸カリウム消費量とTOCの値を比較すると，いくつかのPVC製品とナイロン製器具で両者は大きく食い違った．そのため，有機物総量の指標としてはTOCの方が適していた．

Keywords: consumption of potassium permanganate, total organic carbon

Morita, Y.[*1], Komoda, E.[*1], Boonmar, S.[*2], Markvichitr, K.[*3], Chaunchom, S.[*3], Chanda, C.[*4], Yingsakmongkon, S.[*2], Padungtod, P.[*5], Jha, V.C.[*6], Singh, S.[*7], Yamamoto, S., and Kimura, H.[*8]: **Antimicrobial susceptibility of**

Campylobacter coli isolated from buffaloes in Vientiane, Lao People's Democratic Republic

Nepalese Vet. J., **29**, 42-45 (2009)

A study was conducted on the prevalence of *Campylobcter spp.* in buffaloes and antimicrobial susceptibility of isolates in Vientiane, Lao People's Democratic Republic (Lao PDR). *Campylobcater* was isolated from 3(6%) of th 50 caecum samples and all the isolates were identified as *C. coli*. The resistance profile and MIC of the 3 *C. coli* strains; namely A, B, and C were CP (MIC; 128 mg/liter)-TC (32 mg/liter)-NA(256 mg/liter)- CPFX (128 mg/liter), ABPC (256 mg/liter) -CTRX(64 mg/liter), and ABPC (128 mg/liter) respectively. A quinolone-resistant strain of C. coli has already been isolated in Lao PDR. This study results suggested that a survey on the pervalence of *Campylobacter spp.* in human, food animals, and different types of food products should be performed to determine important sources of *Campylobacter* infection.

Keywords: Campylobacter, buffalo, Lao People's Democratic Republic, drug resistant bacteria

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Hara, H.*1, Ohashi, Y.*1, Sakurai, T.*2, Yagi, K.*3, Fujisawa, T.*1, and Igimi, S.: **Effect of Nisin (Nisaplin) on the Growth of *Listeria monocytogenes* in Karashi-mentaiko (Red-pepper Seasoned Cod Roe)**

食品衛生学雑誌, **50**, 173-177 (2009)

The influence of Nisaplin, which contains 2.5% nisin, on the growth of *Listeria monocytogenes* in Karashi-mentaiko (red-pepper seasoned cod roe) was investigated. The MICs of Nisaplin for *L. monocytogenes* (10^8 CFU/mL) were measured; seven isolates showed a value of 1,600μg/mL and one isolate showed a value of 800μg/mL. All *L. monocytogenes* isolates had a MIC of 800μg/mL at 10^6 CFU/mL. The number of *L. monocytogenes* in Karashi-mentaiko stored at 4 C was decreased by Nisaplin added at 60 and 600μg/g. These results indicated that Nisaplin effectively inhibits the growth of *L. monocytogenes* in Karashi-mentaiko.

Keywords: nisin, *Listeria monocytogenes*, Karashi-mentaiko

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萩原博和*, 露木朝子*, 古川壮一*, 森永　康*, 五十君靜信：**乳児用調製粉乳（PIF）の調乳および保存方法が*Enterobacter sakazakii*の生残と増殖に及ぼす影響**

食品衛生学雑誌, **50**, 109-116 (2009)

The effect of the reconstruction and storage conditions of powdered infant formula (PIF) on the survival and growth of three *Enterobacter sakazakii* strains, ATCC 29004, HT 022 and HT 028, was investigated. D values of *E. sakazakii* ATCC 29004 and HT 022 at 60C were 3.6 and 1.6 min, respectively, and that of HT 028 at 52C was 1.6 min. The effect of the temperature of the water used for the reconstruction of PIF on the inactivation of the three *E. sakazakii* strains was also investigated. One to 2 log order inactivation occurred at 70C, and above 5 log order inactivation at 80C. Storage tests at 5, 10 and 25C showed that none of the strains could grow at 5C, HT 028 grew slightly at 10C, and at 25C all three strains started growth after 4 hr incubation and reached up to 8 log CFU/mL after 16 hr incubation. From the above results, it is concluded that a suitable temperature of the hot water for reconstruction of PIF is above 70C, and the preferred storage temperature of reconstructed PIF, which is recommended to be consumed within 2 hr, is below 5C.

Keywords: powdered infant formula, *Enterobacter sakazakii*, control

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Tamura, A.*1, Yamasaki, M.*2, Okutani, A.*3, Igimi, S., Saitoh, N.*3, Ekawa, T.*4, Ohta, H.*4, Katayama, Y.*5, and Amano, F.*1: **Dry-resistance of *Salmonella enterica* subsp. *enterica* Serovar Enteritidis is regulated by both SEp22, a novel pathogenicity-related factor of *Salmonella*, and nutrients**

Microbes and Environments, **24**, 121-127 (2009)

Environmental isolates of *Salmonella enterica* serover

Enteritidis（*S.* Enteritidis）clones were grown to the logarithmic phase, washed and re-suspended in saline or Luria-Bertani（LB）medium, and then 10-μL aliquots of the suspensions were dried overnight at room temperature. The dried bacteria were mixed with 1 mL of ice-cold PBS, suspended and examined for colony-forming activity. All of the pathogenic clones with high levels of SEp22, identical to *Salmonella* Dps, maintained good viability if suspended in LB medium prior to drying. However, none of the nonvirulent strains, exhibiting low levels of SEp22, survived. Similar results were obtained with sep22-knocked out mutants, suggesting that SEp22 is important for that acquisition of dry-resistance. Nutritional factors, such as LB medium, cabbage extracts, and egg yolk but not egg white, were shown to be necessary for the acquisition of dry-resistance, because none of the clones remained viable irrespective of SEp22 expression if suspended in saline. Scanning electron micrograms also supported the importance of nutrition, showing regrowth of the bacteria after drying in LB but not in saline. These results suggest the importance of both SEp22 expression and nutrients for the acquisition of dry-resistance by S. Enteritidis.

Keywords: Dry-resistance, *Salmonella*, knocked out mutant

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Tamura, A.[*], Nishio, E.[*], Fujimori, K.[*], Igimi, S. and Amano, F.[*]: **Lactoferrin inhibits the acquisition of dry-resistance by *Salmonella* spp.**

Bioscience and Microflora, **28**, 81-88（2009）

An assay method was established for estimation of dry-resistance of Salmonella. Environmental isolates of *Salmonella enterica* spp., including *S.* Enteritidis were grown to the logarithmic phase, washed and re-suspended in saline or Luria-Bertani（LB）medium, followed by drying overnight in an automatic dry-keeper at room temperature. The dried bacteria were recovered by mixing with ice-cold PBS, suspended, and examined for viability by colony-forming activity. A pathogenic clone of S. Enteritidis, SECl#15-1, was not viable in saline alone but maintained its viability in LB medium, suggesting it requires nutrients for the acquisition of dry-resistance. Addition of lactoferrin or apolactoferrin to the bacterial suspension in 20% LB medium prior to the dry-protocol decreased the viability of SECl#15-1 in a dose-dependent manner. However, lactoferrin showed no effect on the growth of SECl#15-1 in liquid culture with LB or M9 medium, suggesting that it exerts bactericidal effects under dry but not under wet conditions. Besides, *Salmonella* spp. other than *S.* Emteritidis, such as *S.* Typhimurium, *S.* Oranienburg, *S.* Weltevreden, *S.* Johannesburg, and *S.* Infantis, also showed dry-resistance, which was significantly inhibited by lactoferrin and almost entirely by apolactoferrin. These results suggest that lactoferrin inhibits the acquisition of dry-resistance by *Salmonella* spp., suggesting that there is a possible use for lactoferrin in the control of *Salmonella* food-poisoning as an additive in dry food.

Keywords: dry-resistance, lactoferrin, *Salmonella*

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Morita, H.[*1], Toh, H.[*2], Oshima, K.[*3], Murakami, M.[*1], Taylor, T.D.[*2], Igimi, S., and Hattori, M.[*3]: **Complete genome sequence of probiotic *Lactobacillus rhamnosus* ATCC 53103**

J. Bacteriol., **191**, 7630-1631（2009）

Lactobacillus rhamnosus is a facultatively heterofermentative lactic acid bacterium and is frequently isolated from human gastrointestinal mucosa of healthy individuals. *L. rhamnosus* ATCC 53103, isolated from a healthy human intestinal flora, is one of the most widely used and well-documented probiotics. Here, we report the finished and annotated genome sequence of this organism.

Keywords: complete genome, probiotic, *Lactobacillus rhamnosus*

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Kajikawa, A., Masuda, K., Katoh, M., and Igimi, S.: **Adjuvant effects for oral immunization provided by recombinant Lactobacillus casei secreting biologically active murine interleukin-1 beta**

Clinical and Vaccine Immunology., **17**, 43-48 (2010)

Vaccine delivery systems using lactic acid bacteria are under development, but their efficiency is insufficient. Autologous cytokines, such as interleukin-1β (IL-1β), are potential adjuvants for mucosal vaccines and can be provided by recombinant lactic acid bacteria. The aim of this study was the construction and evaluation of recombinant *Lactobacillus casei* producing IL-1β as an adjuvant delivery agent. The recombinant strain was constructed using an expression/secretion vector plasmid, including a mature IL-1β gene from mouse. The biological activity of the cytokine was confirmed by IL-8 production from Caco-2 cells. In response to the recombinant *L. casei* secreting IL-1β, expression of IL-6 was detected in vivo using a ligated-intestinal-loop assay. The release of IL-6 from Peyer's patch cells was also detected in vitro. Intragastric immunization with heat-killed *Salmonella enterica* serovar Enteritidis (SE) in combination with IL-1β-secreting lactobacilli resulted in relatively high SE-specific antibody production. In this study, it was demonstrated that recombinant *L. casei* secreting bioactive murine IL-1βprovided adjuvant effects for intragastric immunization.

Keywords: *Lactobacillus*, cytokine, recombinant

Tanaka, Y.[*1], Takahashi, H.[*1], Imai, A.[*1], Asao, T.[*2], Kozaki, S.[*2], Igimi, S., and Kimura, B.[*1]: **Reconsideration of flexibility in verifying rapid alternative food microbiological methods**

Food Control, **21**, 1075-1079 (2010)

Method comparison criteria for validating novel microbiology methods are discussed for aerobic plate count of fish samples using two rapid alternative methods (dissolved oxygen and automated MPN methods) and a reference method (AOAC 966.23, pour plate method). Results revealed that the present AOAC and ISO 16140 criteria such as requiring strict consistency in pairwise comparison may lead to overly strict validation disallowing advantageous rapid alternative methods in food microbiology. Pairwise comparison was not suitable for validating alternative microbiology methods, but the linear regression analysis followed by checking the fit to an ideal regression line was appropriate and easy-to-understand for validating the tested rapid alternative microbiology methods. Further discussion on an international level would benefit the development and use of rapid alternative methods.

Keywords: rapid methods, microbiology, validation

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Kajikawa, A., Ichikawa, E., and Igimi, S.: **Development of a Highly Efficient Protein-secreting System in Recombinant Lactobacillus casei**

Journal of Microbiology and Biotechnology, **20**, 375-382 (2010)

The available techniques for heterologous protein secretion in *Lactobacillus* strains are limited. The aim of the present study was to develop an efficient protein-secretion system using recombinant lactobacilli for various applications such as live delivery of biotherapeutics. For the construction of expression vectors, the *Lactobacillus brevis* slpA promoter, *Lactobacillus casei* prtP signal sequence, and mouse IL-10 sequences were used as a model system. Interestingly, the slpA promoter exhibited strong activity in L. casei, contrary to previous observations. In order to stabilize replication of the plasmid in *E. coli*, a removable terminator sequence was built into the promoter region. For the improvement of secretion efficiency, a DTNSD oligopeptide was added to the cleavage site of signal peptidase. The resulting plasmids provided remarkably efficient IL-10 secretion. Accumulation of the protein in the culture supernatant varied widely according to the pH conditions. By analysis of the secreted protein, formation of homodimers, and biological activity, IL-10 was confirmed to be functional. The presently constructed plasmids could be useful tools for heterologous protein secretion in *L. casei*.

Keywords: vector, *Lactobacillus*, recombinant

Kajikawa, A. and Igimi, S.: **Innate and acquired immune responses induced by recombinant *Lactobacillus casei* displaying flagellin-fusion antigen on the cell-surface**

Vaccine, **28**, 3409-3415 (2010)

Bacterial flagellins are known as antigens that induce innate immune responses through TLR5 and boost immune responses in combination with other antigens. The aim of the present study was to determine the immunological properties of recombinant *Lactobacillus casei* producing flagellin and flagellin-fusion antigens in vitro and in vivo. Recombinant lactobacilli expressing

Salmonella FliC and FliC fused to truncated SipC on the cell-surface were constructed. Fusion and non-fusion flagellin associated with *L. casei* retained the ability to induce IL-8 production by Caco-2 cells. Immunization of mice with these recombinant strains induced antigen-specific antibodies and cytokine production. The results showed that the outside epitope of the heterologous antigen was recognized more easily by the immune system than the inside epitope. The immune responses elicited by the *Lactobacillus*-associated antigens were mainly Th1 while that by the soluble antigen was Th2, although some of the responses were mixed.

Keywords: immune responses, *Lactobacillus*, recombinant

Suzuki, H.: **Differences in Intraepithelial Lymphocytes in the Proximal, Middle, Distal Parts of Small Intestine, Cecum, and Colon of Mice**

Immunological Investigations, **38**, 780-796（2009）

We have previously reported the regional differences in the intraepithelial lymphocytes（IELs）present in the small intestine of mice. In this study, we further investigated these differences on the basis of our previous findings and studied the entire intestine, including the cecum and colon. Most of the significant differences in phenotypic compositions were found between the small and large intestines, although some differences were found among the different parts of the small and large intestines. In particular, the composition of the subsets in $\alpha\beta$ T cells and $\gamma\delta$ T cells clearly differed between the small and large intestines. For example, in $\alpha\beta$ T cells, the percentages of double negative（DN）and CD8$\alpha\alpha^+$ cells were higher in the large intestine, that of CD8 $\alpha\beta^+$ cells was higher in the small intestine, and those of $CD4^+$ and $CD4^+$ CD8alphaalpha(+) double positive（DP）cells were higher in the distal part of the small intestine. In gammadelta T cells, the percentage of CD $\alpha\alpha^+$ cells was higher in the small intestine and that of DN cells was higher in the large intestine. These results indicate that the differences between IELs in the small and large intestines are discontinuous.

Keywords: IEL, small intestine, large intestine

川崎 勝*, 町井研士：**冷凍ホタテホモジネート中の遊離脂肪酸の測定について**

秦野研究所年報，**32**，9-13（2009）

下痢性貝毒マウスバイオアッセイ精度管理調査用試料を安定供給するうえの問題点の一つである，遊離脂肪酸の生成に関し測定法の検討，及び，資料中の生成状況について調査を行なった．その結果，簡便かつ精度の良い，遊離脂肪酸の測定が可能となった．

Keywords: diarrhetic shellfish poison, reference material for quality assurance test, free fatty acid

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Tanaka, H., Takino, M., Sugita-Konishi, Y., Tanaka, T., Toriba, A., Hayakawa, K.: **Determination of nivalenol and deoxynivalenol by liquid chromatography/atmospheric pressure photoionization mass spectrometry**

Rapid Commun. Mass Spectrom., **23**(19), 3119-3124（2009）

Fusarium species, a plant pathogenic fungus of wheat and other cereals, produces toxic metabolites such as nivalenol（NIV）and deoxynivalenol（DON）. Control of contamination by these toxins is very difficult, and a continuous survey of the occurrence is necessary for these toxins. Thus, the accurate and convenient determination of the cereals contaminated with these toxins is important for the supply of safe foods. A selective analytical method based on high-performance liquid chromatography, combined with atmospheric pressure photoionization（APPI）mass spectrometry, has been developed for simultaneous determination of NIV and DON. The parameters investigated for the optimization of APPI were the ion source parameters fragmentor voltage, capillary voltage, and vaporizer temperature, and also mobile phase composition and flow rate. Furthermore, chemical noise and signal suppression of analyte signals due to sample matrix interference were investigated for APPI. The results indicated that APPI provides lower matrix effect and the correlation coefficient of NIV and DON in the range 0.2-100 ng x mL(-1) was above 0.999. Recoveries of NIV and DON in wheat ranged from 86 to 107% and limits of detection of NIV and DON were 0.20 ng x g(-1) and 0.39 ng x g(-1), respectively. In addition, the proposed method was applied for the analysis of naturally contaminated wheat samples. APPI was found to offer lower matrix effect and was a convenient technique for routine analysis of NIV and DON residues in wheat

at trace levels. Copyright (c) 2009 John Wiley & Sons, Ltd.
Keywords: deoxynivalenol, nivalenol, APPI, LC/MS/MS

Mizutani, K., Kumagai, S., Mochizuki, N., Kitagawa, Y., Sugita-Konishi, Y.: **Determination of a yellow rice toxin, luteoskyrin, in rice by using liquid chromatography-tandem mass spectrometry with electrospray ionization**
J. Food Prot., **72**(6), 1321-6 (2009)

Penicillium islandicum produces luteoskyrin (LUT), a yellow rice toxin that has been found frequently in rice. However, conventional analytical methods for determining LUT are limited, are complicated, and exhibit low sensitivity. In this study, an analytical method more sensitive and simple based on high-performance liquid chromatography combined with electrospray ionization mass spectrometry was developed. The cleanup procedure of the method was one step, using a solid-phase extraction cartridge. An isocratic mobile-phase system, consisting of acetonitrile-water-acetic acid (50:49:1 [vol/vol/vol]) at a flow rate of 0.2 ml/min, was utilized to obtain the best resolution. Our method showed good linearity (r = 0.9993, 0.5 to 50 ng/g) and high repeatability (relative standard deviation = 8.9 and 5.1% at levels of 0.5 and 10 ng/g, respectively) in the fortification test. The detection and quantification limits for the method in multiple-reaction monitoring mode were 0.1 and 0.3 ng/g, respectively. The average recovery of LUT in spiked rice at 0.5 and 10 ng/g was 80.7 and 85.2%, respectively. The method developed in this study should be applicable to survey LUT in rice,
Keywords: luteoskyrin, HPLC, LC/MS/MS

Tanaka, T., Sugita-Konishi, Y., Takino, M.[*1], Tanaka, T.[*2], Toriba, A.[*3], Hayakawa, K.[*3]: **A Survey of the Occurrence of *Fusarium* Mycotoxins in Biscuits in Japan by Using LC/MS**
J. of Health Science., **56**(2), 188-194 (2010)

By adopting a rapid and sensitive method for simultaneous detection of nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (FX), 3-acetyl deoxinivalenol (3ADON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenone (Zen), the natural occurrence of these mycotoxins in biscuits made of wheat (201 samples) in Japan was surveyed. Samples were analyzed by LC/MS with atmospheric pressure photo ionization (APPI). Further confirmation was performed by liquid chromatography/time of flight mass spectrometry (LC/TOFMS). The average contamination of each *Fusarium* mycotoxin was 3.1, 23, 0.7, 0.1 and 4.2ng/g for NIV, DON, HT-2, T-2 and ZEN, respectively. Multiple toxins were observed in 120 samples while FX and 3ADON were not detected. The incidence of these toxins was 41% for NIV, 98% for DON, 19% for HT-2, 11% for T-2 and 2% for ZEN. There were no significant differences in the concentration and incidence between conventional biscuits made of wheat and biscuits made of wheat for infants. This is the first report concerning the presence of NIV, DON, HT-2, T-2 and ZEN in biscuits in Japan.
Keywords: *Fusarium* mycotoxin, contamination survey, LC/MS, LC/time of flight mass spectrometry, biscuit, Japan

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Ohnishi, T., Muroi, M.[*], Tanamoto, K.[*]: **Inhibitory effects of soluble MD-2 and soluble CD14 on bacterial growth**
Microbiology and Immunology, **54**, 74-80 (2010)

We studied the effect of the soluble forms of the endotoxin receptor molecules MD-2 (sMD-2) and CD14 (sCD14) on bacterial growth. When *Escherichia coli* and *Bacillus subtilis* were incubated at 37°C for 18 h with either sMD-2 or sCD14, growth of these bacteria was significantly inhibited as evaluated by viable cell counts and NADPH/NADH activity. A mutant of sCD14 (sCD14d57-64) lacking a region essential for LPS binding did not inhibit the growth of *E. coli*, whereas the mutant inhibited the growth of *B. subtilis*. Addition of excess peptidoglycan (PG) in the bacterial culture reversed the inhibitory effect of sMD-2 on the growth of *B. subtilis*, but not on the growth of *E. coli*. Furthermore, when evaluated by ELISA, both sMD-2 and sCD14 bound specifically to PG. Taken together, these results indicate that sMD-2 and sCD14 inhibit the growth of both Gram-positive and Gram-negative bacteria and further suggest that the binding to PG

and LPS is involved in the inhibitory effect of sMD-2 on Gram-positive bacteria and of sCD14 on Gram-negative bacteria, respectively.

Keywords: Endotoxin, LPS, MD-2, CD14

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宮原美知子，田口真澄[*1]，久米田裕子[*1]，神吉政史[*1]，郡司明彦[*2]，森田友美[*2]，太田順司[*2]，高山正彦[*2]，高須一重[*2]，木股裕子[*3]，塚本定三[*1]：**食品からの改良サルモネラ検出法の検討と鶏挽肉および未殺菌液卵でのその評価**

日本食品微生物学会誌，**26**，107-113（2009）

サルモネラは食中毒事件発生を引き起こす件数が日本では年々減少しているが，現在でも危険な食中毒原因菌である．新しい食品からのサルモネラ検出法を検討した．新しいといっても，1998年11月25日に通知された未殺菌液卵の検査法を基に変えたものである．硫化水素産生と非産生の２種類のサルモネラを使って検出感度を検討した．硫化水素産生性のサルモネラでは３個接種での検査であったが，検出することができた．硫化水素非産生株での接種実験では，７個の接種で検出が可能であった．鶏挽肉の汚染率は40/70であったが，汚染菌数はほとんどがMPN 0.3以下/gであった．一方，未殺菌卵液でのサルモネラの汚染率は8/20であったが，汚染菌数は鶏挽肉と比較して格段に高かった．鶏挽肉では*S.* Infantisが，未殺菌液卵では*S.* Enteritidisが主な汚染サルモネラ血清型であった．日本では，*S.* Enteritidisが主なサルモネラ食中毒発生原因血清型であることから，卵に由来するサルモネラが日本の食中毒の主な原因であると推定される．卵のサルモネラ検査は今も今後も検査する必要があると思われる．新しい検査法は鶏挽肉や未殺菌液卵サルモネラ検査に使えることが分かり，少数サルモネラ検出にも充分であることが分かった．

Keywords: サルモネラ検出法，鶏挽肉，未殺菌液卵

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Kimura, J.[*1], Abe, H.[*1], Kamitani, S.[*1], Toshima, H.[*1], Fukui, A.[*1], Miyake, M.[*2], Kamata, Y., Sugita-Konishi Y., Yamamoto, S., Horiguchi, Y.[*1]: ***Clostridium perfringens* Enterotoxin Interacts with Claudins via Electrostatic Attraction**

J. Biol. Chem., **285**, 401-408（2010）

食中毒の原因物質である*Clostridium perfringens*腸管毒素（CPE）は標的細胞の原形質膜の選択的透過性を乱す孔形成毒素であり，結果として細胞は死ぬ．以前，我々はCPEの細胞表面のレセプターとしてクローディンを証明した．タイトジャンクションの構成成分であるクローディンは４回膜貫通蛋白質で20種以上の巨大なファミリーを成しており，それらの全てがCPEのレセプターとして働くわけではない．毒素が感受性クローディンを見分けるメカニズムは不明である．本研究で我々はCPEとの相互作用に関与するクローディンの領域が第２細胞外ループにあること突き止め，感受性クローディンにおけるこの領域の等電点が非感受性クローディンより高いことを発見した．等電点が上昇するようなアミノ酸置換はCPE非感受性クローディンに感受性を授ける一方で，等電点が低下するようなアミノ酸置換は感受性クローディンの間でCPEへの感受性低下をもたらす．CPEのクローディン結合ドメインの立体構造は306番チロシン，310番チロシン，312番チロシン，315番ロイシンによって囲まれた酸性の裂け目を明らかになり，感受性クローディンとの相互作用に必須であると報告された．これらの結果は塩基性のクローディンの領域と酸性のCPEの裂け目の間の静電気引力がこれらの相互作用に関係することを意味している．

Keywords: *Clostridium perfringens*, Enterotoxin, Claudins, Cytotoxicity, Receptors

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工藤由起子，後藤慶一[*1]，尾上洋一[*2]，渡辺麻衣子，李　謙一[*3]，熊谷　進[*3]，小西良子，大西貴弘：**清涼飲料水における微生物を原因とする苦情事例の解析**

食品衛生学雑誌，**50**，315-320（2009）

全国地方自治体に行った消費者からの清涼飲料水の微生物に関連する苦情の調査結果において，茶系飲料と果汁飲料で苦情事例が多く，果汁飲料は生産量に比して発生頻度が高かった．開封前の事例では流通時での容器の破損，開封後では消費者の消費方法が微生物汚染の原因になることが示された．汚染微生物の種類としてはカビが多いことが判明し，カビは制御の対象として重要であると考えられた．製造から消費までの必要な対応を考えると，製造工程では中小製造者の支援，流通過程では製造者による容器の破損防止のための運送・販売業者の啓発，消費では適切な消費方法についての消費者の啓発が

必要であると思われた．これらの支援および啓発によって，清涼飲料水の苦情を減らすことが可能と思われた．
Keywords: soft drink, complain, microbial contamination

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野田裕之[*1]，千須和美母衣[*1]，金子通治[*1]，尾上洋一[*2]，高鳥浩介[*3]，工藤由起子：**ブラックタイガーエビに接種した*Salmonella* WeltevredenおよびS. Senftenbergの冷凍保存下における生残性**

食品衛生学雑誌，**50**，86-90（2009）

冷凍流通している輸入エビのサルモネラ汚染が判明したことから，冷凍保存下のエビにおけるサルモネラの生残性を検討した．ブラックタイガーに血清型WeltevredenおよびSenftenbergを体表および体内の2方法で接種した．冷凍保存温度は－10℃，－20℃および－30℃の３温度で，保存期間は12週間とした．その結果，冷凍保存温度が低下するほど，また，体表接種より体内接種の方がサルモネラの生残性が高かった．さらに，*S.* Senftenbergは*S.* Weltevredenより高い生残性を示した．サルモネラはエビで冷凍保存された場合，特に－30℃ではその接種菌数が長期間維持されることが確認できたことから，エビを解凍する時にはサルモネラの存在も考慮に入れ，衛生的な取扱いが必要である．
Keywords: *Salmonella*, Survival, Shrimp

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Nemoto, J.[*1], Sugawara, C.[*1], Akahane, K.[*1], Hashimoto, K.[*1], Kojima, T.[*1], Ikedo, M.[*1], Konuma, H.[*2] and Hara-Kudo, Y.: **Rapid and specific detection of the thermostable direct haemolysin gene in *Vibrio parahaemolyticus* by Loop-mediated isothermal amplification**

J. Food Prot., **72**, 748-754（2009）

Several investigators have reported that thermostable direct haemolysin（TDH）and TDH-related hemolysin（TRH）are important virulence factors of *Vibrio parahaemolyticus*, but it has been difficult to detect these factors rapidly in seafood and other environmental samples. A novel nucleic acid amplification method, termed the loop-mediated isothermal amplification（LAMP）, which amplifies DNA with high specificity and rapidity under isothermal conditions, was applied. In this study, we designed *tdh* gene-specific LAMP primers for detection of TDH-producing *V. parahaemolyticus*. The specificity of this assay was evaluated with 32 strains of TDH-producing *V. parahaemolyticus*, one strain of TDH-producing *Grimontia hollisae*, 10 strains of TDH-non-producing *V. parahaemolyticus* and 94 strains of TDH-non-producing bacteria, and the sensitivity was sufficient to detect one cell per test. Moreover, to investigate the detection of TDH-producing *V. parahaemolyticus* in oysters, the LAMP assay was performed on enrichment culture in alkaline peptone water of oyster samples inoculated with TDH-producing *V. parahaemolyticus*, TDH-non-producing *V. parahaemolyticus* and *V. alginolyticus* after enrichment in alkaline peptone water. These results suggest that the LAMP assay targeting *tdh* gene has high sensitivity and specificity and is useful to detect TDH-producing *V. parahaemolyticus* in oyster after enrichment.
Keywords: *Vibrio parahaemolyticus*, thermostable direct hemolysin, TDH

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山﨑省吾[*1, 2]，右田雄二[*1]，中村まき子[*1]，浦　伸孝[*1]，工藤由起子，三澤尚明[*2, 3]，岡本嘉六[*2, 4]，髙瀬公三[*2, 4]：**長崎県沿岸における*Vibrio vulnificus*の分布と環境因子**

日本獣医師会雑誌，**62**，649-655（2009）

長崎県沿岸の漁港７地点(有明海５地点，橘湾２地点)における海水中の*Vibrio vulnificus*（*V.v*）菌数をMPN-PCR法を用いて測定し，その菌数の増減に及ぼす各種環境因子（水温，塩分濃度，DO，COD，総窒素量，総リン量，クロロフィルａ量）の影響を明らかにするため両者の順位相関を検討した．海水中の*V.v*の最高値は2.4×10^6MPN/100mℓ示し，環境因子の中で水温，塩分濃度およびDOと菌数の間に相関を認め，その相関値は，それぞれr_s=0.714，r_s=－0.712およびr_s=－0.462であった．*in vitro*の実験においても，DO濃度が低いほど増殖能の高いことが判明した．また，*V.v*の同地域由来魚介類からの分離率は，二枚貝類で90.5%，および魚類で79.2%を示し，その菌数の最高値は10^5MPN/gであった．
Keywords: seawater, prevalence, *Vibrio vulnificus*

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Ui, J.*1, Kondo, K.*, Sawada, T.*2 and Hara-Kudo, Y.: **Survival of foodborne pathogens in grain products and the effect of catechins**

J. Food Hyg. Soc. Jpn., **50**, 126-130（2009）

穀類加工品中の食中毒細菌の生残について検討した．*Salmonella Enteritidis*と*Staphylococcus aureus*は穀類フレーク中で３週間以上生残した．*S.* Enteritidis は穀類フレーク中で7または14日保存後に10^2 CFU/g以下に減少したが，生残した菌は牛乳中で急速に増殖し25時間後には10^9CFU/gに至った．また，カテキン溶液に浸漬後の穀物フレークでは，*S.* Enteritidisおよび*S. aureus*の菌数の減少が認められた．さらに，カテキンの穀類調理品への添加によって*S.* Enteritidis，*S. aureus*および*Bacillus cereus*の生残または増殖が抑制された．以上のように，本研究では*S.* Enteritidis，*S. aureus*および*B. cereus*は 穀類加工中で長期に生残が可能であり，カテキンがそれら食中毒細菌の生残と増殖を抑制することが明らかになった．

Keywords: foodborne pathogens, grain products, catechins

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小沼ルミ*1，渡辺麻衣子，工藤由起子，小西良子，瓦田研介*1，高鳥浩介*2：**糸状菌の流動パラフィン重層法による長期保存後の生存性**

防菌防黴，**38**，75-80（2010）

流動パラフィン重層法を用いて20年以上保存されていた糸状菌12属64種222株（主に*Aspergillus*属，*Penicillium*属および*Fusarium*属）について菌株を継代培養し，生存性を評価した．菌株は集落形成および胞子形成を肉眼および顕微鏡による観察などから確認した．その結果，(1)生存性は供試した12属の間で異なった；(2)生存率は*Aspergillus* 属 で 64%，*Fusarium* 属 で 60% お よ び *Penicillium*属で22%の順に高かった；(3)*Aspergillus*属および*Fusarium*属では30年以上生存した菌種が認められた；(4)*Penicillium*属と好稠菌類では30年以上生存した菌株はなかった；(5)同じ属であっても菌種によって生存率は異なった；以上のことが認められた．これらのことから，流動パラフィン重層法は簡便であるが，長期保存には凍結乾燥法など他の方法も併せて用いることが必要と考えられた．

Keywords: Mineral oil method, Filamentous fungi, Preservation

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Aoyama, K.*1, Nakajima, M.*2, Tabata, S.*3, Ishikuro, E.*4, Tanaka, T.*5, Norizuki, H.*6, Itoh, Y.*7, Fujita, K.*4, Kai, S.*8, Tsutsumi, T.*6, Takahashi, M.*9, Tanaka, H.*10, Iizuka, S.*4, Ogiso, M.*4, Maeda, M.*11, Yamaguchi, S.*11, Sugiyama, K., Sugita-Konishi, Y. and Kumagai, S.*12: **Four-year surveillance for ochratoxin A and fumonisins in retail foods in Japan**

J Food Prot., **73**, 344-352（2010）

Between 2004 and 2007 we examined foods from Japanese retail shops for contamination with ochratoxin A（OTA）and fumonisins B1, B2, and B3. A total of 1,358 samples of 27 different products were examined for OTA, and 831 samples of 16 different products were examined for fumonisins. The limits of quantification ranged from 0.01 to 0.5μg/kg for OTA and 2 to 10μg/kg for the fumonisins. OTA was detected in amounts higher than limits of quantification in wheat flour, pasta, oatmeal, rye, buckwheat flour and dried buckwheat noodles, raisins, wine, beer, coffee beans and coffee products, chocolate, cocoa, and coriander. OTA was found in more than 90%of the samples of instant coffee and cocoa, and the highest concentration of OTA, 12.5μg/kg, was detected in raisins. The concentration of OTA in oatmeal, rye, raisins, wine, and roasted coffee beans varied remarkably from year to year. Fumonisins were detected in frozen and canned corn, popcorn grain, corn grits, cornflakes, corn soups, corn snacks, beer, soybeans, millet, and asparagus. The highest concentrations of fumonisins B1, B2, and B3 were detected in corn grits（1,670, 597, and 281μg/kg, respectively）. OTA and fumonisins were detected in several food products in Japan; however, although Japan has not set regulatory levels for these mycotoxins, their concentrations were relatively low.

Keywords: ochratoxin A, fumonisins, retail foods

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Sugita-Konishi, Y., Sato, T.*1, Saito, S.*1, Nakajima, M.*2, Tabata, S.*3, Tanaka, T.*4, Norizuki, H.*5, Itoh, Y.*6, Kai, S.*7, Sugiyama, K., Kamata, Y., Yoshiike, N.*8 and Kumagai, S.*9: **Exposure to aflatoxins in Japan: Risk assessment for aflatoxin B1**

Food Addit. Contam. Pt. A Chem. Anal. Control Expo. Risk Assess., **27**, 365-372 (2010)

The intake of total aflatoxins (AFT) and aflatoxin B_1 (AFB_1) from food in Japan was estimated from AFT and AFB_1 concentration and frequency data in 24 foods (884 samples) from a 3-year retail market survey from the summer of 2004 to the winter of 2006, and by food consumption data from the National Health and Nutrition Survey performed in 2005. The AFT and AFB_1 survey revealed that peanut, peanut products, cocoa, chocolate, pistachio, white pepper, red pepper, almond, job's tears, buckwheat and corn grits are considered to be contributors of AFT (or AFB_1) intake in Japan (maximum AFB_1 (AFT) levels ranged from 0.21 to 28.0 microg kg^{-1} (from 0.21 to 9.0 microg kg^{-1})) in AFT-contaminated food. A probabilistic approach using the Monte Carlo method was carried out to simulate an estimate of the AFT (or AFB_1) intake distributions in each age group in Japan. In this study, AFB_1 intake ranged from 0.003 to 0.004 ng kg^{-1} body weight day^{-1} (from lower to upper limits), and the potential risk for cancer using a formula devised by the Joint Food and Agricultural Organization/World Health Organization Expert Committee on Food Additives was estimated at 0.00004-0.00005 person/year/100,000 persons, even though this was in the higher levels (95.0th percentile) of the consumer population. The results suggest that the current dietary intake of AFB^{-1} in Japan has no appreciable effect on health.

Keywords: aflatoxins, aflatoxin B_1, risk assessment

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Sugiyama, K., Muroi, M.*1, Tanamoto, K.*1, Nishijima, M.*2 and Sugita-Konishi, Y.: **Deoxynivalenol and nivalenol inhibit lipopolisaccharide-induced nitric oxide production by mouse macrophage cells**

Toxicol. Lett., **192**, 150-154 (2010)

Deoxynivalenol (DON) and nivalenol (NIV), trichothecene mycotoxins, are secondary metabolites produced by Fusarium fungi. Trichothecene mycotoxins cause immune dysfunction, thus leading to diverse responses to infection. The present study evaluated the effect of DON and NIV on nitric oxide (NO) production by RAW264 cells stimulated with lipopolisaccharide (LPS). LPS-induced NO production was reduced in the presence of these toxins. The transcriptional activation and expression of inducible NO synthase (iNOS) by LPS were also repressed by these toxins. DON or NIV inhibited LPS-induced expression of interferon-β (IFN-β). These results indicate that DON and NIV inhibit the LPS-induced NO and IFN-β production, which both play an important role for host protection against invading pathogens, and suggests that the inhibition of these factors may be involved in the immunotoxic effects of these mycotoxins.

Keywords: deoxynivalenol, nivalenol, lipopolisaccharide, nitric oxide

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Fukuhara, K., Nakanishi, I.*1, Ohkubo, K.*2, Obara, Y.*3, Tada, A.*4, Imai, K.*4, Ohno, A., Nakamura, A.*4, Ozawa, T.*5, Urano, S.*4, Saito, S.*3, Fukuzumi, S.*2, Anzai, K.*1, Miyata, N.*6, Okuda, H.: **Intramolecular base-accelerated radical-scavenging reaction of a planar catechin derivative having a lysine moiety**

Chem. Comm., **6180-6182** (2009)

A planar catechin derivatized from naturally-occurring

(+)-catechin was previously shown to have 5-fold increased radical-scavenging activity. The radical-scavenging reaction of phenolic antioxidants is significantly accelerated by the presence of a base. Here, a planar catechin derivative (PCL) having a lysine moiety as a base was synthesized in order to develop a stronger antioxidant which could be developed as a preventative agent for oxidative stress related disease. The scavenging rate constant of galvinoxyl radical (GO·) by PCL was 400-fold larger than that by (+)-catechin, determined by the stopped-flow technique. Strong hydrogen bonding between the amino group in the lysine moiety and the OH-group in the catechol moiety stabilizes the radical cation intermediate ($PCL^{\cdot+}$) generated in the one electron oxidation of PCL by radical scavenging GO·. Such stabilization significantly enhances the radical scavenging activity of PCL.

Keywords: catechin, antioxidant, polyphenol

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Ohno, A., Kawasaki, N., Fukuhara, K., Okuda, H., Yamaguchi, T.: **Time-dependent changes of oxytocin using ^{1}H-NMR coupled with multivariate analysis: A new approach for quality evaluation of protein/peptide biologic drugs**

Chem. Pharm. Bull., **57**, 1396-1399 (2009)

A new method that combines ^{1}H-NMR and principal component analysis (PCA) was employed to obtain the quality evaluation of biopharmaceuticals, with regard to their quality, consistency, and differences in protein modification patterns. To assess the feasibility of the method, three ^{1}H-NMR spectra of oxytocin (OXT) were collected every 7 d (at Day 0, 7 and 14), and time-dependent changes in the spectra were found by PCA of the ^{1}HNMR signals from 0.5－9.0 ppm, excluding the region around the water signal (4.6－5.0 ppm). Although the three OXT spectra seemed similar by simple visual inspection, time-dependent differences among the three spectra were clearly distinguished by a PCA scores plot. Peak changes indicating both OXT decomposition and the emergence of new OXT decomposition products within the timeframe of the experiment were also observed by a PCA loading plot. The results demonstrate that this method can evaluate the consistency of biopharmaceutical quality.

Keywords: quality evaluation, biologic drug, principal component analysis, ^{1}H-NMR, oxytocin

Fukuhara, K., Ohno, A., Nakanishi, I.*1, Imai, K.*2, Nakamura, A.*2, Anzai, K.*1, Miyata, N.*3, Okuda, H.: **Novel ninhydrin adduct of catechin with potent antioxidative activity**

Tetrahedron Letters, **50**, 6989-6992 (2009)

The reaction of ninhydrin with (+)-catechin in the presence of TMSOTf resulted in condensation product 1, which consists of a 2:1 mixture of epimers at the C2 position. The antioxidative radical scavenging activity of 1 against the galvinoxyl radical, acting as an oxyl radical, was significantly enhanced compared to (+)-catechin. Our results offer a new method for chemical modification of a natural phenolic antioxidant.

Keywords: catechin, antioxidant, polyphenol

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Ohno, A., Kawasaki, N., Fukuhara, K., Okuda, H., Yamaguchi, T.: **Complete NMR analysis of oxytocin in phosphate buffer**

Magn. Reson. Chem., **48**, 168-172 (2010)

Complete NMR analysis of oxytocin (OXT) in phosphate buffer was elucidated by one-dimensional (1D)- and two-dimensional (2D)-NMR techniques, which involve the assignment of peptide amide NH protons and carbamoyl NH_2 protons. The $^1H-^{15}N$ correlation of seven amide NH protons and three carbamoyl NH_2 protons were also shown by HSQC NMR of OXT without ^{15}N enrichment.

Keywords: oxytocin, ^{1}H NMR, ^{13}C NMR, ^{15}N NMR, phosphate buffer

Hishikawa, K.*, Nakagawa, H.*, Furuta, T.*, Fukuhara, K., Tsumoto, H.*, Suzuki, T.*, Miyata, N.*: **Multiple bond-conjugated photoinduced nitric oxide releaser working with two-photon excitation**

Bioorg. Med. Chem. Lett., **20**, 302-5 (2010)

Four novel nitric oxide (NO) releasers working via

two-photon excitation (TPE), based on an acceptor-donor-acceptor (A-D-A) molecular design, were synthesized. Their decomposition and NO release in response to one-photon excitation, and their decomposition in response to two-photon excitation were examined. Their photoinduced decomposition characteristics are discussed.

Keywords: nitric oxide, stilbene, photon excitation

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Oba, M.[*1], Demizu, Y., Yamagata, N., Sato, Y., Doi, M.[*2], Tanaka, M.[*3], Suemune, H.[*1], Okuda, H., Kurihara, M.: **Solid-state Conformation of Diastereomeric -Pro-Pro-(Aib)$_4$ Sequences**

Tetrahedron, **66**, 2293-2296 (2010)

The crystal structures of two diastereomeric -Pro-Pro-(Aib)$_4$- sequences, Cbz-L-Pro-L-Pro-(Aib)$_4$-OMe (1) and Cbz-D-Pro-L-Pro-(Aib)$_4$-OMe (2), have been determined by X-ray crystallographic analysis. The crystals of the two compounds were characterized by the following parameters: (1) monoclinic, $P2_1$, a = 10.543 Å, b = 8.103 Å, c = 22.642 Å, β = 97.679, Z = 2, R_1 = 0.104, and R_w = 0.327; (2) orthorhombic, $P2_12_12_1$, a = 10.470 Å, b = 10.953 Å, c = 32.405 Å, Z = 4, R_1 = 0.040, and R_w = 0.046. In the asymmetric unit of 1, the homochiral L-Pro^1-L-Pro^2 adopts a polyproline II structure, which induces a left-handed (M) 3_{10}-helical structure in the following-(Aib)$_4$-sequence. The preferred conformation of diastereomeric 2, which contains heterochiral D-Pro^1-L-Pro^2 segments, was similar to that of 1 with differences at the N-terminal D-Pro residue

Keywords: peptide, helix, secondary structure

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Demizu, Y., Yamagata, N., Sato, Y., Doi, M.[*1], Tanaka, M.[*2], Okuda, H., Kurihara, M.: **Controlling the Helical Screw Sense of Peptides with C-Terminal L-Valine**

J. Pept. Sci., **16**, 153-158 (2010)

One chiral L-valine (L-Val) was inserted into the C-terminal position of achiral peptide segments constructed from α-aminoisobutyric acid (Aib) and α,β-dehydrophenylalanine (Δ^ZPhe) residues. The IR, ^{1}H NMR, and CD spectra indicated that the dominant conformations of the pentapeptide Boc-Aib-ΔPhe-(Aib)$_2$-L-Val-NH-Bn 3 and the hexapeptide Boc-Aib-ΔPhe-(Aib)$_3$-L-Val-NH-Bn 4 in solution were both right-handed (P) 3_{10}-helical structures. X-ray crystallographic analyses of 3 and 4 revealed that only a right-handed (P) 3_{10}-helical structure was present in their crystalline states. The conformation of 4 was also studied by molecular-mechanics calculations.

Keywords: α-aminoisobutyric acid, α,β-dehydrophenylalanine, conformational analysis

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Kurihara, M., Sato, Y., Yamagata, N., Demizu, Y., Okuda, H., Nagano, M.[*1], Doi, M.[*2], Tanaka, M.[*3], Suemune, H.[*1]: **Computational Study on Helical Structure of Chiral α,α-Disubstituted Oligopeptides**

Peptide Science 2009, **384-385** (2010)

Prediction of the conformation of oligopeptides using computational simulation presents an interesting challenge to design functionalized and bioactive peptides. Computational simulation using conformational search calculations with AMBER* force field is most useful for conformational analysis of oligopeptides containing α,α-disubstituted α-amino acids.

Keywords: α,α-disubstituted α-amino acid, oligopeptide, conformational search

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Yamagata, N., Demizu, Y., Sato, Y., Oba, M.[*1], Tanaka, M.[*2], Doi, M.[*3], Nagasawa, K.[*4], Suemune, H.[*1], Okuda, H., Kurihara, M.: **Controlling the Helical Screw Sense of Peptides by N-Terminal Proline**

Peptide Science 2009, **383-384** (2010)

Three types of N-blocked peptides; each containing achiral α-aminoisobutyric acid (Aib) residues and chiral L-Pro, L-Pro-L-Pro, or D-Pro-L-Pro residues in the N-terminal position of the sequence, have been synthesized by solution-phase methods. The IR, CD, and NOESY spectra indicated that the dominant conformations of three peptides in solution were helical structures. The preferred secondary structures of each peptide were a left-handed (M) 3_{10}-helix in the crystal

state. The conformation of peptides was also studied by molecular mechanics calculations.

Keywords: α,α-disubstituted α-amino acid, proline, peptide conformation

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Demizu, Y., Tanaka, M.[*1], Suemune, H.[*2], Doi, M.[*3], Sato, Y., Okuda, H., Kurihara, M.: **Conformational Analysis of Water-soluble Oligopeptides Composed of Chiral Cyclic α,α-Disubstituted α-Amino Acids**

Peptide Science 2009, **381-382**（2010）

Chiral cyclic α,α-disubstituted amino acids; (*S,S*)-Ac_5c^{dOP} (P = MOM, H) were synthesized starting from dimethyl L-(+)-tartrate, and their homo-oligomers were prepared by solution-phase methods. The preferred secondary structure of (*S,S*)-Ac_5c^{dOMOM} homopeptide was a left-handed (*M*) 3_{10}-helix both in solution and in the crystal state. The (*S,S*)-Ac_5c^{dOH} hexapeptide could be well dissolved in water, and was more helical in water than in 2,2,2-trifluoroethanol solution.

Keywords: α,α-disubstituted α-amino acid, side-chain chiral center, peptide conformation

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Demizu, Y., Moriyama, A.[*], Onomura, O.[*]: **Non-enzymatic Kinetic Resolution of Racemic alpha-Hydroxyalkanephosphonates with Chiral Copper Catalyst**

Tetrahedron Lett., **37**, 5241-5244（2009）

Kinetic resolution of a-hydroxyalkanephosphonates was efficiently performed by benzoylation in the presence of copper (II) triflate and (*R,R*)-Ph-BOX as a catalyst with excellent s value of up to 286.

Keywords: kinetic resolution, asymmetric benzoylation, molecular recognition

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Kurihara, M., Sato, Y., Yamagata, N., Okuda, H., Nagano, M.[*1], Demizu, Y., Doi, M.[*2], Tanaka, M.[*3], 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 α-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, oligopeptide, conformational search

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Sugiyama, T.[*1], Ninomiya, K.[*2], Imamura, Y.[*3], Kurihara, M., Takano, M.[*4], Kittaka, A.[*4]: **Sequence-specific cleavage of DNA by peptide nucleic acids conjugated with metal complexes**

Peptide Science 2009, **425-426**（2010）

Two 12-base peptide nucleic acids (PNAs) conjugated with a bleomycin model and a bipyridine derivative (bpy) were synthesized and their DNA cleavage activity was evaluated. The bpy-PNA conjugate cleaved DNA in the presence of Cu^{2+} and reducing agent. Mass spectrometry was employed to determine the cleavage sites.

Keywords: antigene, DNA cleavage, peptide nucleic acid

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Tanaka, H.[*1], Hoshikawa, Y.[*2], Oh-Hara, T.[*1], Koike, S.[*1], Naito, M., Noda, T.[*2], Arai, H.[*3], Tsuruo, T.[*1] and Fujita, N.[*1]: **PRMT5, a Novel TRAIL Receptor-Binding Protein, Inhibits TRAIL-Induced Apoptosis via Nuclear Factor-{kappa}B Activation**

Mol. Cancer Res., **7**, 557-569（2009）

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and has selective antitumor activity.

Although TNF-alpha-induced intracellular signaling pathways have been well studied, TRAIL signaling is not fully understood. Here, we identified a novel TRAIL receptor-binding protein, protein arginine methyltransferase 5 (PRMT5), as a result of proteomic screening. PRMT5 selectively interacted with death receptor 4 and death receptor 5 but not with TNF receptor 1 or Fas. PRMT5 gene silencing sensitized various cancer cells to TRAIL without affecting TRAIL resistance in nontransformed cells. PRMT5 contributed to TRAIL-induced activation of inhibitor of kappaB kinase (IKK) and nuclear factor-kappaB (NF-kappaB), leading to induction of several NF-kappaB target genes. Although IKK inhibition increased sensitivity to both TRAIL and TNF-alpha, PRMT5 knockdown potentiated TRAIL-mediated cytotoxicity alone. PRMT5 had no effect on TNF-alpha-mediated NF-kappaB signaling. These results show the selectivity of PRMT5 for TRAIL signaling. The PRMT5 small interfering RNA-mediated susceptibility to TRAIL was rescued by ectopic expression of active IKKbeta, confirming the involvement of PRMT5 in TRAIL resistance by activating the NF-kappaB pathway. Collectively, our findings suggest the therapeutic potential of PRMT5 in TRAIL-based cancer treatments.
Keywords: TRAIL, apoptosis, NF-kB

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Sippel, M.[*1], Rajala, R.[*1], Korhonen, L.[*1], Bornhauser, B.[*1], Sokka, A.L.[*2], Naito, M. and Lindholm, D.[*1]: **Dexamethasone regulates expression of BRUCE/Apollon and the proliferation of neural progenitor cells**

FEBS Lett., **583**, 2213-2217 (2009)

Glucocorticoid hormones (GHs) regulate cell proliferation of neural progenitor cells (NPCs) contributing to reduction of neurogenesis after stress. We show here that dexamethasone (Dex) decreases BRUCE/Apollon (BRUCE) in cultured NPCs in a GH-receptor-dependent manner. Downregulation of BRUCE by Dex or using silencing RNA reduced the number of proliferating NPCs, whilst overexpression of BRUCE counteracted the effect of Dex. Dex also elevated the deubiquitinating enzyme, Usp8/Ubpy, which via Nrdp1 decreases BRUCE. The results show that BRUCE is a target for GHs in the NPCs, and that BRUCE controls cell division of NPCs and possibly of other stem cells.
Keywords: dexamethasone, Apollon, neural progenitor

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Katayama, R.[*1], Ishioka, T.[*2], Takada, S.[*3], Takada, R.[*3], Fujita, N.[*1], Tsuruo, T.[*1] and Naito, M.: **Modulation of Wnt signaling by the nuclear localization of cellular FLIP-L**

J. Cell Sci., **123**, 23-28 (2010)

Cellular FLIP (cFLIP) inhibits the apoptosis signaling initiated by death receptor ligation. We previously reported that a long form of cFLIP (cFLIP-L) enhances Wnt signaling via inhibition of beta-catenin ubiquitylation. In this report, we present evidence that cFLIP-L translocates into the nucleus, which could have a role in modulation of Wnt signaling. cFLIP-L has a functional bipartite nuclear localization signal (NLS) at the C-terminus. Wild-type cFLIP-L (wt-FLIP-L) localizes in both the nucleus and cytoplasm, whereas NLS-mutated cFLIP-L localizes predominantly in the cytoplasm. cFLIP-L also has a nuclear export signal (NES) near the NLS, and leptomycin B, an inhibitor of CRM1-dependent nuclear export, increases the nuclear accumulation of cFLIP-L, suggesting that it shuttles between the nucleus and cytoplasm. Expression of mutant cFLIP-L proteins with a deletion or mutations in the NLS and NES confers resistance to Fas-mediated apoptosis, as does wt-FLIP-L, but they do not enhance Wnt signaling, which suggests an important role of the C-terminus of cFLIP-L in Wnt-signaling modulation. When wt-FLIP-L is expressed in the cytoplasm by conjugation with exogenous NES (NES-FLIP-L), Wnt signaling is not enhanced, whereas the NES-FLIP-L increases cytoplasmic beta-catenin as efficiently as wt-FLIP-L. cFLIP-L physically interacts with the reporter plasmid for Wnt signaling, but not with the control plasmid. These results suggest a role for nuclear cFLIP-L in the modulation of Wnt signaling.
Keywords: Wnt signaling, FLIP-L, NLS

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Nakamura, A.[*1], Naito, M., Arai, H[*2]. and Fujita, N.[*1]: **Mitotic phosphorylation of Aki1 at Ser208 by cyclin B1-Cdk1 complex**

Biochem. Biophys. Res. Commun., **393**, 872-876 (2010)

Akt kinase-interacting protein 1 (Aki1)/Freud-1/CC2D1A is localized in the cytosol, nucleus, and centrosome. Aki1 plays distinct roles depending on its localization. In the cytosol, it acts as a scaffold protein in the phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway. In the nucleus, it is a transcriptional repressor of the serotonin-1A (5-HT1A) receptor. In the centrosome, it regulates spindle pole localization of the cohesin subunit Scc1, thereby mediating centriole cohesion during mitosis. Although the function of Aki1 has been well clarified, the regulatory machinery of Aki1 is poorly understood. We previously found that Aki1 in mitotic cells displayed reduced mobility on immunoblot analysis, but the reason for this was unclear. Here we show that the electrophoretic mobility shift of Aki1 is derived from mitotic phosphorylation. The cyclin B1-cyclin-dependent kinase 1 (Cdk1) complex was found to be one of the kinases responsible for Aki1 phosphorylation during mitosis. We identified the Ser (208) residue of Aki1 as a cyclin B1-Cdk1 phosphorylation site. Furthermore, cyclin B1-Cdk1 inhibitor treatment was shown to attenuate the level of Aki1 in complex with Scc1, suggesting that Aki1 phosphorylation by cyclin B1-Cdk1 contributes to Aki1-Scc1 complex formation. Our results indicate that cyclin B1-Cdk1 is a kinase of Aki1 during mitosis and that its phosphorylation of Aki1 may regulate mitotic function.

Keywords: phosphorylation, Aki1, cyclin B1-Cdk1

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Lu, R.[*], Ito, J.[*], Iwamoto, N.[*], Nishimaki-Mogami, T. and Yokoyama, S.[*]: **FGF-1 induces expression of LXRalpha and production of 25-hydroxycholesterol to upregulate the apoE gene in rat astrocytes**

J. Lipid Res., **50**, 1156-1164 (2009)

Fibroblast growth factor 1 (FGF-1) enhances apolipoprotein E (apoE) expression and apoE-HDL biogenesis in autocrine fashion in astrocytes associated with healing of brain injury. FGF-1 stimulates mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) to increase cholesterol biosynthesis and phosphatidylinositol 3-OH kinase (PI3K)/Akt to enhance apoE-HDL secretion. We investigated the mechanism for FGF-1 to upregulate apoE transcription. FGF-1 increased apoE and liver X receptor alpha (LXRalpha) mRNAs in rat astrocytes. Increase of LXRalpha mRNA was suppressed by inhibition of the FGF-1 receptor-1 and MEK/ERK but not by inhibition of PI3K/Akt. The increases of apoE mRNA and apoE-HDL secretion were both inhibited by downregulation or inhibition of LXRalpha, while they were partially suppressed by inhibiting cholesterol biosynthesis. We identified the liver X receptor element responsible for activation of the rat apoE promoter by FGF-1 located between -450 and -320 bp, and the direct repeat 4 (DR4) element in this region (-448 to -433 bp) was responsible for the activation. Chromatin immunoprecipitation analysis supported that FGF-1 enhanced association of LXR with the rat apoE promoter. FGF-1 partially activated the apoE promoter even in the presence of an MEK inhibitor that inhibits the FGF-1-mediated enhancement of cholesterol biosynthesis. On the other hand, FGF-1 induced production of 25-hydroxycholesterol by MEK/ERK as an sterol regulatory element-dependent reaction besides cholesterol biosynthesis. We concluded that FGF-1-induced apoE expression in astrocytes depends on LXRalpha being mediated by both LXRalpha expression and an LXRalpha ligand biosynthesis.

Keywords: LXR, apoE, FGF-1

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Suzuki, K.[*1], Takahashi, K.[*1], Nishimaki-Mogami, T., Kagechika, H.[*2], Yamamoto, M.[*1] and Itabe, H.[*1]: **Docosahexaenoic acid induces adipose differentiation-related protein through activation of retinoid x receptor in human choriocarcinoma bewo cells**

Biol. Pharm. Bull., **32**, 1177-1182 (2009)

Adipose differentiation-related protein (ADRP) is associated with intracellular lipid droplets that accumulate neutral lipids. Here we report that ADRP expression in a human choriocarcinoma cell line, BeWo, is regulated through activation of retinoid X receptor (RXR) and peroxisome proliferator-activated receptor-gamma (PPARgamma). Incubation with docosahexaenoic acid (DHA) or oleic acid (OA) induced accumulation

of triacylglycerol (TG) and ADRP in BeWo cells. DHA-induced ADRP expression was suppressed by RXR-antagonists, PA452 and HX531. However, oleic acid-induced ADRP expression was not blocked by the RXR-antagonists but by a PPARgamma-antagonist. Treatment of the cells with RXR-agonists, HX630 and PA024, increased Adrp transcripts, however, they alone did not change the levels of ADRP protein and TG in BeWo cells. Induction of ADRP protein was observed in the presence of a proteasome inhibitor, suggesting that ADRP is degraded under lipid-poor conditions. These results suggest that expression of ADRP is in part regulated by RXR and PPARgamma transcription factors, and DHA induces ADRP by acting as an endogenous agonist of RXR.

Keywords: ADRP, RXR, DHA

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Iguchi, Y.[*], Kihira, K.[*], Nishimaki-Mogami, T. and Une, M.[*]: **Structure-activity relationship of bile alcohols as human farnesoid X receptor agonist**
Steroids, **75**, 95-100 (2009)

FXR (farnesoid X receptor) is a bile acid-activated nuclear receptor that regulates not only the biosynthesis and enterohepatic circulation of bile acids, but also triglyceride, cholesterol and glucose metabolism. FXR-mediated signaling pathways have become promising novel drug targets for the treatment of common metabolic and hepatic diseases. With the aim of uncovering novel modulators of FXR and further elucidating the molecular basis of FXR activation, we investigated the structure-activity relationships of a variety of naturally occurring sterols structurally related to bile acids in terms of their FXR agonist activity. Here, we report that the ability of bile alcohols to activate FXR varied with the position and number of hydroxyl groups existing in the steroid side chain of bile alcohols. In addition, we showed that the shortening of the steroid side chain of bile acids as well as bile alcohols resulted in a decline of the ability of these agents to activate FXR. Thus, we provide new insights into the structure-activity relationships of bile acids and bile alcohols as FXR agonists.

Keywords: FXR, bile alcohol, bile acid

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Ohoka, N., Sakai, S.[*1], Onozaki, K.[*1], Nakanishi, M.[*2] and Hayashi, H.[*1]: **Anaphase-promoting complex/cyclosome-cdh1 mediates the ubiquitination and degradation of TRB3**
Biochem. Biophys. Res. Commun., **392**, 289-294 (2010)

We have recently demonstrated that TRB3, a novel endoplasmic reticulum (ER) stress-inducible protein, is induced by CHOP and ATF4 to regulate their function and ER stress-induced cell death; however, the regulation of TRB3 function has not been well characterized. Here we demonstrate that TRB3 is an unstable protein regulated by the ubiquitin-proteasome system. The carboxyl-terminal domain of TRB3 is necessary for protein degradation, and in this region, we found the typical D-box motif, which is a critical sequence for the anaphase-promoting complex/cyclosome (APC/C) dependent proteolysis. TRB3 proteins were stabilized by deletion of its D-box motif and interacted with APC/C coactivator proteins, Cdc20 and Cdh1. The expression level of TRB3 protein is down-regulated by over-expression of Cdh1 but not by that of Cdc20. In addition, knockdown of Cdh1 enhanced the endogenous TRB3 expression level and suppressed its ubiquitination level. These results suggest that APC/C (Cdh1) is involved in ubiquitination and down-regulating the stability of TRB3 protein.

Keywords: TRB3, APC/C, Cdh1

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Gay, S.C.[*1], Sun, L.[*2], Maekawa, K., Halpert, J.R.[*1] and Stout, C.D.[*3]: **Crystal structures of cytochrome P450 2B4 in complex with the inhibitor 1-biphenyl-4-methyl-1H-imidazole:ligand-induced structural response through alpha-helical repositioning**
Biochemistry, **48**, 4762-4771 (2009)

Two different ligand occupancy structures of cytochrome P450 2B4 (CYP2B4) in complex with 1-biphenyl-4-methyl-1H-imidazole (1-PBI) have been determined by X-ray crystallography. 1-PBI belongs to a series of tight binding, imidazole-based CYP2B4 inhibitors. 1-PBI binding to CYP2B4 yields a type II spectrum with a Ks value of 0.23μM and inhibits enzyme activity with an IC50 value of 0.035μM. Previous CYP2B4 structures

have shown a large degree of structural movement in response to ligand size. With two phenyl rings, 1-PBI is larger than 1-(4-chlorophenyl)imidazole (1-CPI) and 4-(4-chlorophenyl)imidazole (4-CPI) but smaller than bifonazole, which is branched and contains three phenyl rings. The CYP2B4-1-PBI complex is a structural intermediate to the closed CPI and the open bifonazole structures. The B/C-loop reorganizes itself to include two short partial helices while closing one side of the active site. The F-G-helix cassette pivots over the I-helix in direct response to the size of the ligand in the active site. A cluster of Phe residues at the fulcrum of this pivot point allows for dramatic repositioning of the cassette with only a relatively small amount of secondary structure rearrangement. Comparisons of ligand-bound CYP2B4 structures reveal trends in plastic region mobility that could allow for predictions of their position in future structures based on ligand shape and size.

Keywords: X-ray crystal structures, CYP2B4, inhibitor

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Maekawa, K., Harakawa, N., Sugiyama, E., Tohkin, M., Kim, S.R., Kaniwa, N., Katori, N., Hasegawa, R., Yasuda, K.*1, Kamide, K.*2, Miyata, T.*3, Saito, Y. and Sawada, J.: **Substrate-dependent functional alterations of seven CYP2C9 variants found in Japanese subjects**

Drug Metab. Dispos., **37**, 895-903 (2009)

CYP2C9 is a polymorphic enzyme that metabolizes a number of clinically important drugs. In this study, catalytic activities of seven alleles found in Japanese individuals, *CYP2C9*3* (I359L), **13* (L90P), **26* (T130R), **28* (Q214L), **30* (A477T), **33* (R132Q), and **34* (R335Q), were assessed using three substrates (diclofenac, losartan, and glimepiride). When expressed in a baculovirus-insect cell system, the holo and total (apo and holo) CYP2C9 protein expression levels were similar among the wild type (CYP2C9.1) and six variants except for CYP2C9.13. A large part of CYP2C9.13 was present in the apo form P420. Compared with CYP2C9.1, all variants except for CYP2C9.34 exhibited substrate-dependent changes in K_m, V_{max}, and intrinsic clearance (V_{max}/K_m). For diclofenac 4-hydroxylation, the intrinsic clearance was decreased markedly (by >80%) in CYP2C9.13, CYP2C9.30, and CYP2C9.33 and variably (63－76%) in CYP2C9.3, CYP2C9.26, and CYP2C9.28 due to increased K_m and/or decreased V_{max} values. For losartan oxidation, CYP2C9.13 and CYP2C9.28 showed 2.5- and 1.8-fold higher K_m values, respectively, and all variants except for CYP2C9.34 showed >77% lower V_{max} and intrinsic clearance values. For glimepiride hydroxylation, the K_m of CYP2C9.13 was increased 7-fold, and the V_{max} values of all variants significantly decreased, resulting in reductions in the intrinsic clearance by >80% in CYP2C9.3, CYP2C9.13, CYP2C9.26, and CYP2C9.33 and by 56 to 75% in CYP2C9.28 and CYP2C9.30. These findings suggest the necessity for careful administration of losartan and glimepiride to patients bearing these six alleles.

Keywords: genetic polymorphism, CYP2C9, function

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Gay, S.C.*1, Shah, M.B.*1, Talakad, J.C.*1, Maekawa, K., Roberts, A.G.*1, Wilderman, P.R.*1, Sun, L.*2, Yang, J.Y.*1, Huelga, S.C.*1, Hong, W.X.*3, Zhang, Q.*2, Stout, C.D.*3 and Halpert, J.R.*1: **Crystal Structure of a Cytochrome P450 2B6 Genetic Variant in Complex with the Inhibitor 4-(4-Chlorophenyl)imidazole at 2.0 A Resolution**

Mol Pharmacol., **77**, 529-38 (2010)

The structure of the K262R genetic variant of human cytochrome P450 2B6 in complex with the inhibitor 4-(4-chlorophenyl)imidazole (4-CPI) has been determined using X-ray crystallography to 2.0- resolution. Production of diffraction quality crystals was enabled through a combination of protein engineering, chaperone coexpression, modifications to the purification protocol, and the use of unique facial amphiphiles during crystallization. The 2B6-4-CPI complex is virtually identical to the rabbit 2B4 structure bound to the same inhibitor with respect to the arrangement of secondary structural elements and the placement of active site residues. The structure supports prior P450 2B6 homology models based on other mammalian cytochromes P450 and is consistent with the limited site-directed mutagenesis studies on 2B6 and extensive studies on

P450 2B4 and 2B1. Although the K262R genetic variant shows unaltered binding of 4-CPI, altered binding affinity, kinetics, and/or product profiles have been previously shown with several other ligands. On the basis of new P450 2B6 crystal structure and previous 2B4 structures, substitutions at residue 262 affect a hydrogen-bonding network connecting the G and H helices, where subtle differences could be transduced to the active site. Docking experiments indicate that the closed protein conformation allows smaller ligands such as ticlopidine to bind to the 2B6 active site in the expected orientation. However, it is unknown whether 2B6 undergoes structural reorganization to accommodate bulkier molecules, as previously inferred from multiple P450 2B4 crystal structures.

Keywords: X-ray crystal structures, CYP2B6, ticlopidine

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Sai, K., Saito, Y., Maekawa, K., Kim, S.R., Kaniwa, N., Nishimaki-Mogami, T., Sawada, J., Shirao, K.*, Hamaguchi, T.*, Yamamoto, N.*, Kunitoh, H.*, Ohe, Y.*, Yamada, Y.*, Tamura, T.*, Yoshida, T.*, Matsumura, Y.*, Ohtsu, A.*, Saijo, N.* and Minami, H.*: **Additive effects of drug transporter genetic polymorphisms on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients**

Cancer Chemother. Pharmacol., **66**, 95-105 (2010)

Aim: Effects of genetic polymorphisms/variations of *ABCB1*, *ABCC2*, *ABCG2* and *SLCO1B1* in addition to "*UGT1A1*28* or **6*" on irinotecan pharmacokinetics/pharmacodynamics in Japanese cancer patients were investigated. ***Methods***: Associations between transporter haplotypes/variations along with *UGT1A1*28* or **6* and SN-38 area under the time-concentration curve (AUC) or neutropenia were examined in irinotecan monotherapy (55 patients) and irinotecan-cisplatin-combination therapy (62 patients). ***Results***: Higher SN-38 AUC values were observed in *ABCB1*2677G>T (A893S) (**2* group) for both regimens. Associations of grade 3/4 neutropenia were observed with *ABCC2*-1774delG (**1A*), *ABCG2* [421C>A (Q141K) and IVS12 + 49G>T (*#IIB*)] and *SLCO1B1*521T>C (V174A) (**15・17*) in the irinotecan monotherapy, while they were evident only in homozygotes of *ABCB1*2*, *ABCG2#IIB*, *SLCO1B1*15・17* in the cisplatin-combination therapy. With combinations of haplotypes/variations of two or more genes, neutropenia incidence increased, but their prediction power for grade 3/4 neutropenia is still unsatisfactory. ***Conclusion***: Certain transporter genotypes additively increased irinotecan-induced neutropenia, but their clinical importance should be further elucidated.

Keywords: Transporter, irinotecan, genetic polymorphism

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Akiyama, H., Nakamura, F., Yamada, C.*1, Nakamura, K., Nakajima, O., Kawakami, H.*1, Harikai, N.*2, Furui, S.*3, Kitta, K.*3, Teshima, R.: **A screening method for the detection of the 35S promoter and the nopaline synthase terminator in genetically modified organisms in a real-time multiplex polymerase chain reaction using high-resolution melting-curve analysis**

Biol. Pharm. Bull., **32**, 1824-1829 (2009)

To screen for unauthorized genetically modified organisms (GMO) in the various crops, we developed a multiplex real-time polymerase chain reaction high-resolution melting-curve analysis method for the simultaneous qualitative detection of 35S promoter sequence of cauliflower mosaic virus (35SP) and the nopaline synthase terminator (NOST) in several crops. We selected suitable primer sets for the simultaneous detection of 35SP and NOST and designed the primer set for the detection of spiked ColE1 plasmid to evaluate the validity of the polymerase chain reaction (PCR) analyses. In addition, we optimized the multiplex PCR conditions using the designed primer sets and EvaGreen® as an intercalating dye. The contamination of unauthorized GMO with single copy similar to NK603 maize can be detected as low as 0.1% in a maize sample. Furthermore, we showed that the present method would be applicable in identifying GMO in various crops and foods like authorized GM soybean, authorized GM potato, the biscuit which is contaminated with GM soybeans and the rice which is contaminated with unauthorized GM rice. We consider this method to be a simple and reliable assay for screening for unauthorized GMO in crops and the processing food products.

Keywords: nopaline synthase terminator, genetically modified organism, real-time multiplex polymerase chain reaction

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橋本博之[*]，伊藤歌奈子[*]，田中裕之[*]，穐山　浩，手島玲子，眞壁裕樹[*]，中西希代子[*]，宮本文夫：**モデル加工食品を用いた特定原材料（小麦）検査におけるネステッドPCR法の検討**

食品衛生学雑誌，**50**，178-183（2009）

小麦のスクリーニング検査陽性モデル加工食品を11種類作製し，通知法PCRおよびネステッドPCR法による検出状況を調査し，また鋳型DNAの増量効果について検討を行った．現行の通知法PCRでは３種類が，ネステッドPCR法では１種類のモデル加工食品が検出不可能であった．これらのモデル加工食品では，鋳型DNAを増量させることにより両PCR法で検出可能となった．しかし，通知法ではモデル加工食品ごとに適した鋳型DNAの増量範囲が異なっており，過剰増量によるPCR反応阻害により増幅が不可能となることが，かまぼこおよびゼリーで確認された．以上の結果から，加工食品を対象としたPCR検査法を実施する際には，DNAの抽出方法などを検討することによりPCR阻害物質の低減を図り，PCRに用いる鋳型DNA量を適切に増量することが正確な結果を導き出すための有効な手段の一つになると考えられた．

Keywords: allergenic substance, wheat, ELISA

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Nakamura, K., Akiyama, H., Yamada, C.[*1], Satoh, R., Makiyama, D., Sakata, K., Kawakami, H.[*1], Mano, J.[*2], Kitta, K.[*2], Teshima, R.: **Novel method to detect a construct-specific sequence of the acetolactate synthase gene in genetically-modified flax CDC Triffid (FP967)**

Biol. Pharm. Bull., **33**, 532-534 (2010)

During the fall of 2009, a trace of unauthorized genetically modified (GM) flax (*Linum usitatissimum* L.) line, CDC Triffid, which is resistant to sulfonylurea herbicides, was detected in many countries including Japan. A method to reliably identify the CDC Triffid line was urgently required. We developed a novel construct-specific real-time PCR method to identify the mutant acetolactate synthase gene in the CDC Triffid line. We confirmed that the method can detect 0.001% GM flax in DNA mixing solution. The study shows that the developed method is specific, sensitive and reliable way to monitor a trace of CDC Triffid.

Keywords: genetically modified organism, flax, polymerase chain reaction

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Mano, J.[*], Yanaka, Y.[*], Akiyama, H., Teshima, R., Furui, S.[*], Kitta, K.[*]: **Improvement of polymerase chain reaction-based BT11 maize detection method by reduction of non-specific amplification**

Shokuhin Eiseigaku Zasshi, **51**, 32-36 (2010)

The Bt11 maize-specific qualitative detection method based on polymerase chain reaction (PCR) is one of the standard methods for analyzing genetically modified (GM) crops described in the Japanese Agricultural Standard (JAS) analytical test handbook. This method has been broadly used for administrative monitoring of GM crops and quality control of grains in commercial distribution. In the present investigation, possible false-positive detections were observed in the assays with the Bt11 maize-specific method, and these erroneous detections were proved to be caused by non-specific DNA amplification. We improved the detection method to reduce these non-specific amplification by decreasing the concentration of magnesium ions in the PCR mixtures. The subsequent evaluation of analytical performances demonstrated no remarkable differences between the commonly used and the improved methods, except for the reduced non-specific amplification. The results exhibited that the conventional method should be replaced with the improved method for the reliable detection of Bt11 maize.

Keywords: qualitative detection method, genetically modified organism, Bt11

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清水えり[*1]，布藤　聡[*1]，増淵友子[*2]，峯岸恭孝[*3]，日野明寛[*2]，穐山　浩，手島玲子，古井　聡[*3]，橘田和美[*3]：**ポリプロピレン製品DNA検査に及ぼす影響：PCR検査に好適なマイクロチューブの選択方法について**

食品衛生学雑誌，**51**，43-47（2010）

遺伝子組換え食品（GMO）検査を行う際，サンプルを保存や希釈など，様々な場面でディスポーザブルのマイクロチューブ（以下，チューブと略す）を使用する．チューブの品質はPCR反応後の定量値に大きな影響を与える可能性がある．様々なチューブを用いて試験した結果，DNAの吸着現象や溶出物がみられることが明らかとなった．我々は，チューブに起因するDNAの吸着現象を解明し．DNA検査に好適な品質のチューブを選択可能とするための品質管理基準及び手法を確立した．

Keywords: microtube, genetically modified organism (GMO), DNA binding

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Harikai, N.[*1], Saito, S.[*2], Abe, M.[*2], Kondo, K., Kitta, K.[*3], Akiyama, H., Teshima, R., Kinoshita, K.[*1]: **Optical detection of specific genes for genetically modified soybean and maize using multiplex PCR coupled with primer extension on a plastic plate**

Biosci. Biotechnol. Biochem., **73**, 1886-1889（2009）

A detection method for one line of genetically modified (GM) soybean and five lines of GM maize was developed using multiplex PCR Coupled with primer extension on a plastic plate. Multiplex PCR products were applied on an extension primer-immobilized plastic plate and the spots corresponding to the DNA sequences were visualized. This method would be a rapid and simple way to optically detect GM soybean and GM maize.

Keywords: arrayed primer extension, genetically modified organism, multiple primer extension

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Suzuki, Y.[*], Kassai, M.[*], Hirose, T.[*], Katayama, S.[*], Nakamura, K., Akiyama, H., Teshima, R., Nakamura, S.[*]: **Modulation of Immunoresponse in BALB/c Mice by Oral Administration of Fag e 1-Glucomannan Conjugate**

J. Agric. Food Chem., **57**, 9787-9792（2009）

Maillard-type glycosylation was applied to preparation of hypoallergenic agents from a major buckwheat allergen, Fag e 1. Conjugation with arabinogalactan (AG), xyloglucan (XG), or yeast glucomannan (YGM) successfully decreased in vitro allergenicity of Fag e 1. Determination of IgE titer in the tested allergic mice revealed that YGM was the most effective for in vivo allergenicity of Fag e 1 among these water-soluble polysaccharides. Real-time PCR analysis using a set of primer for IL-4 (a typical Th2 cytokine) or IFN-γ (a typical Th1 cytokine) showed that expressed mRNA for IL-4 in splenocytes drastically decreased with increasing with Fag e 1-YGM conjugate feeding. In addition, based on a flow-cytometric analysis of T cell subsets in the splenocytes, it was confirmed that the feeding led to an improvement of Th1/Th2 balance in the allergic mice where population of Th1 increased from 2.91% to 4.02%, while that of Th2 decreased from 3.75% to 2.72%. Furthermore, it was revealed that differentiation ratio of regulatory T cell (Treg) in the splenocytes increased from 14.5% to 18.7% by the oral administration. These results indicated that Fag e 1-YGM conjugate can be available for an immunomodulating agent for buckwheat allergy.

Keywords: Fag e 1, buckwheat allergy, yeast glucomannan

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Mano, J.[*], Oguchi, T.[*], Akiyama, H., Teshima, R., Hino, A.[*], Furui, S.[*], Kitta, K.[*]: **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 recombi-

nant 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: multiplex PCR, genetically modified (GM), ligase chain reaction (LCR)

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Oguchi, T.[*1], Onishi, M.[*2], Minegishi, Y.[*3], Kurosawa, Y.[*1], Kasahara, M.[*4], Akiyama, H., Teshima, R., Futo, S.[*2], Furui, S.[*1], Hino, A.[*1], Kitta, K.[*1]: **Development of quantitative duplex real-time PCR method for screening analysis of genetically modified maize**

Shokuhin Eiseigaku Zasshi, **50**, 117-125 (2009)

A duplex real-time PCR method was developed for quantitative screening analysis of GM maize. The duplex real-time PCR simultaneously detected two GM-specific segments, namely the cauliflower mosaic virus (CaMV) 35S promoter (P35S) segment and an event-specific segment for GA21 maize which does not contain P35S. Calibration was performed with a plasmid calibrant specially designed for the multiplex PCR. The result of an in-house evaluation suggested that the analytical precision of the developed method was almost equivalent to those of simplex real-time PCR methods, which have been published as ISO standard methods for the analysis of GMOs in foodstuffs and have also been employed for the analysis of GMOs in Japan. The high analytical performance demonstrated in the current study would be useful for the quantitative screening analysis of GM maize.

Keywords: genetically modified organism (GMO), quantitative analysis, duplex (multiplex) real-time PCR

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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.)**

Shokuhin Eiseigaku Zasshi, **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)

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穐山 浩，佐々木伸大[*1]，大木果林，中村文美，坂田こずえ，中村公亮，大森清美[*2]，中島安基江[*3]，古井聡[*4]，橘田和美[*4]，小関良宏[*1]，手島玲子：**PCR法を用いた米加工品の安全性未審査遺伝子組換え米の検知法**

日本食品化学会誌，**16**，147-151（2009）

安全性未審査中国産遺伝子組換え米の二系統についてPCRを用いた検知法を確立した．二系統は，両系統ともBacillus thuringiensis（Bt）由来のcry遺伝子が挿入されたBt米（Bt63米とNNBt米）である．両系統に発現しているBtトキシンは害虫抵抗性を示す．両系統に共通のcry遺伝子を検知するプライマー対を設計した．また我々が明らかにした挿入配列に基づいてBt63米とNNBt米の各々特異的なプライマー対を設計した．確立した方法を用いてビーフン陽性検体及びもち米擬陽性検体から，各々混入している当該系統のBt米が検出された．確立した方法は，米加工品中の２系統のBt米を監視目的に検査する方法として有用であると思われる．

Keywords: 遺伝子組換えコメ，PCR，もち米

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Kondo, K., Obitsu, S., Ohta, S., Matsunami, K.*, Otsuka, H.*, Teshima, R.: **Poly（ADP-ribose）polymerase（PARP）-1- independent apoptosis-inducing factor（AIF）release and cell death are induced by eleostearic acid and blocked by alpha- tocopherol and MEK inhibition**

J. Biol. Chem., **285**, 13079-13091（2010）

ESA induced the caspase-independent and AIF-initiated apoptotic death of neuronal cell lines, independently of PARP-1 activation. The cell death was inhibited by the MEK inhibitor U0126 and by knockdown of MEK. AIF was translocated to the nucleus after the induction of apoptosis by -ESA in differentiated PC12 cells without activating caspase-3 and PARP-1. The eleostearic acid（ESA）-mediated cell death was not inhibited by PARP inhibitor DPQ and by knockdown of PARP-1 using small interfering RNA. Unlike MNNG, histonephosphorylated histone 2AX was not phosphorylated by ESA, which suggests no DNA damage. Overexpression of Bcl-2 did not inhibit the cell death. ESA caused a small quantity of superoxide production in the mitochondria, resulting in the reduction of mitochondrial membrane potential, both of which were blocked by a trace amount of tocopherol localized in the mitochondria.

Keywords: Apoptosis, PARP-1, Eleostearic Acid

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酒井信夫，安達玲子，中村　厚，柴原裕亮*1，上坂良彦*1，清木興介*2，織田浩司*2，穐山　浩，手島玲子：**いわゆる健康食品に含まれる甲殻類様たんぱく質量の実態調査**

日本食品化学学会誌，**16**，118-122（2009）

わが国ではエビ・カニ等の甲殻類に対するアレルギー患者が増加しており，平成20年度にエビとカニは特定原材料に指定された．本研究では，90種類の健康食品中の甲殻類（様）タンパク質量について２種のELISAキットを用いて調査を行った。キトサン及びグルコサミン関連健康食品（甲殻類外殻を原材料とする）49検体中48検体では甲殻類タンパク質は検出されなかった．１検体については甲殻類タンパク質が検出されたが，これは原材料として使用されている魚肉練り製品に由来する可能性が高いと考えられ，キトサン及びグルコサミン関連健康食品については、甲殻類タンパク質が混入する可能性は低いと考えられた。蜂の子健康食品21検体及び蟻健康食品20検体ではELISAキットで陽性となるものが高頻度に見られ，これは，ELISAキットの検出標的タンパク質である甲殻類トロポミオシンの昆虫類トロポミオシンとの相同性が高いため、キットの抗体が昆虫類トロポミオシンに反応したためと考えられた．

Keywords: allergenic protein, crustaceans, insects, tropomyosin, health foods

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Sakai, S., Adachi, R., Akiyama, H., Teshima, R., Morishita, N.*1, Matsumoto, T.*1, Urisu, A.*2: **Enzyme-linked immunosorbent assay kit for the determination of soybean protein in processed foods: interlaboratory evaluation**

J. AOAC Int., **93**, 243-248（2010）

The labeling of foods containing ingredients derived from soybean is recommended in Japan because of an increasing number of patients who are allergic to soybeans. To ensure proper labeling, a novel sandwich ELISA kit for the determination of soybean protein in processed foods（FASTKIT Ver. II, “Soybean,” Nippon Meat Packers, Inc.; “soy kit”）has been developed. Five types of incurred samples（model processed foods: rice gruel, sausage, sweet adzuki bean soup, sweet potato cake, and tomato sauce）containing 10μg soybean soluble protein/g food were prepared for use in interlaboratory evaluations of the soy kit. The soy kit displayed a sufficient RSDR value（interlaboratory precision: 9.3－13.4% RSDR）and a high level of recovery（97－114%）for all the incurred samples. The RSDr value for the incurred samples was mostly <4.8%. The results of this interlaboratory evaluation suggest that the soy kit can be used as a precise and reliable tool for the determination of soybean proteins in processed foods.

Keywords: food allergen, interlaboratory studies, ELISA, soybean

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Nakajima, O., Akiyama, H., Teshima, R.: **Real-Time PCR Method for Detecting Contamination of Beef by Material from Genetically Engineered Cattle**

Biol. Pharm. Bull., **32**, 1313-1316（2009）

Prion protein knockout (PRNP-/-) cattle have been developed and may be used to produce bovine material such as serum, collagen, and gelatin. However, genetically engineered animals (GE animals) must not be imported or made commercially available in Japan, because they are not authorized for food use in Japan. We used real-time PCR to develop method of detection for neomycin- and the puromycin-resistance genes in beef samples. Plasmids containing the neomycin- resistance gene and the puromycin-resistance gene were used as standard reference molecules. The results clearly showed that the method we developed is capable of quantitatively detecting the neomycin- and the puromycin-resistance genes in the plasmids in the presence of genomic DNA extracted from a beef sample. We also applied the method to testing of beef samples imported from the United States (US). This method will make it possible to monitor beef for contamination by material from GE cattle to assure food safety.

Keywords: GE animal, PRNP knockout cattle, real-time PCR

Nakajima, O., Koyano, S.*, Akiyama, H., Sawada, J.*, Teshima, R.: **Confirmation of a predicted lack of IgE binding to Cry3Bb1 from genetically modified (GM) crops**

Regul. Tixicol. Pharmacol., **56**, 306-311 (2009)

Some GM crops including MON863 corn and stack varieties contain Cry3Bb1 protein. Cry3Bb1 is very important from the standpoint of assessing the safety of GM crops. In this study Cry3Bb1 was assessed from the standpoint of possible binding to IgE from allergy patients. First, an ELISA that was improved in our laboratory was used to test serum samples from 13 corn allergy patients in the United States with recombinant Cry3Bb1 expressed in Escherichia coli, and serum samples from 55 patients in Japan with various food allergies were also assayed. Two samples from the Japanese allergy patients were suspected of being positive, but Western blotting analysis with purified Cry3Bb1 indicated that the binding between IgE and Cry3Bb1 was nonspecific. Ultimately, no specific binding between IgE and recombinant Cry3Bb1 was detected. Next, all proteins extracted from MON863 corn and non-GM corn were probed with IgE antibodies in serum samples from the corn allergy patients by Western blotting, but the staining patterns of MON863 and non-GM corn were similar, meaning that unintended allergic reactions to MON863 are unlikely to occur. Our study provides additional information that confirms the predicted lack of IgE binding to Cry3Bb1 in people with existing food allergies

Keywords: Cry3Bb1, genetically modified food, safety assessment, IgE binding test

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Nakamura, R., Uchida, Y., Higuchi, M. and Teshima, R.: **Development of a novel allergy test using a cultured mast cell line**

ImmunoTox Lett., **14**, 12-13 (2009)

ラットマスト細胞株RBL-2H3細胞に，ヒトの高親和性IgE受容体および転写因子NF-ATによりルシフェラーゼの発現が誘導されるレポーター遺伝子を導入したRS-ATL8細胞を作製し，新規アレルギー試験法としての有用性を検証した.

Keywords: allergy test, luciferase, mast cell

Nakamura, R., Satoh, R., Nakajima, Y., Kawasaki, N., Yamaguchi, T., Sawada, J., Nagoya, H.* and Teshima, R.: **Comparative study of GH-transgenic and non-transgenic amago salmon (*Oncorhynchus masou ishikawae*) allergenicity and proteomic analysis of amago salmon allergens**

Regul. Toxicol. Pharmacol., **55**, 300-308 (2009)

Genetically modified (GM) foods are beneficial from the standpoint of ensuring a constant supply of foodstuffs, but they must be tested for safety before being released on the market, including by allergenicity tests to ensure that they do not contain new allergens or higher concentrations of known allergens than the same non-GM foods. In this study we used GM-amago salmon into which a growth hormone gene had been introduced and compared the allergens contained in the GM and the non-GM-amago salmons. We used a combination of Western blotting with allergen-specific antibodies and a proteomic analysis of their allergens with patients' sera, a so-called allergenome analysis, to analyze allergens. Western blotting with specific antibodies showed no increase in the content of the known allergens fish parvalbumin and fish type-I collagen in GM-amago salmon, in comparison with their content in non-GM-amago salmon. The allergenome analysis of

two fish-allergic patients allowed us to identify several IgE-binding proteins in amago salmon, including parvalbumin, triose-phosphate isomerase, fructose-bisphosphate aldolase A, and serum albumin, and there were no qualitative differences in these proteins between GM and non-GM-amago salmons. These results indicate that amago salmon endogenous allergen expression does not seem to be altered by genetic modification.

Keywords: Fish, Allergenome, Genetically modified foods

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Nakamura, R., Nakano, M.[*1], Arisawa, K.[*2], Ezaki, R.[*2], Horiuchi, H.[*1] and Teshima, R.: **Allergenicity study of EGFP-transgenic chicken meat by serological and 2D-DIGE analysis**

Food Chem. Toxicol., **48**, 1302-1310 (2010)

Genetically modified (GM) foods must be tested for safety, including by allergenicity tests to ensure that they do not contain new allergens or higher concentrations of known allergens than the same non-GM foods. In this study experimentally developed EGFP-transgenic chickens were used and evaluated the allergenicity of meat from the chicken based on a serological and two-dimensional difference gel electrophoresis (2D-DIGE) analysis. For the serological analysis, a Western blotting with allergen-specific antibodies and a proteomic analysis of chicken meat allergens with patients' sera, a so-called allergenome analysis, were used. The allergenome analysis allowed us to identify five IgE-binding proteins in chicken meat, including a known allergen, chicken serum albumin, and no qualitative difference in their expressions between the GM and non-GM chicken meat was found. Results of the 2D-DIGE analysis showed that none of the IgE-binding proteins in chicken meat were significantly changed in expression levels between non-GM and GM chicken, and only 3 of the 1500 soluble protein spots including green fluorescence protein were markedly different as a result of gene transfer. These above results showed that the combination of serological and 2D-DIGE analysis is a valid method of evaluating quality and quantity of allergens in GM foods.

Keywords: Allergenicity, Chicken meat, 2D-DIGE

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Asakawa, N.[*], Sakiyama, N.[*], Teshima, R., Mitaku, S.[*]: **Characteristic amino acid distribution around segments unique to allergens**

J. Biochem., **147**, 127-133 (2010)

Epitopes are located at the surface of allergens with which antibodies specifically bind. On the assumption that fragments unique to allergens have common, characteristic amino acid sequences, we compared the amino acid sequences of allergens with those of non-allergens. Segments around fragments unique to allergens showed wavelet-like distributions for several amino acids. Charged residues, alanine and glycine had positive peaks at the centre of the unique segments with small valleys on both sides, while aromatic residues, proline and cysteine showed the inverse distribution. Furthermore, the wavelet-like distribution of amino acids could be represented by a universal distribution function together with an index characterizing the intensity of the wavelet. Using the universal distribution function and the novel index of amino acids, we developed a simple method for extracting segments and fragments that are unique to allergens. The significance of the universal distribution function and the novel index is also discussed, by comparing the plot of the allergen-unique fragments index and dynamic fluctuation in the three dimensional structure of birch pollen allergen as both a single molecule and a complex with the corresponding antibody.

Keywords: allergen, unique sequence fragment, bioinformatics

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Kezuka, Y.[*], Itagaki, T.[*], Satoh, R., Teshima, R., Nonaka, T.[*]: **Purification, crystallization and preliminary X-ray analysis of a deletion mutant of a major buckwheat allergen**

Acta Cryst., **F65**, 1267-1270 (2009)

A 16 kDa buckwheat protein (BWp16) is a major allergen responsible for immediate hypersensitivity reactions including anaphylaxis. A deletion mutant of BWp16 (rBWp16DeltaN) was overproduced and purified and was shown to be immunologically active. A three-wavelength MAD data set was collected from a crystal

of selenomethionine-labelled rBWp16DeltaN. The crystal belonged to the triclinic space group P1, with unit-cell parameters a = 28.39, b = 31.54, c = 32.20 A, alpha = 111.92, beta = 108.91, gamma = 98.74 degrees. One monomer was expected to be present in the asymmetric unit based on the calculated Matthews coefficient of 1.76 A(3) Da(-1).

Keywords: buckwheat allergen, 2S albumin, BWp16

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Satoh, R., Koyano, S., Takagi, K., Nakamura, R., Teshima, R.: **Identification of an IgE-Binding Epitope of a Major Buckwheat Allergen, BWp16, by SPOTs Assay and Mimotope Screening**

Int. Arch. Allergy Immunol., **153**(2), 133-140 (2010)

Background: The buckwheat 16-kDa protein (BWp16), as reported in our previous study, is a major allergen in buckwheat; however, the IgE-binding epitopes of BWp16 have not as yet been identified. Methods: We screened candidates for IgE-binding epitopes on BWp16 by using arrays of overlapping peptides synthesized on activated cellulose membranes (SPOTs membrane). The mimotope method was also used to analyze IgE-binding epitopes of BWp16. Nine single alanine (Ala) mutants of BWp16 expressed in Escherichia coli were used to confirm the epitopes of BWp16. The IgE-binding activity of single Ala mutants of BWp16 was determined by ELISA with mouse anti-BWp16 polyclonal antiserum or ELISA inhibition with sera from buckwheat allergic patients. Results: The SPOTs assay identified amino acid residues 99-110, i.e. EGVRDLKELPSK, as a candidate for the linear IgE-binding epitope of BWp16. The mimotope method indicated that peptides similar to EGVRDLKE were candidate sequences for epitopes of BWp16. Ala scanning of rBWp16 revealed that all EGVRDLKE peptides containing a single amino acid mutation had weaker IgE-binding activity than rBWp16 WT. An ELISA inhibition assay for rBWp16 WT revealed the inhibitory effect of rBWp16 D103A to be less than that of rBWp16 WT. Conclusions: We identified the peptide EGVRDLKE as a very likely candidate for the IgE-binding epitope of BWp16, and Asp103 as the critical amino acid in BWp16. This is the first report on the identification of IgE-binding epitopes of BWp16. Our findings will contribute to the production of BWp16 hypoallergens, and to allergen-specific immunotherapy for buckwheat allergy.

Keywords: Buckwheat, 2S albumin, mimotope

Morita, T., Hayashi, M.[*1], Nakajima, M.[*1], Tanaka, N[*2], Tweats, D.J.[*3], Morikawa, K. and Sofuni, T.[*4]: **Practical Issues on the Application of the GHS Classification Criteria for Germ Cell Mutagens**

Regul. Toxicol. Pharmacol., **55**, 52-68 (2009)

The Globally Harmonized System of Classification and Labeling of Chemicals (GHS) requires classification of chemicals on germ cell mutagenicity. The Japanese government has conducted GHS classification on about 1400 chemicals in a 2-year project (J-GHS) for implementing GHS domestically. Prior to the classification work, the technical guidance for classification of germ cell mutagens was prepared. This guidance introduces the concept of heritable mutagenicity, and presents detailed criteria for germ cell mutagens, test data to be used, and a practical decision tree for classification. These practical guidance and supporting explanations are useful for non-expert Classifiers (scientists applying the classification criteria). Several issues, however, were identified during the course of J-GHS and in re-evaluating the classification results. These include: (1) the information sources when available data are limited; (2) lack of understanding GHS classification criteria or insufficient review of the information by Classifiers; (3) varying opinions of experts on data quality and weight of evidence, and; (4) decision tree approaches, e.g., inadequacy for use in overall evaluation in some cases. Ideally, classification should be performed by Classifiers with high expertise using high quality information sources. Genetic toxicologists as experts should consider data quality and reliability, and give a critical review of all available information for support of classification. A weight of evidence approach is also required to assess mutagenic potential of chemicals. Critical points for suitable classification for GHS are discussed.

Keywords: GHS, germ cell mutagens, hazard classification

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Kim, S.R., Saito, Y., Itoda, M., Maekawa, K., Kawamoto, M.[*], Kamatani, N.[*], Ozawa, S., Sawada, J.: **Genetic variations of the ABC transporter gene *ABCB11* encoding the human bile salt export pump (BSEP) in a Japanese population**

Drug Metab. Pharmacokinet., **24**, 277-281 (2009)

The bile salt export pump (BSEP) encoded by *ABCB11* is located in the canalicular membrane of hepatocytes and mediates the secretion of numerous conjugated bile salts into the bile canaliculus. In this study, 28 *ABCB11* exons (including non-coding exon 1) and their flanking introns were comprehensively screened for genetic variations in 120 Japanese subjects. Fifty-nine genetic variations, including 19 novel ones, were found: 14 in the coding exons (6 nonsynonymous and 8 synonymous variations), 4 in the 3'-UTR, and 41 in the introns. Three novel nonsynonymous variations, 361C>A (Gln121Lys), 667C>T (Arg223Cys), and 1460G>T (Arg487Leu), were found as heterozygotes and at 0.004 allele frequencies. These data provide fundamental and useful information for genotyping *ABCB11* in the Japanese and probably other Asian populations.

Keywords: bile salt export pump, genetic variation, Japanese

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Fukushima-Uesaka, H., Saito, Y., Maekawa, K., Kurose, K., Sugiyama, E., Katori, N., Kaniwa, N., Hasegawa, R., Hamaguchi, T.[*1], Eguchi-Nakajima, T.[*1], Kato, K.[*1], Yamada, Y.[*1], Shimada, Y.[*1], Yoshida, T.[*1], Yamamoto, N.[*1], Nokihara, H.[*1], Kunitoh, H.[*1], Ohe, Y.[*1], Tamura, T.[*1], Ura, T.[*2], Saito, M.[*2], Muro, K.[*2], Doi, T.[*1], Fuse, N.[*1], Yoshino, T.[*1], Ohtsu, A.[*1], Saijo, N.[*1], Matsumura, Y.[*1], Okuda, H., Sawada, J.: **Genetic polymorphisms of copper- and platinum drug-efflux transporters *ATP7A* and ATP7B in Japanese cancer patients**

Drug Metab. Pharmacokinet., **24**, 565-574 (2009)

ATP7A and *ATP7B* are involved in cellular resistance to platinum compounds such as cisplatin. By sequencing *ATP7A*, 38 genetic variations, including 30 novel ones were detected from 203 Japanese cancer patients. Of these, seven nonsynonymous variations were found: novel 1030A>G (R344G), 2111A>G (Q704R), 2200C>A (Q734K), 2948C>T (T983M) and 3112G>A (V1038I) at 0.004 frequencies and known 2299G>C (V767L) and 4390A>G (I1464V) at 0.351 and 0.075 frequencies, respectively. Regarding *ATP7B*, 28 novel and 33 known genetic variations were detected including 13 nonsynonymous ones: novel 1258A>G (M420V), 1426G>A (A476T), and 2401A>C (T801P) were found at 0.002, 0.005, and 0.002, respectively and known 1216G>T (A406S), 1366G>C (V456L), 2495A>G (K832R), 2785A>G (I929V), 2855G>A (R952K), 2871delC (P957PfsX9), 3419T>C (V1140A), 3836A>G (D1279G), 3886G>A (D1296N) and 3889G>A (V1297I) at 0.483, 0.463, 0.387, 0.005, 0.384, 0.005, 0.387, 0.002, 0.012, and 0.015 frequencies, respectively. Linkage disequilibrium between detected variations was also analyzed. Our results would provide fundamental and useful information for genotyping *ATP7A* and *ATP7B* in the Japanese and probably other Asian populations.

Keywords: copper transporter, genetic variation, Japanese

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Sugiyama, E., Lee, S.J.[*1], Lee, S.S.[*1], Kim, W.Y.[*1], Kim, S.R., Tohkin, M., Hasegawa, R., Okuda, H., Kawamoto, M.[*2], Kamatani, N.[*2], Sawada, J., Kaniwa, N., Saito, Y. and Shin, J.G.[*1]: **Ethnic differences of two non-synonymous single nucleotide polymorphisms in CDA gene**

Drug Metab. Phatmacokinet., **24**, 553-559 (2009)

Cytidine deaminase, encoded by the *CDA* gene, catalyzes anti-cancer drugs gemcitabine and ara-C into their respective inactive metabolites. In *CDA*, two functionally significant non-synonymous polymorphisms, 79A>C (Lys27Gln) and 208G>A (Ala70Thr), have been found and their minor allele frequencies (MAFs) were reported in Japanese and Chinese patients, and relatively small numbers of healthy volunteers in Caucasians and Africans. In this study, we determined the MAFs of both polymorphisms in 200 healthy volunteers of Koreans, along with 206 Japanese, 200 Chinese-Americans, 150 Caucasian-Americans and 150 African-Americans in order to reveal the ethnic differences. MAFs of 79A>C (Lys27Gln) were 0.153 in Koreans and 0.327 in Caucasian-Americans, 0.204 in

Japanese, 0.155 in Chinese-Americans, and 0.087 in African-Americans. MAFs of 208G>A（Ala70Thr）were 0.005 in Koreans and 0.022 in Japanese, and the minor allele was not detected in Chinese-Americans, Caucasian-Americans or African-Americans. Thus possibly, MAF of 208G>A in Japanese is likely to be somewhat higher than in Koreans and Chinese-Americans. These data would provide fundamental and useful information for pharmacogenetic studies on cytidine deaminase-catalyzing drugs.

Keywords: *CDA*, allele frequency, ethnic-difference

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Matsubara, J.[*1,5], Ono, M.[*1], Honda, K.[*1], Negishi. A.[*1], Ueno, H.[*2], Okusaka, T.[*2], Furuse, J.[*3], Furuta, K.[*3], Sugiyama, E., Saito, Y., Kaniwa, N., Sawada, J., Shoji, A.[*4], Sakuma, T.[*4], Chiba, T.[*5], Saijo, N.[*3], Hirohashi, S.[*1] and Yamada, T.[*1]: **Survival Prediction for Pancreatic Cancer Patients Receiving Gemcitabine Treatment**

Mol. Cell. Proteomics, **9**, 695-704（2010）

Although gemcitabine monotherapy is the standard treatment for advanced pancreatic cancer, patient outcome varies significantly, and a considerable number do not benefit adequately. We therefore searched for new biomarkers predictive of overall patient survival. Using LC-MS, we compared the base-line plasma proteome between 29 representative patients with advanced pancreatic cancer who died within 100 days and 31 patients who survived for more than 400 days after receiving at least two cycles of the same gemcitabine monotherapy. Identified biomarker candidates were then challenged in a larger cohort of 304 patients treated with the same protocol using reverse-phase protein microarray. Among a total of 45,277 peptide peaks, we identified 637 peaks whose intensities differed significantly between the two groups ($p < 0.001$, Welch's t test). Two MS peaks with the highest statistical significance ($p = 2.6 \times 10^{-4}$ and $p = 5.0 \times 10^{-4}$) were revealed to be derived from α1-antitrypsin and α1-antichymotrypsin, respectively. The levels of α1-antitrypsin ($p = 8.9 \times 10^{-8}$) and α1-antichymotrypsin ($p = 0.001$) were significantly correlated with the overall survival of the 304 patients. We selected α1-antitrypsin ($p = 0.0001$), leukocyte count ($p = 0.066$), alkaline phosphatase ($p = 8.3 \times 10^{-8}$), and performance status ($p = 0.003$) using multivariate Cox regression analysis and constructed a scoring system (nomogram) that was able to identify a group of high risk patients having a short median survival time of 150 days (95% confidence interval, 123－187 days; $p = 2.0 \times 10^{-15}$, log rank test). The accuracy of this model for prognostication was internally validated and showed good calibration and discrimination with a bootstrap-corrected concordance index of 0.672. In conclusion, an increased level of α1-antitrypsin is a biomarker that predicts short overall survival of patients with advanced pancreatic cancer receiving gemcitabine monotherapy. Although an external validation study will be necessary, the current model may be useful for identifying patients unsuitable for the standardized therapy.

Keywords: gemcitabine, proteomics, biomarker

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Komeiji, Y.[*1], Mochizuki, Y.[*2], Nakano, T., Fedorov, D. G.[*1]: **Fragment Molecular Orbital-based Molecular Dynamics（FMO-MD）, a quantum simulation tool for large molecular systems**

J. Mol. Struct.（*Theochem*）, **898**, 2-7（2009）

Fragment Molecular Orbital-based Molecular Dynamics（FMO-MD）is an ab initio molecular dynamics method based on the Fragment Molecular Orbital method. FMO-MD is a general tool for quantummechanical MD simulations of large molecular systems that works because of the high parallel efficiency and accuracy of FMO. We review the methodology of FMO-MD and its applications to the conformation sampling of formaldehyde in a solvent, the direct simulation of hydrolysis of methyl diazonium cation, and the comparison of free energy profiles of the Menschutkin reaction in the presence and absence of the solvent. Based on these studies, we compare FMO-MD with other MD methods and discuss the future prospects of the FMO-MD method.

Keywords: FMO-MD, hydrolysis, Menschutkin reaction

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Inoue, T. and Hirabayashi, Y.: **Hematopoietic neoplastic diseases develop in C3H/He and C57BL/6 mice after benzene exposure: strain differences in bone marrow tissue responses observed using microarrays**

Chem. Biol. Interact., **184**, 240-245 (2010)

In this study, Trp53-deficient and wild-type mice of both C57BL/6 and C3H/He strains were exposed to benzene (33, 100, and 300 ppm; 6h/day, 5 days/week for 26 weeks) and then observed for lifetime. As results, first, the incidence of nonthymic lymphomas in C57BL/6 mice and acute myeloid leukemias (AMLs) in C3H/He mice showed linear responses at the lower exposure level in Trp53-deficient mice; second, the incidence of thymic lymphomas in C57BL/6 mice and nonthymic lymphomas in C3H/He mice increased without a plateau-like ceiling; thus, the former equivocal induction of hematopoietic neoplasms (HPNs) in the case of low-dose benzene exposure was assumed to be based on the DNA repair potential in wild-type mice, and the latter limited increase in HPNs in the case of high-dose benzene exposure was considered to be due to excessive apoptosis in wild-type mice. Concerning the incidence of AMLs, though a dose of 300 ppm benzene inhalation induced 9% AMLs in wild-type C3H/He mice-AML-prone, it induced AMLs in 38% of Trp53-deficient C3H/He mice. Because AMLs were also observed in Trp53-deficient mice, including in the C57BL/6 mice, benzene exposure may also be a potent inducer of AMLs in mice with some strain differences. In the present study, to elucidate the hematopoietic stem cell-specific, aryl hydrocarbon-receptor-related low-dose adverse effect, global gene expression in the bone marrow was analyzed at 28 days after 2-week-intermittent exposure to 150 mg/kg b.w. benzene, by gavage, i.e., equivalent to the above inhalation protocol with 300 ppm. We observed two conceptually different gene expression profiles; "common gene profiles" (CGPs) shared among mice in each group, and "stochastic gene profiles" (SGPs), i.e., unique union genes from one individual mouse to another. The CGPs of the experimental group and the SGPs of each individual mouse were separately characterized by individual assay. Concerning the CGPs, reciprocal strain differences between C3H/He and C57BL/6 mice in expression gene profiles, both plausible for leukemogenesis, were identified; namely, dominant downmodulations of Sltm and Cryl1, related to suppression of apoptosis and genomic instability in C3H/He mice, respectively, and dominant downmodulations of Atrx/rad54 and Kdm2a, related to a decrease in DNA repair and genomic instability, respectively, in C57BL/6 mice. These findings imply that these reciprocal gene expression differences induced by benzene exposure may lead each strain to undergo different hematopoietic neoplastic pathways. In contrast, each individual mouse often shows a unique SGP. SGPs often include transcription factors, which regulate reciprocal signaling pathways including further SGPs. Among them, apoptosis-related genes expressed in C57BL/6 mice and those in C3H/He mice were attributable to different combinations of SGPs. Such stochastic case-by-case gene expression may be in good agreement with the individual and strain differences observed following benzene exposure. Because gene chip microarray techniques can elucidate stochastic changes in gene expression profiles, possible stochastic toxicology and its future role are discussed.

Keywords: stochastic gene profiles, common gene profiles, Trp53-deficient mice

Yi, J. Y.*, Hirabayashi, Y., Choi, Y. K.*, Kodama, Y., Kanno, J., Han, J. H.*, Inoue, T. and Yoon, B.I.*: **Benzene activates caspase-4 and -12 at the transcription level, without an association with apoptosis, in mouse bone marrow cells lacking the p53 gene**

Arch Toxicol, **83**, 795-803 (2009)

Benzene is a well-known environmental pollutant that can induce hematotoxicity, aplastic anemia, acute myelogenous leukemia, and lymphoma. However, although benzene metabolites are known to induce oxidative stress and disrupt the cell cycle, the mechanism underlying lympho/leukemogenicity is not fully understood. Caspase-4 (alias caspase-11) and -12 are inflammatory caspases implicated in inflammation and endoplasmic reticulum stress-induced apoptosis. The objectives of this study were to investigate the altered expression of caspase-4 and -12 in mouse bone marrow after benzene exposure and to determine whether their alterations are associated with benzene-induced bone marrow toxicity, especially cellular apoptosis. In addition, we evaluated whether the p53 gene is involved in

regulating the mechanism, using both wild-type (WT) mice and mice lacking the p53 gene. For this study, 8-week-old C57BL/6 mice [WT and p53 knockout (KO)] were administered a benzene solution (150 mg/kg diluted in corn oil) via oral gavage once daily, 5 days/week, for 1 or 2 weeks. Blood and bone marrow cells were collected and cell counts were measured using a Coulter counter. Total mRNA and protein extracts were prepared from the harvested bone marrow cells. Then qRT-PCR and Western blotting were performed to detect changes in the caspases at the mRNA and protein level, respectively. A DNA fragmentation assay and Annexin-V staining were carried out on the bone marrow cells to detect apoptosis. Results indicated that when compared to the control, leukocyte number and bone marrow cellularity decreased significantly in WT mice. The expression of caspase-4 and -12 mRNA increased significantly after 12 days of benzene treatment in the bone marrow cells of benzene-exposed p53KO mice. However, apoptosis detection assays indicated no evidence of apoptosis in p53KO or WT mice. In addition, no changes of other apoptosis-related caspases, such as caspase-3 and -9, were found in WT or p53KO mice at the level of mRNA and proteins. These results indicated that upregulation of caspase-4 and -12 in mice lacking the p53 gene is not associated with cellular apoptosis. In conclusion, caspase-4 and -12 can be activated by benzene treatment without inducing cell apoptosis in mouse bone marrow, which are partly under the regulation of the p53 gene.

Keywords: Gene Expression Regulation, Bone Marrow Cells, Benzene

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Kawasaki, Y., Hirabayashi, Y., Kaneko, T., Kanno, J., Kodama, Y., Matsushima, Y., Ogawa, Y., Saitoh, M., Sekita, K., Uchida, O., Umemura, T., Yoon, B.I. and Inoue, T.: **Benzene-induced hematopoietic neoplasms including myeloid leukemia in Trp53-deficient C57BL/6 and C3H/He mice**

Toxicol. Sci., **110**, 293-306 (2009)

This research focused on three major questions regarding benzene-induced hematopoietic neoplasms (HPNs). First, why are HPNs induced equivocally and at only threshold level with low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses both in experiments and in epidemiology? Second, why is there no linear increase in incidence at high-dose exposure despite a lower acute toxicity (LD (50) > 1000 mg/kg body weight; WHO, 2003, Benzene in drinking-water. Background document for development of WHO Guidelines for Drinking-Water Quality)? Third, why are particular acute myeloid leukemias (AMLs) not commonly observed in mice, although AMLs are frequently observed in human cases of occupational exposure to benzene? In this study, we hypothesized that the threshold-like equivocal induction of HPNs at low-dose benzene exposure is based on DNA repair potential in wild-type mice and that the limited increase in HPNs at a high-dose exposure is due to excessive apoptosis in wild-type mice. To determine whether Trp53 deficiency satisfies the above hypotheses by eliminating or reducing DNA repair and by allowing cells to escape apoptosis, we evaluated the incidence of benzene-induced HPNs in Trp53-deficient C57BL/6 mice with specific regard to AMLs. We also used C3H/He mice, AML prone, with Trp53 deficiency to explore whether a higher incidence of AMLs on benzene exposure might explain the above human-murine differences. As a result, heterozygous Trp53-deficient mice of both strains showed a nonthreshold response of the incidence of HPNs at the lower dose, whereas both strains showed an increasing HPN incidence up to 100% with increasing benzene exposure dose, including AMLs, that developed 38% of heterozygous Trp53-deficient C3H/He mice compared to only 9% of wild-type mice exposed to the high dose. The detection of AMLs in heterozygous Trp53-deficient mice, even in the C57BL/6 strain, implies that benzene may be a potent inducer of AMLs also in mice with some strain differences.

Keywords: Acute Myeloid Leukemia, strain differences, Benzene

Kuzumaki, N.[*1], Ikegami, D.[*1], Tamura, R.[*1], Sasaki, T.[*1], Niikura, K.[*1], Narita, M.[*1], Miyashita, K.[*1], Imai, S.[*1], Takeshima, H.[*1], Ando, T.[*1], Igarashi, K., Kanno, J., Ushijima, T.[*2], Suzuki, T.[*1], Narita, M.[*1]: **Hippocampal epigenetic modification at the doublecortin gene is involved in the impairment of neurogenesis with aging**

Synaps., [Epub ahead of print] (2010)

Recent research has suggested that epigenetic mechanisms, which exert lasting control over gene expression without altering the genetic code, could mediate stable changes in brain function. A growing body of evidence supports the idea that epigenetic changes play a role in the etiology of aging and its associated brain dysfunction. The present study was undertaken to evaluate the age-related changes in the expression of doublecortin, which is a marker for neuronal precursors, along with epigenetic modification in the hippocampus of aged mice. In the present study, the doublecortin-positive cells were almost completely absent from the dentate gyrus of the hippocampus of 28-month-old mice. Furthermore, the expression level of doublecortin mRNA was significantly decreased in the hippocampus of aged mice. Under these conditions, a significant decrease in H3K4 trimethylation and a significant increase in H3K27 trimethylation at doublecortin promoters were observed with aging without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. These findings suggest that aging produces a dramatic decrease in the expression of doublecortin along with epigenetic modifications in the hippocampus.

Keywords: epigenetic modification, Neurogenesis, hippocampus

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Kuzumaki, N.[*1], Ikegami, D.[*1], Tamura, R.[*1], Hareyama, N.[*1], Imai, S.[*1], Narita, M.[*1], Torigoe, K.[*1], Niikura, K.[*1], Takeshima, H.[*1], Ando, T.[*1], Igarashi, K., Kanno, J., Ushijima, T.[*2], Suzuki, T.[*1], Narita, M.[*1]: **Hippocampal epigenetic modification at the brain-derived neurotrophic factor gene induced by an enriched environment**

Hippocampus, [Epub ahead of print] (2010)

Environmental enrichment is an experimental paradigm that increases brain-derived neurotrophic factor (BDNF) gene expression accompanied by neurogenesis in the hippocampus of rodents. In the present study, we investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice. Exposure to an enriched environment for 3-4 weeks caused a dramatic increase in the mRNA expression of BDNF, but not platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), epidermal growth factor (EGF), or glial fibrillary acidic protein (GFAP), in the hippocampus of mice. Under these conditions, exposure to an enriched environment induced a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters, in contrast to significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. The expression levels of several microRNAs in the hippocampus were not changed by an enriched environment. These results suggest that an enriched environment increases BDNF mRNA expression via sustained epigenetic modification in the mouse hippocampus.

Keywords: epigenetic modification, brain-derived neurotrophic factor, hippocampus

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[*2] Carcinogenesis Division, National Cancer Center Research Institute

Suzuki, A.[*1], Igarashi, K., Aisaki, K., Kanno, J., Saga, Y.[*2]: **NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs**

Proc. Natl. Acad. Sci. USA., **107**(8), 3594-3599 (2010)

Nanos is one of the evolutionarily conserved proteins implicated in germ cell development. We have previously shown that NANOS2 plays an important role in both the maintenance and sexual development of germ cells. However, the molecular mechanisms underlying these events have remained elusive. In our present study, we found that NANOS2 localizes to the P-bodies, known centers of RNA degradation that are abundantly accumulated in male gonocytes. We further identified byimmunoprecipitation that the components of the CCR4-NOT deadenylation complex areNANOS2-interacting proteins and found that NANOS2 promotes the locali-

zation of CNOT proteins to P-bodies in vivo. We also elucidated that the NANOS2/CCR4-NOT complex has deadenylase activity in vitro, and that some of the RNAs implicated in meiosis interact with NANOS2 and are accumulated in its absence. Our current data thus indicate that the expression of these RNA molecules is normally suppressed via a NANOS2-mediated mechanism. We propose from our current findings that NANOS2-interacting RNAs may be recruited to P-bodies and degraded by the enzymes contained therein through NANOS2-mediated deadenylation.

Keywords: NANOS2, germ cell development, suppression of RNA

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Saegusa, Y.*1, Woo, GH.*1, Fujimoto, H.*1, Inoue, K., Takahashi, M., Hirose, M.*2, Igarashi, K., Kanno, J., Mitsumori, K.*1, Nishikawa, A., Shibutani, M.*1: **Gene expression profiling and cellular distribution of molecules with altered expression in the hippocampal CA1 region after developmental exposure to anti-thyroid agents in rats**

J. Vet. Med. Sci., **72**(2), 187-195 (2010)

To determine whether developmental hypothyroidism causes permanent disruption of neuronal development, we first performed a global gene expression profiling study targeting hippocampal CA1 neurons in male rats at the end of maternal exposure to anti-thyroid agents on weaning (postnatal day 20). As a result, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were up-regulated and those for myelination were often down-regulated. We, then, examined immunohistochemical cellular distribution of Ephrin type A receptor 5 (EphA5) and Tachykinin receptor (Tacr)-3, those selected based on the gene expression profiles, in the hippocampal formation at the adult stage (11-week-old) as well as at the end of exposure. At weaning, both EphA5- and Tacr3-immunoreactive cells with strong intensities appeared in the pyramidal cell layer or stratum oriens of the hippocampal CA1 region. Although the magnitude of the change was decreased at the adult stage, Tacr3 in the CA1 region showed a sustained increase in expressing cells until the adult stage after developmental hypothyroidism. On the other hand, EphA5-expressing cells did not show sustained increase at the adult stage. The results suggest that developmental hypothyroidism caused sustained neuronal expression of Tacr3 in the hippocampal CA1 region, probably reflecting a neuroprotective mechanism for mismigration.

Keywords: developmental exposure, anti-thyroid agents, hippocampal CA1 region

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Sekine, H.*1, Mimura, J.*1, Oshima, M.*1, Okawa, H.*1, Kanno, J., Igarashi, K., Gonzalez, FJ.*2, Ikuta, T.*3, Kawajiri, K.*3, Fujii-Kuriyama, Y.*4: **Hypersensitivity of aryl hydrocarbon receptor-deficient mice to lipopolysaccharide-induced septic shock**

Mol. Cell. Biol., **29**(24), 6391-6400 (2010)

Aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, is known to mediate a wide variety of pharmacological and toxicological effects caused by polycyclic aromatic hydrocarbons. Recent studies have revealed that AhR is involved in the normal development and homeostasis of many organs. Here, we demonstrate that AhR knockout (AhR KO) mice are hypersensitive to lipopolysaccharide (LPS)-induced septic shock, mainly due to the dysfunction of their macrophages. In response to LPS, bone marrow-derived macrophages (BMDM) of AhR KO mice secreted an enhanced amount of interleukin-1beta (IL-1beta). Since the enhanced IL-1beta secretion was suppressed by supplementing Plasminogen activator inhibitor-2 (Pai-2) expression through transduction with Pai-2-expressing adenoviruses, reduced Pai-2 expression could be a cause of the increased IL-1beta secretion by AhR KO mouse BMDM. Analysis of gene expression revealed that AhR directly regulates the expression of Pai-2 through a mechanism involving NF-kappaB but not AhR nuclear translocator (Arnt), in an LPS-dependent manner. Together with the result that administration of the AhR ligand 3-methylcholanthrene partially protected mice with wild-type AhR from endotoxin-induced death, these results raise the possibility that an appropriate AhR ligand may be useful for treating

patients with inflammatory disorders.
Keywords: aryl hydrocarbon receptor, septic shock, hypersensitivity

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Oginuma, M.*, Takahashi, Y., Kitajima, S., Kiso, M.*, Kanno, J., Kimura, A.*, Saga, Y.*: **The oscillation of Notch activation, but not its boundary, is required for somite border formation and rostral-caudal patterning within a somite**
Development, **137**, 1515-1522 (2010)

Notch signaling exerts multiple roles during different steps of mouse somitogenesis. We have previously shown that segmental boundaries are formed at the interface of the Notch activity boundary, suggesting the importance of the Notch on/off state for boundary formation. However, a recent study has shown that mouse embryos expressing Notch-intracellular domain (NICD) throughout the presomitic mesoderm (PSM) can still form more than ten somites, indicating that the NICD on/off state is dispensable for boundary formation. To clarify this discrepancy in our current study, we created a transgenic mouse lacking NICD boundaries in the anterior PSM but retaining Notch signal oscillation in the posterior PSM by manipulating the expression pattern of a Notch modulator, lunatic fringe. In this mouse, clearly segmented somites are continuously generated, indicating that the NICD on/off state is unnecessary for somite boundary formation. Surprisingly, this mouse also showed a normal rostral-caudal compartment within a somite, conferred by a normal Mesp2 expression pattern with a rostral-caudal gradient. To explore the establishment of normal Mesp2 expression, we performed computer simulations, which revealed that oscillating Notch signaling induces not only the periodic activation of Mesp2 but also a rostral-caudal gradient of Mesp2 in the absence of striped Notch activity in the anterior PSM. In conclusion, we propose a novel function of Notch signaling, in which a progressive oscillating wave of Notch activity is translated into the rostral-caudal polarity of a somite by regulating Mesp2 expression in the anterior PSM. This indicates that the initial somite pattern can be defined as a direct output of the segmentation clock.
Keywords: Notch signal, segmentation clock, lunatic fringe

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Tanemura, K., Igarashi, K., Matsugami, TR., Aisaki, K., Kitajima, S., Kanno, J.: **Brain structure impairment and behavioral disturbance induced in male mice offspring by a single intraperitoneal administration of domoic acid (DA) to their dams**
J. Toxicol. Sci., **34**, Suppl. 2, SP279-286 (2009)

To demonstrate induction of delayed central nervous toxicity by disturbing neuronal activities in the developing brain, we administered a single intraperitoneal dose of domoic acid (DA; 1 mg/kg), a potent glutamate receptor agonist, to pregnant female mice at the gestational day of 11.5, 14.5 or 17.5. The dams had recovered from acute symptoms within 24 hr, followed by normal delivery, feeding and weaning. All male offspring mice after weaning were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. At the age of 11 weeks, our neurobehavior testing battery revealed severe impairment of learning and memory with serious deviances of anxiety-related behaviors. The developed brain of prenatally exposed mice showed myelination failure and the overgrowth of neuronal processes of the limbic cortex neurons. This study indicates that the temporal disturbance of neurotransmission of the developing brain induces irreversible structural and functional damage to offspring which becomes monitorable in their adulthood by a proper battery of neurobehavioral tests.
Keywords: domoic acid, prenatal exposure, behavior

Sekiyama, K.*1, Hashimoto, O.*1, Ushiro, Y.*1, Adachi, C.*1, Kikusui, T.*2, Tanemura, K., Hasegawa, Y.*1: **Abnormalities in aggression and anxiety in transgenic mice overexpressing activin E**
Biochem. Biophys. Res. Commun., **385**(3), 319-323 (2009)

To study the function of activin E, a TGF-beta superfamily member, in the regulation of affective behavior,

we investigated the behavior of transgenic mice overexpressing activin E (TgActbetaE mice). Male TgActbetaE mice showed aggressive behavior in resident-intruder tests. In elevated plus-maze tests, the percentage of open arm entries was significantly increased in female TgActbetaE mice compared with that in wild-type mice. Furthermore, female TgActbetaE mice stayed in the central area for a significantly longer time than wild-type mice in open field tests. These results indicated that TgActbetaE mice had less anxiety-like behavior. The number of restraint-stress-evoked c-Fos-positive cells in the hypothalamic paraventricular nucleus in TgActbetaE mice was significantly decreased compared with that in wild-type mice. This suggests that synthesis of corticotrophin-releasing hormone induced by stress was decreased in TgActbetaE mice. Taking these results together, activin E may act as a regulator of the hypothalamic-pituitary-adrenal axis.

Keywords: activin E, aggression, anxiety

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Hirabayashi, Y., and Inoue, T.: **Benzene-induced bone-marrow toxicity: a hematopoietic stem-cell-specific, aryl hydrocarbon receptor-mediated adverse effect**

Chem. Biol. Interact., **184**, 252-258 (2010)

Benzene-induced hematopoietic toxicity is an aryl hydrocarbon receptor (AhR)-related adverse effect that is not exhibited in AhR-knockout (KO) mice. In the hematopoietic system, the steady-state expression of AhRs is limited in the hematopoietic progenitor cells; thus, a hierarchical hematopoietic impairment starts from hematopoietic progenitor cells after benzene exposure. When one looks at wild-type recipient mice that have been lethally irradiated and repopulated with AhR-KO bone marrow cells, owing to reconstruction by the marrow from AhR-KO mice, no impairment is observed in the assay of granulo-macrophage colony-forming units (CFU-GMs) in the bone marrow after benzene exposure of the reconstituted mice. In contrast, in mature white blood cells concern, benzene-induced hematopoietic cytotoxicity is observed in the same reconstituted mice; however, this benzene-induced hematopoietic cytotoxicity in mature white blood cells is not induced in the case of AhR-KO mice repopulated with wild-type bone marrow cells after a lethal dose of irradiation. The mechanism of benzene-induced hematopoietic toxicity in the mature blood cells in AhR-KO mice is assumed to be based on metabolites such as phenol and hydroquinone derived from hepatic AhR. Thus, the former toxicity in mature white blood cells is assumed to be based on the metabolites of the wild-type hepatic AhR, whereas the latter lack of toxicity in mature blood cells in AhR-KO mice is due to the lack of benzene-induced metabolism in the liver. Global gene expression analysis of bone marrow cells after benzene exposure reveals that MEF2c, the functions of which are known to maintain lymphocyte differentiation and promote proliferation of hematopoietic progenitor cells, is commonly downmodulated not only in C57BL/6 but also in C3H/He mice. In response to these impairments of the hematopoietic progenitor cells and the niches, stochastic and reciprocal upregulations of integrin beta 2 and the Runx family are observed, which are known to stabilize hematopoietic niches during the steady-state. Direct observation of the hematopoietic progenitor cells, particularly the Lin(-)c-kit(+)Sca-1(+) (LKS) fraction, after benzene exposure revealed an increased amount of intracytoplasmic reactive oxygen species (ROS) detected by ROS-reacting dye as compared with other blood cell fractions.

Keywords: aryl hydrocarbon receptor, hematopoietic niches, MEF2c

Yi, J.Y.*, Hirabayashi, Y., Choi, Y.K.*, Kodama, Y., Kanno, J., Han, J.H.*, Inoue, T., and Yoon, B.I.*: **Benzene activates caspase-4 and -12 at the transcription level, without an association with apoptosis, in mouse bone marrow cells lacking the p53 gene**

Arch. Toxicol., **83**, 795-803 (2009)

Benzene is a well-known environmental pollutant that can induce hematotoxicity, aplastic anemia, acute myelogenous leukemia, and lymphoma. However, although benzene metabolites are known to induce oxidative stress and disrupt the cell cycle, the mechanism underlying lympho/leukemogenicity is not fully understood. Caspase-4 (alias caspase-11) and -12 are inflammatory caspases implicated in inflammation and endoplasmic reticulum stress-induced apoptosis. The

objectives of this study were to investigate the altered expression of caspase-4 and -12 in mouse bone marrow after benzene exposure and to determine whether their alterations are associated with benzene-induced bone marrow toxicity, especially cellular apoptosis. In addition, we evaluated whether the p53 gene is involved in regulating the mechanism, using both wild-type (WT) mice and mice lacking the p53 gene. For this study, 8-week-old C57BL/6 mice [WT and p53 knockout (KO)] were administered a benzene solution (150 mg/kg diluted in corn oil) via oral gavage once daily, 5 days/week, for 1 or 2 weeks. Blood and bone marrow cells were collected and cell counts were measured using a Coulter counter. Total mRNA and protein extracts were prepared from the harvested bone marrow cells. Then qRT-PCR and Western blotting were performed to detect changes in the caspases at the mRNA and protein level, respectively. A DNA fragmentation assay and Annexin-V staining were carried out on the bone marrow cells to detect apoptosis. Results indicated that when compared to the control, leukocyte number and bone marrow cellularity decreased significantly in WT mice. The expression of caspase-4 and -12 mRNA increased significantly after 12 days of benzene treatment in the bone marrow cells of benzene-exposed p53KO mice. However, apoptosis detection assays indicated no evidence of apoptosis in p53KO or WT mice. In addition, no changes of other apoptosis-related caspases, such as caspase-3 and -9, were found in WT or p53KO mice at the level of mRNA and proteins. These results indicated that upregulation of caspase-4 and -12 in mice lacking the p53 gene is not associated with cellular apoptosis. In conclusion, caspase-4 and -12 can be activated by benzene treatment without inducing cell apoptosis in mouse bone marrow, which are partly under the regulation of the p53 gene.

Keywords: gene expression regulation, bone marrow cells, benzene

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Kawasaki, Y., Hirabayashi, Y., Kaneko, T., Kanno, J., Kodama, Y., Matsushima, Y., Ogawa, Y., Saitoh, M., Sekita, K., Uchida, O., Umemura, T., Yoon, B.I. and Inoue, T.: **Benzene-induced hematopoietic neoplasms including myeloid leukemia in Trp53-deficient C57BL/6 and C3H/He mice**

Toxicol. Sci., **110**, 293-306 (2009)

This research focused on three major questions regarding benzene-induced hematopoietic neoplasms (HPNs). First, why are HPNs induced equivocally and at only threshold level with low-dose benzene exposure despite the significant genotoxicity of benzene even at low doses both in experiments and in epidemiology? Second, why is there no linear increase in incidence at high-dose exposure despite a lower acute toxicity (LD (50) > 1000 mg/kg body weight; WHO, 2003, Benzene in drinking-water. Background document for development of WHO Guidelines for Drinking-Water Quality)? Third, why are particular acute myeloid leukemias (AMLs) not commonly observed in mice, although AMLs are frequently observed in human cases of occupational exposure to benzene? In this study, we hypothesized that the threshold-like equivocal induction of HPNs at low-dose benzene exposure is based on DNA repair potential in wild-type mice and that the limited increase in HPNs at a high-dose exposure is due to excessive apoptosis in wild-type mice. To determine whether Trp53 deficiency satisfies the above hypotheses by eliminating or reducing DNA repair and by allowing cells to escape apoptosis, we evaluated the incidence of benzene-induced HPNs in Trp53-deficient C57BL/6 mice with specific regard to AMLs. We also used C3H/He mice, AML prone, with Trp53 deficiency to explore whether a higher incidence of AMLs on benzene exposure might explain the above human-murine differences. As a result, heterozygous Trp53-deficient mice of both strains showed a nonthreshold response of the incidence of HPNs at the lower dose, whereas both strains showed an increasing HPN incidence up to 100% with increasing benzene exposure dose, including AMLs, that developed 38% of heterozygous Trp53-deficient C3H/He mice compared to only 9% of wild-type mice exposed to the high dose. The detection of AMLs in heterozygous Trp53-deficient mice, even in the C57BL/6 strain, implies that benzene may be a potent inducer of AMLs also in mice with some strain differences.

Keywords: acute myeloid leukemia, strain differences, benzene

Upham, BL.[*1], Park, JS.[*1], Babica, P.[*1], Sovadinova, I.[*1], Rummel, AM.[*1], Trosko, JE.[*1], Hirose, A.[*2], Hasegawa,

R.[*2], Kanno, J., Sai, K.[*3]: **Structure-activity-dependent regulation of cell communication by perfluorinated fatty acids using in vivo and in vitro model systems**

Environ. Health Perspect., **117**(4), 545-551 (2009)

Perfluoroalkanoates, [e.g., perfluorooctanoate (PFOA)], are known peroxisome proliferators that induce hepatomegaly and hepatocarcinogenesis in rodents, and are classic nongenotoxic 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. 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: gap-junctional intercellular communication, perfluorooctanoate, tumor promotion

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Kanno, J.: **Overview: "Children's toxicology", a renovating study field of irreversible "early exposure-delayed effects"**

J. Toxicol. Sci., **34**, Suppl 2, SP199-200 (2009)

"Children are not small adults". This is a well-known phrase, especially in the clinics for diagnosis, efficacy of treatment, side effect, and prognosis. However, in the field of toxicology, this issue has long been a challenge. The knowledge has been limited to the differences in metabolism and other physiological factors. Currently available test guidelines for fetuses and immature animals are teratogenicity and reproductive toxicity studies. These tests look for straight-forward (essentially macroscopic) outcomes established within a rather short period of exposure to the test substances. However, recent advances in molecular toxicology allow combination of in vitro and in vivo studies at molecular levels. The target molecules and receptors can be identified in quantitative fashion and at the fine structure levels around and below the resolution of normal light microscopy. Such expansion of the knowledge lead us to consider a rather new category of "receptor mediated toxicity" or "signal toxicity". Such non-organic insults would merely induce transient effects on adults. However, there are growing evidences that such slight insults on the developing and maturating organisms can leave irreversible effects that become overt in adulthood. As an overview, toxicology has entered a new phase where children's toxicology becomes a renovating study field of the irreversible "early exposure-delayed effects".

Keywords: children's toxicology, receptor-mediated toxicity, early exposure-delayed effect

Xu, J.[*1], Futakuchi, M.[*1], Iigo, M.[*1], Fukamachi, K.[*1], Alexander, DB.[*1], Shimizu, H.[*1], Sakai, Y.[*1], Tamano, S.[*1], Furukawa, F.[*1], Uchino, T.[*2], Tokunaga, H.[*2], Nishimura, T.[*2], Hirose, A.[*3], Kanno, J., Tsuda, H.[*1]: **Involvement of macrophage inflammatory protein 1alpha (MIP1alpha) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intra-pulmonary spraying**

Carcinogenesis, **31**(5), 927-935 (2010)

Titanium dioxide (TiO(2)) is evaluated by World Health Organization/International Agency for Research on Cancer as a Group 2B carcinogen. The present study was conducted to detect carcinogenic activity of nanoscale TiO(2) administered by a novel intrapulmonary spraying (IPS)-initiation-promotion protocol in the rat lung. Female human c-Ha-ras proto-oncogene transgenic rat (Hras128) transgenic rats were treated first with N-nitrosobis (2-hydroxypropyl) amine (DHPN) in the drinking water and then with TiO(2) (rutile type, mean diameter 20 nm, without coating) by IPS. TiO(2) treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas, confirming the effectiveness of the IPS-initiation-promotion protocol. TiO(2) aggregates were localized exclusively in alveolar macrophages and had a mean diameter of 107.4 nm. To investigate the underlying mechanism of its carcinogenic effects, TiO(2) was administered to wild-type rats by IPS five times over 9 days. TiO(2) treatment significantly increased

8-hydroxydeoxy guanosine level, superoxide dismutase activity and macrophage inflammatory protein 1alpha (MIP1alpha) expression in the lung. MIP1alpha, detected in the cytoplasm of TiO(2)-laden alveolar macrophages in vivo and in the media of rat primary alveolar macrophages treated with TiO(2) in vitro, enhanced proliferation of human lung cancer cells. Furthermore, MIP1alpha, also detected in the sera and mammary adenocarcinomas of TiO(2)-treated Hras128 rats, enhanced proliferation of rat mammary carcinoma cells. These data indicate that secreted MIP1alpha from TiO(2)-laden alveolar macrophages can cause cell proliferation in the alveoli and mammary gland and suggest that TiO(2) tumor promotion is mediated by MIP1alpha acting locally in the alveoli and distantly in the mammary gland after transport via the circulation.

Keywords: macrophage inflammatory protein 1alpha, nanoscale titanium dioxide particles, tumor promotion

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Takahashi, K.*, Ishii-Nozawa, R.*, Takeuchi, K.*, Nakazawa, K., Sato, K.: **Two NSAIDs, niflumic acid and diclofenac, inhibit the human glutamate transporter EAAT1 through different mechanisms**

J. Pharmacol. Sci., **112**, 113-117 (2010)

We investigated the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on substrate-induced currents of L-Glutamate (L-Glu) transporter EAAT1 expressed in *Xenopus laevis* oocytes. Niflumic acid (NFA) and diclofenac inhibited L-Glu-induced current through EAAT1 in a non-competitive manner. NFA produced a leftward shift in reversal potential (E_{rev}) of L-Glu-induced current and increased current amplitude at the potentials more negative than -100 mV. Diclofenac had no effects on E_{rev} and inhibited the current amplitude to the same extent at all negative potentials. These results indicate that NFA and diclofenac inhibit the L-Glu-induced EAAT1 current via different mechanisms.

Keywords: L-Glutamate transporter, niflumic acid, diclofenac

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Legendre, C.*, Hori, T., Loyer, P.*, Aninat, C.*, Ishida, S., Glaise, D.*, Lucas-Clerc, C.*, Boudjema, K.*, Guguen-Guillouzo, C.*, Corlu, A.*, Morel, F.*: **Drug-metabolising enzymes are down-regulated by hypoxia in differentiated human hepatoma HepaRG cells: HIF-1alpha involvement in CYP3A4 repression**

Eur. J. Cancer., **45**, 2882-2892 (2009)

Weak blood irrigation within solid tumors including hepatocellular carcinomas (HCCs) plays an important role in resistance to anticancer drugs by decreasing accessibility of cytotoxic agents to tumour cells. Reduced oxygen levels, or hypoxia, also contribute to drug resistance because many anticancer drugs require molecular oxygen to be cytotoxic. Our aim was to develop a new in vitro model mimicking hypoxic cells within HCCs in order to further explore the molecular responses to hypoxia, including regulation of drug-metabolising enzymes (DMEs) expression. For this purpose, we used the highly differentiated human hepatoma HepaRG cells cultured under either normoxic or hypoxic conditions. HepaRG cells cultured under hypoxia might mimic metabolic changes occurring within poorly irrigated differentiated HCCs. Furthermore, hypoxia down-regulates hepatic DMEs, a phenomenon that might compromise chemotherapy effectiveness in HCC treatment. Thus, HepaRG cells might represent a new in vitro model to test anticancer agents in hypoxic versus normoxic conditions. In addition, a new role for HIF-1alpha in the repression of CYP3A4 is demonstrated

Keywords : HepaRG cells, drug-metabolising enzymes, hypoxia

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* INSERM U522

Kasuga, J.*, Ishida, S., Yamasaki, D.*, Makishima, M., Doi, T.*, Hashimoto, Y.*, Miyachi, H.*: **Novel biphenylcarboxylic acid peroxisome proliferator-activated receptor (PPAR) delta selective antagonists**

Bioorg. Med. Chem. Lett, **19**, 6595-6599 (2009)

We designed and synthesized novel PPARdelta antagonists based on the crystal structure of the PPARdelta full agonist TIPP-204 bound to the PPARdelta ligand-binding domain, in combination with our nuclear receptor helix 12 folding modification

hypothesis. Representative compound 3a exhibits PPARdelta-preferential antagonistic activity.
Keywords : PPAR delta, selective antagonist

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Miyajima, A., Sunouchi, M., Mitsunaga, K.[*1], Yamakoshi, Y.[*2], Nakazawa, K., Usami, M.: **Sexing of postimplantation rat embryos in stored two-dimensional electrophoresis samples by polymerase chain reaction of an *Sry* sequence**

J. Toxicol. Sci., **34**, 681-685（2009）

Proteomic analysis of developmental toxicity by two-dimensional electrophoresis（2-DE）may detect gender-related toxic effects in embryos without visible gender characteristics. In the present study, we explored sexing of rat embryo stored in frozen 2-DE samples by polymerase chain reaction（PCR）of a male-specific gene sequence, sex determining region Y（*Sry*）. The embryo proper and yolk sac membrane at gestation day 11 from Wistar rats were used for stored embryonic 2-DE samples. The embryonic 2-DE samples were desalted and their total DNA was extracted. The *Sry* sequence in the extracted DNA was amplified by PCR and the product was analyzed by agarose gel electrophoresis. The embryos with the PCR product of *Sry* were determined as male, and those without the product were determined as female. It was concluded that stored embryonic 2-DE samples could be used for retrospective examination of gender-related effects in proteomic analysis of developmental toxicity.
Keywords: developmental toxicity, embryo, sexing, 2-DE

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Usami, M., Nakajima, M.[*1], Mitsunaga, K.[*2], Miyajima, A., Sunouchi, M. and Doi, O.[*3]: **Proteomic analysis of indium embryotoxicity in cultured postimplantation rat embryos**

Reprod. Toxicol., **28**, 477-488（2009）

Indium embryotoxicity was investigated by proteomic analysis with two-dimensional electrophoresis of rat embryos cultured from day 10.5 of gestation for 24h in the presence of 50 microM indium trichloride. In the embryo proper, indium increased quantity of several protein spots including those identified as serum albumin, phosphorylated cofilin 1, phosphorylated destrin and tyrosyl-tRNA synthetase. The increased serum albumin, derived from the culture medium composed of rat serum, may decrease the toxicity of indium. The increase of phosphorylated cofilin 1 might be involved in dysmorphogenicity of indium through perturbation of actin functions. In the yolk sac membrane, indium induced quantitative and qualitative changes in the protein spots. Proteins from appeared spots included stress proteins, and those from decreased or disappeared spots included serum proteins, glycolytic pathway enzymes and cytoskeletal proteins, indicating yolk sac dysfunction. Thus, several candidate proteins that might be involved in indium embryotoxicity were identified.
Keywords: indium, embryotoxicity, rat

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Tasaki, M., Umemura, T., Kijima, A., Inoue, T., Okamura, T., Kuroiwa, Y., Ishii, Y., Nishikawa, A.: **Simultaneous induction of non-neoplastic and neoplastic lesions with highly proliferative hepatocytes following dietary exposure of rats to tocotrienol for 2 years**

Arch. Toxicol., **83**, 1021-1030（2009）

Focusing attention on the pathological intrinsic property of nodular hepatocellular hyperplasia（NHH）, a 104-week carcinogenicity study was performed in male and female Wistar Hannover rats given tocotrienol（TT）at concentrations of 0, 0.4 or 2% in the diet. At necropsy, multiple cyst-like nodules were observed, but were further enlarged in size, which consequently formed a protuberant surface with a partly pedunculated shape in the liver at the high dose in both sexes. NHH was not always accompanied by spongiosis, and instead angiectasis was prominent in some nodules. However, several findings in the affected hepatocytes implied that NHH did not harbor neoplastic characteristics from increased exposure despite sustained high cell proliferation. On the other hand, in the high-dose females, the incidence of hepatocellular adenomas was significantly higher than in the control. The overall data clearly suggested that NHH is successively enlarged by further long-term exposure to TT, but does not be-

come neoplastic. In contrast, TT induces low levels of hepatocellular adenomas in female rats.
Keywords: tocotrienol, nodular hepatocellular hyperplasia

Ishii, Y., Okamura, T., Inoue, T., Tasaki, M., Umemura, T., Nishikawa, A.: **Dietary catechol causes increased oxidative DNA damage in the livers of mice treated with acetaminophen**
Toxicology., **263**, 93-99 (2009)

We have shown that direct reaction of catechol with NO results in generation of reactive oxygen and nitrogen species (RNS) through semiquinone radical formation, leading to oxidative DNA damage in rat forestomach. In the present study, we investigated whether dietary catechol systemically exerts the same effects under NO-rich circumstances. Male ICR mice were treated with or without 0.8% catechol in the diet for 2 weeks followed by acetaminophen (APAP) administration at a dose of 300mg/kg by single i.p. injection. 8-OHdG was significantly increased at 24h in the co-treatment group, but not with either catechol or APAP alone. In view of the finding of positive hepatocytes for NO_2Tyr prior to generation of 8-OHdG, the process of oxidative DNA damage might involve RNS formation. Precise quantitative analysis of NO_2Tyr by means of LC-MS/MS confirmed increase of RNS due to the reaction of catechol with NO produced after APAP-induced hepatitis. The overall data imply that antioxidants with a catechol structure can cause oxidative DNA damage under inflammatory conditions.
Keywords: NO, catechol, oxidative stress

Umemura, T., Tasaki, M., Kijima, A., Okamura, T., Inoue, T., Ishii, Y., Suzuki, Y., Masui, N.*, Nohmi, T., Nishikawa, A.: **Possible participation of oxidative stress in causation of cell proliferation and in vivo mutagenicity in kidneys of gpt delta rats treated with potassium bromate**
Toxicology., **257**, 46-52 (2009)

In the present study, utilizing the antioxidative effects of alpha-tocopherol (alpha-TP) or sodium ascorbic acid (SAA) to attenuate oxidative stress, alterations in BrdU-LIs and reporter gene mutations in kidneys of male and female gpt delta rats given $KBrO_3$ were examined. Five male and female gpt delta rats in each group were given $KBrO_3$ in the drinking water for 9 weeks, with 1% of alpha-TP or SAA administered in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Increases in 8-OHdG levels in kidney DNA by $KBrO_3$ were significantly inhibited by SAA. While BrdU-LIs in the proximal tubules of female rats were also significantly reduced by SAA, those in the males and gpt mutant frequencies in kidney DNA of both sexes were not affected by SAA or alpha-TP. Alpha2u-globulin suggested that induction of cell proliferation observed in the males might primarily result from accumulation of this protein. The overall data indicated that while oxidative stress well correlates with induction of cell proliferation in females, its role in males and in generation of in vivo mutagenicity by $KBrO_3$ in both sexes is limited.
Keywords: $KBrO_3$, oxidative stress, gpt delta rats

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* Japan SLC, Inc.

Dewa, Y.*, Nishimura, J.*, Muguruma, M.*, Jin, M.*, Kawai, M.*, Saegusa, Y.*, Okamura, T., Umemura, T., Mitsumori, K.*: **Involvement of oxidative stress in hepatocellular tumor-promoting activity of oxfendazole in rats**
Arch Toxicol., **83**, 503-511 (2009)

The tumor-promoting effects of oxfendazole (OX), a benzimidazole anthelmintic, were investigated using a medium-term rat hepatocarcinogenesis model. Six-week-old male F344 rats received an intraperitoneal injection of DEN and were given a powdered diet containing 0 or 500 ppm OX for 6 weeks from 2 weeks after DEN treatment. All animals were subjected to two-thirds partial hepatectomy 1 week after OX treatment. The numbers and areas of GST-P-positive foci were significantly increased in the livers of rats treated with OX, with concomitantly increased cell proliferation, compared with those in the livers of the DEN alone group. Quantitative real-time RT-PCR analysis revealed that OX induced mRNA expression of Nrf2-regulated phase II enzymes. Reactive oxygen species production increased in microsomes isolated from the livers of OX-treated rats. Furthermore, OX enhanced oxidative DNA damage and lipid peroxidation. These results suggest that administration of OX at a high dose and for a long term enhances oxidative stress responses, which may contribute to its tumor-promoting potential in rats.
Keywords: oxfendazole, oxidative stress, DEN

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Inoue, K., Yoshida, M., Takahashi, M., Shibutani, M.[*1], Takagi, H., Hirose, M.[*2], Nishikawa, A.: **Induction of kidney and liver cancers by the natural food additive madder color in a two-year rat carcinogenicity study**

Food Chem Toxicol., **47**, 184-191（2009）

Madder color（MC）extracted from the roots of Rubia tinctorum（madder root）has been used as a food coloring in Japan. Our previous studies revealed MC to have obvious subchronic and chronic toxicity and potent carcinogenicity targeting rat liver and kidney. In the present two-year carcinogenicity study, conducted to further elucidate the long-term effects of MC and its target organs, male and female F344 rats were fed diet containing 0%, 2.5%, and 5.0% MC for 104 weeks. Body weights were significantly decreased in treated groups of both sexes throughout the feeding period. However, survival rates at week 104 were higher in treated groups of both sexes than in controls. Relative weights of the kidneys and liver were significantly increased in treated groups of both sexes. Histopathologically, karyomegaly and atypical tubules/hyperplasias, as well as renal cell adenomas and carcinomas were significantly increased in treated groups of both sexes with dose-dependence. Moreover, the incidence of hepatocellular adenomas and/or carcinomas was increased significantly with a dose-relation in treated groups of both sexes. These data provide clear evidence that MC exerts unequivocal carcinogenicity against renal tubule cells and hepatocytes in rats.

Keywords: Carcinogenicity, Madder color, Liver, Kidney, F344 rats

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[*2] Food Safety Commission

Inoue, K., Yoshida, M., Takahashi, M., Fujimoto, H., Ohnishi, K.[*1], Nakashima, K.[*1], Shibutani, M.[*2], Hirose, M.[*3], Nishikawa, A.: **Possible contribution of rubiadin, a metabolite of madder color, to renal carcinogenesis in rats**

Food Chem Toxicol., **47**, 752-759（2009）

Madder color（MC）has been shown to exert carcinogenic potential in the rat kidney in association with degeneration, karyomegaly, increased cell proliferation of renal tubule cells and increased renal 8-OHdG levels. To clarify the causal relationship of components and metabolites of MC to renal carcinogenesis, male F344 rats were fed lucidin-3-O-primeveroside（LuP）or alizarin（Alz）, and the genotoxic LuP metabolites lucidin（Luc）or rubiadin（Rub）for up to 26 weeks. After one week and four weeks, Luc did not induce any renal changes. In contrast, after one week, cortical tubule degeneration was apparent in the Alz and LuP groups, and cytoplasmic swelling with basophilic change and karyomegaly in the outer medulla was observed only in the Rub group. LuP and Rub increased the proliferative activity of tubule cells in the outer medulla, and Alz and LuP increased renal 8-OHdG levels. After 26 weeks, Rub but not Alz induced atypical tubules, a putative preneoplastic lesion, and karyomegaly in the outer medulla. These results indicate that Rub may be a potent carcinogenic metabolite of MC, targeting proximal tubule cells in the outer medulla, although oxidative stress increased by Alz or LuP might also be involved in renal carcinogenesis by MC.

Keywords: Alizarin, Lucidin-3-*O*-primeveroside, Lucidin, Rubiadin, Kidney, F344 rats

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Inoue, K., Yoshida, M., Takahashi, M., Fujimoto, H., Shibutani, M.[*1], Hirose, M.[*2], Nishikawa, A.: **Carcinogenic potential of alizarin and rubiadin, components of madder color, in a rat medium-term multi-organ bioassay**

Cancer Sci., **100**, 2261-2267（2009）

Madder color（MC）, a food coloring extracted from roots of Rubia tinctorum L., has been proven to exert carcinogenicity in the rat kidney and liver. Furthermore, it induces DNA adducts in the kidney, liver, and colon. MC is in fact composed of anthraquinones such as lucidin-3-O-primeveroside and alizarin. To clarify which of these might be responsible for the carcinogenicity, a rat medium-term multi-organ carcinogenesis bioassay was performed focusing on the kidney, liver, and colon. Male 6-week-old F344 rats after receiving five different carcinogens were fed a diet containing either 0.008%

or 0.04% of alizarin or rubiadin, a metabolite of lucidin-3-O-primeveroside, for 23 weeks. Treatment with 0.04% rubiadin significantly increased atypical renal tubules/hyperplasias and induced renal cell adenomas and carcinomas. Renal cell tumors were also increased with 0.04% alizarin, although at lower incidence than with rubiadin. In addition, glutathione S-transferase placental form-positive liver cell foci and large intestinal dysplasias were significantly increased with 0.04% rubiadin. These results indicate that both rubiadin and alizarin can increase renal preneoplastic lesions, the potential of the latter being weaker. Rubiadin may also target the liver and large intestine, suggesting a major role in madder color-induced carcinogenicity.

Keywords: Alizarin, Rubiadin, a rat medium-term multi-organ bioassay

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[*2] Food Safety Commission

Inoue, K., Yoshida, M., Takahashi, M., Cho, Y.M., Takami, S., Nishikawa, A.: **Rhabdomyosarcoma in the Abdominal Cavity of a 12-Month-Old Female Donryu Rat**

J. Toxicol. Pathol., **22**, 195-197 (2009)

Neoplasms of skeletal muscle origin are very rare in the rat. Recently, we experienced a case of rhabdomyosarcoma as a white mass involving the junction of the esophagus and stomach in the abdominal cavity of a 12-month-old female Donryu rat. Histopathologically, the neoplastic cells composing the mass invasively spreaded from the lamina propia to the tunica serosa in the stomach as well as the esophagus. Although the neoplastic cells varied in appearance, pleomorphic atypical cells with abundant eosinophilic cytoplasm were prominent. Some tumor cells were stained blue with phosphotungstic acid hematoxylin. The nuclei of spindle-shaped neoplastic cells were arranged longitudinally like beads. Multinucleate giant cells and mitotic figures were also frequently observed. Immunohistochemically, these neoplastic cells were positive for desmin and myoglobin, whereas they were negative for alpha-smooth muscle actin. Taken together these findings, this tumor was diagnosed as a pleomorphic rhabdomyosarcoma, probably derived from the muscle layer of the lower part of the esophagus. This is the first report of rhabdomyosarcoma in a Donryu rat.

Keywords: Rhabdomyosarcoma, Donryu rat

Woo, G.H., Takahashi, M., Inoue, K., Fujimoto, H., Igarashi, K., Kanno, J., Hirose, M.[*1], Nishikawa, A., Shibutani, M.[*2]: **Cellular distributions of molecules with altered expression specific to thyroid proliferative lesions developing in a rat thyroid carcinogenesis model**

Cancer Sci., **100**, 617-625 (2009)

To identify differentially regulated molecules related to early and late stages of tumor promotion in a rat two-stage thyroid carcinogenesis model by an antithyroid agent, sulfadimethoxine, microarray-based microdissected lesion-specific gene expression profiling was carried out. Proliferative lesions for profiling were divided into two categories: (i) focal follicular cell hyperplasias (FFCH) and adenomas (Ad) as early lesions; and (ii) carcinomas (Ca) as more advanced. In both cases, gene expression was compared with that in surrounding non-tumor follicular cells. Characteristically, upregulation of cell cycle-related genes in FFCH + Ad, downregulation of genes related to tumor suppression and transcription inhibitors of inhibitor of DNA binding (Id) family proteins in Ca, and upregulation of genes related to cell proliferation and tumor progression in common in FFCH + Ad and Ca, were detected. The immunohistochemical distributions of molecules included in the altered expression profiles were further examined. In parallel with microarray data, increased localization of ceruloplasmin, cyclin B1, and cell division cycle 2 homolog A, and decreased localization of poliovirus receptor-related 3 and Id3 were observed in all types of lesion. Although inconsistent with the microarray data, thyroglobulin immunoreactivity appeared to reduce in Ca. The results thus suggest cell cycling facilitation by induction of M-phase-promoting factor consisting of cyclin B1 and cell division cycle 2 homolog A and generation of oxidative responses as evidenced by ceruloplasmin accumulation from an early stage, as well as suppression of cell adhesion involving poliovirus receptor-related 3 and inhibition of cellular differentiation regulated by Id3. Decrease of thyroglobulin in Ca may reflect dedifferentiation with progression.

Keywords: A rat thyroid carcinogenesis model, sulfadimethoxine, FFCH, Adenoma, Carcinoma

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Shibutani, M.*1, Woo, G.H., Fujimoto, H., Saegusa, Y.*1, Takahashi, M., Inoue, K., Hirose, M.*2, Nishikawa, A.: **Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents**

Reprod Toxicol., **28**, 297-307（2009）

To clarify the developmental effects of hypothyroidism and to establish a detection system of resultant brain retardation, pregnant rats were administered 3 or 12 ppm of 6-propyl-2-thiouracil（PTU）or 200 ppm of methimazole（MMI）in the drinking water from gestation day 10 to postnatal day 20 and maintained after weaning until 11 weeks of age（adult stage）. Offspring displayed evidence of growth retardation lasting into the adult stage, which was particularly prominent in males. Except for hypothyroidism-related thyroid follicular cell hypertrophy, most histopathological changes that appeared at the end of chemical exposure were related to growth retardation and reversed by the adult stage. A delayed onset of puberty and an adult stage gonadal enlargement occurred by exposure to anti-thyroid agents, both being especially evident in males, and this effect might be related to gonadal growth suppression during exposure. At the adult stage, the distribution variability of hippocampal CA1 pyramidal neurons reflecting mismigration could be detected in animals receiving both thyrotoxins, with a dose-dependent effect by PTU. Similarly, a reduction in the area of the corpus callosum and oligodendroglial cell numbers in the cerebral deep cortex, both reflecting impaired oligodendroglial development, were detected in rats administered both chemicals. Thus, all effects, except for impaired brain development, might be linked to systemic growth retardation, and the brain morphometric methods employed in this study may be useful to evaluate the potency of chemicals to induce hypothyroidism-related brain retardation.

Keywords: Developmental hypothyroidism, 6-Propyl-2-thiouracil, Methimazole, Growth retardation, Neuronal migration, Oligodendroglial development

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Saegusa, Y.*1, Fujimoto, H., Woo, G.H., Inoue, K., Takahashi, M., Mitsumori, K.,*1 Hirose, M.*2, Nishikawa, A., Shibutani, M.*1: **Developmental toxicity of brominated flame retardants, tetrabromobisphenol A and 1,2,5,6,9,10-hexabromocyclododecane, in rat offspring after maternal exposure from mid-gestation through lactation**

Reprod Toxicol., **28**, 456-467（2009）

To evaluate developmental exposure effects of two brominated flame retardants, tetrabromobisphenol A（TBBPA）and 1,2,5,6,9,10-hexabromocyclododecane（HBCD）, pregnant Sprague-Dawley rats were administered either chemical at doses of 100, 1000 or 10,000 ppm in a soy-free diet from gestation day 10 until the day 20 after delivery. Offspring exposed to TBBPA showed dose-unrelated slight decreases of serum triiodothyronine（T(3)）concentration at postnatal day 20, and there was no evidence of hypothyroidism-related neuronal mismigration and impaired oligodendroglial development as judged by morphometric analyses of NeuN-immunoreactive neuronal distribution in the hippocampal CA1, and area of corpus callosum as well as density of 2',3'-cyclic nucleotide 3'-phosphodiesterase（CNPase）-immunoreactive oligodendrocytes in the cingulate deep cortex at the adult stage. On the other hand, HBCD exerted a weak hypothyroidism evident with increases in thyroid weight, thyroid follicular cell hypertrophy and serum concentrations of thyroid-stimulating hormone as well as decreases of serum T(3) concentrations in offspring at 10,000 ppm at weaning. Increased thyroid weights and decreased serum T(3) concentrations were also observed in the adult stage from 1000 ppm. With regard to the effect on brain development, HBCD reduced density of CNPase-positive oligodendrocytes at 10,000 ppm, suggesting an impaired oligodendroglial development. Results thus suggest that TBBPA did not exert developmental brain effects, while HBCD did, and 100 ppm was determined to be the no-observed-adverse-effect level of HBCD from changes in thyroid parameters at the adult stage by maternal exposure, translating into 8.1-21.3mg/kg-d.

Keywords: Tetrabromobisphenol A（TBBPA）, 1,2,5,6,9,10-Hexabromocyclododecane（HBCD）, Brominated flame retardants（BFRs）, Developmental toxicity, Maternal exposure, Thyroid hormones, Brain retardation

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*2 Food Safety Commission

Takahashi, M., Yoshida, M., Inoue, K., Morikawa, T., Nishikawa, A.: **A ninety-day toxicity study of semicarbazide hydrochloride in Wistar Hannover GALAS rats**

Food Chem Toxicol., **47**, 2490-2498 (2009)

A ninety-day toxicity study of semicarbazide hydrochloride (SEM-HCl) was conducted in male and female Wistar Hannover GALAS rats fed diet containing the compound at concentration of 0, 250, 500 and 1000 ppm. Suppression of body weight gain and food consumption was found in both sexes at 1000 ppm throughout the study. Enlargement and deformation of knee joints were obvious at 500 and 1000 ppm from week 3, together with deformation of the thorax and tail. Histopathologically, disarrangement of chondrocytes and fissures in the cartilage matrix were apparent at all doses tested in epiphyseal and articular cartilage. The severity of these lesions increased dose-dependently, accompanied by increased connective tissues and bone deformation at high doses. Additionally, compact bones at 1000 ppm became thin, suggesting loss of bone mass. In the thoracic aorta, the edges of elastic laminae became rough and the interlaminar spaces were altered from a fibrillar to a rod or globular appearance. No abnormalities were detected in any other organs. Taken together, toxicological effects of subchronic exposure to SEM-HCI were mainly observed in bone, cartilage and the aorta, with the no-observed-adverse-effect-level estimated from the present histopathological examination of less than 250 ppm in both sexes.

Keywords: Semicarbazide hydrochloride, Osteolathyrism, Food contaminant, 90-day study, GALAS rats

Takahashi, M., Shibutani, M.[*1], Nakahigashi, J.[*2], Sakaguchi, N.[*2], Inoue, K., Morikawa, T., Yoshida, M., Nishikawa, A.: **Limited lactational transfer of acrylamide to rat offspring on maternal oral administration during the gestation and lactation periods**

Arch Toxicol., **83**, 785-793 (2009)

To evaluate the developmental exposure effects of acrylamide (ACR) on the nervous and male reproductive systems, pregnant Sprague-Dawley rats were given ACR at 0, 25, 50 or 100 ppm in the drinking water from gestational day 6 to postnatal day (PND) 21 and histopathological assessment was performed at PND 21. Exposure levels in offspring were examined by measurement of free ACR and hemoglobin (Hb)-ACR adducts on PND 14, and compared with maternal levels on PND 21. Additionally, a group of offspring that received ACR at 50 mg/kg by intraperitoneal injections directly three times a week from PND 2 to 21 was subjected to analysis for comparison with maternal exposure groups. Although maternal neurotoxicity was evident at 100 ppm, no changes suggestive of neurotoxicity or testicular toxicity were observed in their offspring except for growth retardation evident as lowered body weights. In contrast, offspring given ACR intraperitoneally exhibited obvious neurotoxicity, but not testicular damage. Free ACR in serum and milk was detected in neither dams nor their offspring. The level of ACR-Hb adducts in offspring was one tenth or less than that in dams. In summary, although preweaning rats have susceptibility to ACR-induced neurotoxicity, the internal level of ACR in offspring exposed through maternal oral administration is insufficient to induce neurotoxicity and testicular toxicity due to limited lactational transfer.

Keywords: Acrylamide, Hemoglobin adduct, Neurotoxicity, Testicular toxicity, Rat

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Takahashi, M., Inoue, K., Yoshida, M., Morikawa, T., Shibutani, M.[*], Nishikawa, A.: **Lack of chronic toxicity or carcinogenicity of dietary N-acetylglucosamine in F344 rats**

Food Chem Toxicol., **47**, 462-471 (2009)

Chronic toxicity and carcinogenicity of N-acetylglucosamine (GlcNAc) were examined in male and female F344 rats. GlcNAc was given in the diet at levels of 0%, 1.25%, 2.5% or 5% to groups of 10 rats of each sex for 52 weeks in the chronic toxicity study and 0%, 2.5% or 5% to groups of 50 rats of each sex for 104 weeks in the carcinogenicity study. GlcNAc exerted no toxic effects with regard to clinical signs, mortality, hematology, serum biochemistry and histopathological assessment. Slight suppression of body weight gain was observed at more than 2.5%, but this appeared to be due to slight reduction of caloric intake with the high concentration of test compound, rather than any

toxicity. Thus, it was concluded that GlcNAc has neither toxic nor carcinogenic effects in F344 rats, the no observed adverse effect levels (NOAEL) estimated from the chronic toxicity study being 5% in both sexes, equivalent to 2323 and 2545 mg/kg/day in males and females, respectively.

Keywords: N-acetylglucosamine, Chronic toxicity, Carcinogenicity, F344 rats

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Taniai, E.*, Kawai, M.*, Dewa, Y.*, Nishimura, J.*, Harada, T.*, Saegusa, Y.*, Matsumoto, S.*, Takahashi, M., Mitsumori, K.*, Shibutani, M.*: **Crosstalk between PTEN/Akt2 and TGFβ signaling involving EGF receptor down-regulation during the tumor promotion process from the early stage in a rat two-stage hepatocarcinogenesis model**

Cancer Sci., **100**, 813-820 (2009)

The present study investigated the involvement of signaling of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) /protein kinase B (Akt) and transforming growth factor-beta (TGFbeta) as well as receptor tyrosine kinases in the tumor promotion processes in a two-stage hepatocarcinogenesis model using male F344 rats. The cellular localization of related molecules was examined in liver cell foci expressing glutathione S-transferase placental form (GST-P) at the early stage of tumor promotion by fenbendazole (FB), piperonyl butoxide, or thioacetamide. Distribution in the liver cell foci and neoplastic lesions positive for GST-P was also examined at the later stage of FB promotion. In contrast to the initiation-alone cases, subpopulations of GST-P-positive foci induced by promotion for 6 weeks, regardless of the promoting chemicals used, enhanced down-regulation of PTEN and up-regulation of phosphorylated (active) Akt2 and phosphorylated substrate(s) of Akt-kinase activity. Also, up-regulation of TGFbeta receptor I and down-regulation of epidermal growth factor receptor (EGFR) were enhanced in the subpopulation of GST-P-positive foci in all promoted cases. A similar pattern of cellular distribution of these molecules was also observed in the neoplastic lesions at the late stage. These results suggest a crosstalk between Akt2 and TGFbeta signaling that involves a mechanism requiring EGFR down-regulation during the entire tumor promotion process starting from the early stage. In particular, a shift in subcellular localization of phosphorylated substrate(s) of Akt from the cell membrane in liver cell foci to the cytoplasm in carcinomas was observed, suggesting an alteration of the function or activity of the corresponding molecule(s).

Keywords: PTEN, Akt2, TGFb, EGF receptor, a rat two-stage hepatocarcinogenesis model

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Yoshida, M., Watanabe, G.*1, Suzuki, T.*2, Inoue, K., Takahashi, M., Maekawa, A*3, Taya, K.*1, Nishikawa, A.: **Long-term treatment with bromocriptine inhibits endometrial adenocarcinoma development in rats**

J Reprod Dev., **55**, 105-109 (2009)

The effects of long-term blockade of prolactin (PRL) action by bromocriptine (BRC) treatment on uterine carcinogenesis and on related ovarian physiology were investigated using a rat uterine cancer model. Ten-week-old cycling female Donryu rats, a high yield strain for uterine corpus tumors (endometrial adenocarcinomas), were treated with N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), as a tumor initiator, and injected with 1 mg/kg body weight BRC subcutaneously 4 times per week until 14.5 months of age to block the proestrus PRL surge. The study was terminated at 15 months of age, and the results showed that long-term BRC treatment significantly inhibited endometrial adenocarcinoma development in terms of both incidence (34.6% to 13.0% with significant difference at 5%) and multiplicity (0.35 to 0.18 with significant difference at 5%), which indicates the number of adenocarcinomas per animals. While BRC did not affect estrous cyclicity in the treated animals, a significant decline was evident in the serum 17β-estradiol (E2) to progesterone (P) ratio (E: P ratio), and the serum E2 level showed a decreased tendency at 15 months of age. While the precise pathway to the inhibitory effect could not be determined; the pathway by which ovarian hormonal imbalance decreases the serum E: P ratio most likely plays a crucial role.

Keywords: Bromocripchne, Long-term treatment, Prolactin blockade, Rat, Uteirne carcinogenesis

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Sato, I.*1, Kawamoto, K.*1, Nishikawa, Y.*1, Tsuda, S.*1, Yoshida, M., Yaegashi, K.*2, Saito, N.*2, Liu, W.*3, Jin, Y.*3: **Neurotoxicity of perfluorooctane sulfonate (PFOS) in rats and mice after single oral exposure**
J Toxicol Sci., **34**, 569-574 (2009)

Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are widely used in industrial fields and consumer products, and are ubiquitously found in the environment and animal tissues. In the present study, their neurotoxicity was examined using rats and mice by means of neurobehavioral observation, histopathological inspection and chemical assays. PFOS and PFOA alone did not cause any neurotoxic symptoms up to their sublethal doses (PFOS: 500 mg/kg, PFOA: 1,000 mg/kg). However, tonic convulsions were caused in the PFOS-treated rats (≥ 250 mg/kg) and mice (≥ 125 mg/kg) when ultrasonic stimulus was applied to the animals. The same ultrasonic stimulus never induced convulsions in the control animals and in the animals treated with PFOA. Concentration of PFOS in the brain was considerably lower than in other tissue, but it seemed to increase gradually with time after exposure. No morphological changes were detected by histopathological examination of the brain. There were also no changes in concentrations of norepinephrine, dopamine, serotonin, glycine, 4-aminobutylic acid and glutamic acid in the brain. The present study revealed neurotoxic effects of PFOS in animals. Convulsive effect of PFOS may not be attributed to the quantitative alterations of neurotransmitters or lesions of nerve cells in the brain, although the mechanism of its neurotoxicity has not been cleared.
Keywords: PFOS, PFOA, Neurotoxicity, Convulsion

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Imai, T., Takami, S., Cho, Y.-M., Hirose, M.*, Nishikawa, A.: **Modifying effects of prepubertal exposure to potassium perchlorate and tetrabromobisphenol A on susceptibility to N-bis(2-hydroxypropyl)nitrosamine- and 7,12-dimethylbenz(a)anthracene-induced carcinogenesis in rats**
Toxicol Lett., **185**, 160-167 (2009)

Early life exposure to certain kinds of chemicals is of concern because of a possible increase in cancer risk, but relevant data are limited. In the present experiment, modifying effects of prepubertal administration of potassium perchlorate (KClO(4)) and tetrabromobisphenol A (TBBPA) on susceptibility to multi-organ carcinogenesis were evaluated. F344 dam rats were administered 0% (control), 0.01%, 0.1% or 1% TBBPA in diet or 0.01% KClO(4) in drinking water after parturition. Their weaned offspring in each group were treated for 2 weeks in the same manner. From 6 weeks of age, all offspring were treated with N-bis(2-hydroxypropyl) nitrosamine in drinking water for 4 weeks. In addition the females at 7 weeks of age were gavaged once with 7,12-dimethylbenz(a)anthracene. At weeks 39 and 47 of age, the males and females, respectively, were euthanized and the liver, kidney, lung, esophagus, thyroid, urinary bladder, testis, epididymis, ovary and mammary gland were histopathologically examined. The incidences of thyroid follicular adenomas in 1% TBBPA females ($p<0.05$) and of transitional cell papillomas in the urinary bladder of 0.01%, 0.1% and 1% TBBPA females were increased ($p<0.05$) as compared to the controls. These results indicate that prepubertal exposure to TBBPA raises susceptibility to thyroid and urinary bladder tumorigenesis in rats. Although causes of the effect on thyroid carcinogenesis might be direct and/or indirect hormonal actions, further studies are needed for confirmation.
Keywords: Potassium perchlorate, Tetrabromobisphenol A, Rat, Carcinogenicity, Juvenile toxicity

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* Food Safety Commission

Ota, Y., Imai, T.*1, Onose, J., Takami, S., Cho, Y.-M., Hirose, M.*2, Nishikawa, A.: **A 55-week chronic toxicity study of dietary administered kojic acid (KA) in male F344 rats**
J Toxicol Sci., **34**, 305-313 (2009)

A chronic toxicity study of kojic acid (KA) was performed using male F344 rats by dietary administration at concentrations of 0 (control), 0.5 and 2.0% for 55 weeks. Body weight gain was suppressed in the 2.0% group. The major hematological findings were decreased red blood cell (RBC) count and hematocrit (Ht) values

at both 0.5 and 2.0%. In serum biochemistry, increased aspartate transaminase (AsT), alanine transaminase (AlT), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (gamma-GTP) levels were detected in the 0.5 and 2.0% groups. Histopathologically, single cell necrosis of hepatocytes and proliferation of bile ductules in both treatment groups, and hypertrophy of hepatocytes, granulomas and proliferation of bile ducts in the 2.0% group were increased in incidence, and numbers and areas of glutathione-S-transferase placental-form (GST-P) positive foci were increased in the liver of the 2.0% group. In the thyroids, diffuse follicular cell hyperplasia at 0.5 and 2.0% and focal follicular cell hyperplasia and follicular adenoma at 2.0% were increased. A thyroid follicular carcinoma was also observed at 2.0%. Additionally, increased incidences of hyaline casts and basophilic tubules in the kidneys at 2.0% and microgranulomas containing crystals in the lung in both treatment groups were noted. At 2.0%, hypertrophy of cortical cells in zona fasciculata was also increased in the adrenals. In conclusion, no observed adverse effect level of KA was below 0.5%, which is equivalent to 227 mg/kg body weight/day in male rats.

Keywords: Kojic acid, Chronic toxicity, F344 rats

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Cho, Y.-M., Imai, T.*1, Ito, Y., Takami, S., Hasumura, M., Yamazaki, T., Hirose, M.*2, Nishikawa, A.: **A 13-week subchronic toxicity study of dietary administered saponin-rich and isoflavones-containing soybean extract in F344 rats**

Food Chem Toxicol., **47**, 2150-2156 (2009)

A subchronic toxicity study of soybean extract was performed in F344 rats with dietary administration at concentrations of 0%, 1.25%, 2.5% and 5% for 13 weeks. No mortality or abnormal clinical signs in any group were observed. Body weight gains were decreased with a tendency for reduction of feed intake in the 1.25% and above female and 5% male groups. In males, absolute and relative liver weights were increased in the 1.25% and above groups. In females relative kidney weights were increased in the 1.25% and above groups. Other significant changes such as decreased RBC and hematocrit and increased urea nitrogen were detected in the 2.5% and/or 5% groups. On histopathological observation, atrophy of the ventral prostate was observed in all animals in the 5% male group. Mucification and atrophy of the vaginal epithelium and increased atretic follicles in ovaries were noted in 2.5% and 5% female rats. Based on the above findings the lowest-observed-adverse-effect level for male and female rats was estimated to be 1.25% (707.2 and 751.8 mg/kg b. w./day, respectively).

Keywords: Soybean extract, F344 rats, Subchronic toxicity

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*2 Food Safety Commission

Imai, T.*1, Hasumura, M., Cho, Y.-M., Ota, Y., Takami, S., Hirose, M.*2, Nishikawa, A.: **Inhibitory effects of aminoguanidine on thyroid follicular carcinoma development in inflamed capsular regions of rats treated with sulfadimethoxine after N-bis (2-hydroxypropyl) nitrosamine-initiation**

Cancer Sci., **100**, 1794-1800 (2009)

We have reported that thyroid capsular thickening with inflammation induced by an antithyroidal agent, sulfadimethoxine (SDM), might play a role in the development of invasive follicular carcinomas in rats initiated with N-bis (2-hydroxypropyl) nitrosamine (DHPN). Inducible nitric oxide synthase (iNOS) expressed in the inflamed capsular regions further appeared to be implicated in the tumor progression. In the present study, the effects of an iNOS inhibitor, aminoguanidine (AG), on thyroid carcinogenesis were examined. F344 male rats were treated with SDM in drinking water (0.1%) with or without concomitant dietary administration of AG (0.2%) for 4 and 10 weeks after subcutaneous injection of DHPN at 2800 mg/kg bodyweight. At week 4, thyroid capsular thickening with inflammation was observed and iNOS-positive foci were found in the inflamed regions. In addition, single-strand DNA-positive inflammatory cells were scattered among neighboring follicular cells, indicating some cellular damage, at least partly in association with iNOS induction. Concurrent dietary administration of AG with SDM treatment slightly decreased the number of single-strand DNA-positive cells but did not alter the incidence and multiplicity of iNOS-positive foci in the inflamed capsular regions at week 4. At week 10,

however, invasive follicular carcinomas predominantly arose in the thickened capsule in the DHPN-SDM-treated rats, and AG administration decreased ($P < 0.05$) their multiplicity. The carcinoma cells were partly positive for iNOS. These results thus suggested that iNOS induction in both inflammatory and tumor cells might play pivotal roles in tumor progression in this DHPN-SDM rat model.

Keywords: Aminoguanidine, Thyroid follicular carcinoma, Inflamed capsular regions, Rats, Sulfadimethoxine

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Cho, Y.-M., Imai, T.[*1], Hasumura, M., Watanabe, N.[*3], Ushijima, T.[*3], Hirose, M.[*2], Nishikawa, A.: **Increased H-ras mutation frequency in mammary tumors of rats initiated with N-methyl-N-nitrosourea (MNU) and treated with acrylamide**

J Toxicol Sci., **34**, 407-412 (2009)

We recently demonstrated the incidence and multiplicity of N-methyl-N-nitrosourea (MNU) -induced mammary tumors to be increased by administration of acrylamide (AA) in post-initiation in rats. In the present study, to clarify the mechanisms of enhancement, H-ras gene mutations in mammary tumors induced in MNU-initiated rats with or without subsequent AA administration were investigated. Frequencies of mutations in codon 12 from GGA to GAA were significantly ($p < 0.05$) higher in rats with AA administration (82%, 23 out of 28 tumors) as compared to those without AA (50%, 9 out of 18 tumors), but the latency and volume of H-ras mutation-harboring tumors were similar to those of the mutation-lacking tumors. No mutations in codons 13 or 61 were detected in either treatment groups. The results thus indicate that H-ras gene mutations in codon 12 play a pivotal role in initiation of carcinogenesis and it appears possible that AA administration may selectively co-stimulate and/or maintain initiated cells via other genomic or non-genomic events in MNU-treated rats.

Keywords: Acrylamide, Mammary tumors, H-ras gene, Rat

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Totsuka, Y.[*1], Higuchi, T.[*1], Imai, T.[*1], Nishikawa, A., Nohmi, T., Kato, T.[*2], Masuda, S.[*2], Kinae, N.[*2], Hiyoshi, K.[*2], Ogo, S.[*3], Kawanishi, M.[*3], Yagi, T.[*3], Ichinose, T.[*4], Fukumori, N.[*5], Watanabe, M.[*6], Sugimura, T.[*1], Wakabayashi, K.[*1]: **Genotoxicity of nano/microparticles in in vitro micronuclei, in vivo comet and mutation assay systems**

Part Fibre Toxicol., **6**, 23 (2009)

Background

Recently, manufactured nano/microparticles such as fullerenes (C60), carbon black (CB) and ceramic fiber are being widely used because of their desirable properties in industrial, medical and cosmetic fields. However, there are few data on these particles in mammalian mutagenesis and carcinogenesis. To examine genotoxic effects by C60, CB and kaolin, an in vitro micronuclei (MN) test was conducted with human lung cancer cell line, A549 cells. In addition, DNA damage and mutations were analyzed by in vivo assay systems using male C57BL/6J or gpt delta transgenic mice which were intratracheally instilled with single or multiple doses of 0.2 mg per animal of particles.

Results

In in vitro genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C60, CB and kaolin in a dose-dependent manner. These three nano/microparticles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Moreover, single or multiple instillations of C60 and kaolin, increased either or both of gpt and Spi- mutant frequencies in the lungs of gpt delta transgenic mice. Mutation spectra analysis showed transversions were predominant, and more than 60% of the base substitutions occurred at G:C base pairs in the gpt genes. The G:C to C:G transversion was commonly increased by these particle instillations.

Conclusion

Manufactured nano/microparticles, CB, C60 and kaolin, were shown to be genotoxic in in vitro and in vivo assay systems.

Keywords: Nano/microparticles, *In vitro* and *in vivo* mutation assay system, Genotoxicity

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Valenti, A.*, Perugino, G.*, Nohmi, T., Rossi, M.* and Ciaramella, M.*: **Inhibition of translesion DNA polymerase by archaeal reverse gyrase**

Nucleic Acids Res., **37**, 4287-4295（2009）

Reverse gyrase is a unique DNA topoisomerase endowed with positive supercoiling activity. It is typical of microorganisms living at high temperature and is likely to play a role in maintenance of genome stability. We have identified the translesion DNA polymerase Sso PolY（also named Dpo4）as one partner of reverse gyrase in the archaeon *Sulfolobus solfataricus*. In cell extracts, PolY and reverse gyrase co-immunoprecipitate with each other and with the single strand binding protein, SSB. The interaction is confirmed *in vitro* with purified proteins. Reverse gyrase inhibits PolY activity and inhibition depends on the intact positive supercoiling activity. *In vivo*, reverse gyrase and PolY are both degraded after induction of DNA damage. We suggest that, modifying the template structure, reverse gyrase acts as a brake for PolY preventing its mutagenic activity when undesired. Inhibition of a translesion polymerase by topoisomerase-induced modification of DNA structure may represent a previously unconsidered mechanism of regulation of these enzymes.

Keywords: Inhibition of PolY by reverse gyrase DNA damage, DNA topology, protein-protein interaction

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Sui, H.*, Kawakami, K.*, Sakurai, N.*, Hara, T.* and Nohmi, T.: **Improvement and evaluation of high throughput fluctuation Ames test using 384-well plate with *Salmonella typhimurium* TA100 and TA98**

Genes and Environ., **31**, 47-55（2009）

Recently, it has become necessary to increase the progress of research studies into drug discovery because of the introduction of combinatorial chemistry and robotics; therefore, genotoxicity screening assays which can be conducted with a small amount of compound, in a short time, and which can predict the results of regulatory genotoxicity tests for pharmaceuticals are required in the early stage of research. The bacterial reverse mutation test（Ames test）is a regulatory genotoxicity test and is conducted in the early stage of non-clinical safety studies. Morita established a high throughput fluctuation Ames test using 384-well plates with *Salmonella typhimurium* TA100 and TA98（Environ. Mutagen Res., 2003, 25: 23-31）, which is referred to as original FAT in this study. Here, we report an improved high throughput fluctuation Ames test（i.e., improved FAT）. The improved FAT indicated a higher positive response than the original FAT in several mutagens. Furthermore, we evaluated the improved FAT with *S. typhimurium* TA100 and TA98 using 40 National Toxicology Program（NTP）chemicals. As a result, there was 80.0%（32/40）concordance between the Ames test and the improved FAT. In conclusion, the improved FAT can predict the results of the Ames test with high concordance（especially its negative specificity）. The improved FAT requires a much smaller amount of test chemicals than the Ames test（i.e., 5 mg vs 100 mg when using two tester strains）and is able to be automated. Thus, the improved FAT is considered to be useful as a screening test in the early stage of drug discovery.

Keywords: High throughput fluctuation Ames test, screening, *Salmonella typhimurium*, TA100 and TA98

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Galhardo, R.S.*1, Do, R.*1, Yamada, M., Friedberg, E.C.*2, Hastings, P.J.*1, Nohmi, T. and Rosenberg, S.M.*1: **DinB up-regulation is the sole role of the SOS response in stress-induced mutagenesis in *Escherichia coli***

Genetics, **182**, 55-68（2009）

Stress-induced mutagenesis is a collection of mechanisms observed in bacterial, yeast and human cells in which adverse conditions provoke mutagenesis, often under the control of stress responses. Control of mutagenesis by stress responses may accelerate evolution specifically when cells are maladapted to their environments, *i.e.*, are stressed. It is therefore important to understand how stress responses increase mutagenesis. In the *Escherichia coli* Lac assay, stress-induced point mutagenesis requires induction of at least two stress responses: the RpoS-controlled general/starvation-stress-response, and the SOS DNA-damage response, both of which upregulate DinB

error-prone DNA polymerase, among other genes required for Lac mutagenesis. We show that up-regulation of DinB is the only aspect of the SOS response needed for stress-induced mutagenesis. We constructed two *dinB*O^c (operator-constitutive) mutants. Both produce SOS-induced levels of DinB constitutively. We find that both *dinB*O^c alleles fully suppress the phenotype of constitutively SOS-"off" *lexA*(Ind$^-$) mutant cells, restoring normal levels of stress-induced mutagenesis. Thus, *dinB* is the only SOS gene induction of which is required for stress-induced point mutagenesis. Further, although spontaneous SOS induction has been observed to occur only in a small fraction of cells, upregulation of *dinB* by the *dinB*O^c alleles in all cells does not promote an increase in mutagenesis, implying that SOS induction of DinB, though necessary, is insufficient to differentiate cells into a hypermutable condition.

Keywords: *dinB*, evolution, stress responses, stress-induced mutagenesis, adaptive mutation, SOS response

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Shibata, A.*1, 2, Maeda, D.*1, Ogino, H.*1, Tsutsumi, M.*3, Nohmi, T., Nakagama, H.*1, Sugimura, T.*1, Teraoka, H.*2 and Masutani, M.*1: **Role of Parp-1 in suppressing spontaneous deletion mutation in the liver and brain of mice atadolescence and advanced age**

Mutat. Res., **664**, 20-27 (2009)

Poly(*ADP-ribose*) *polymerase-1* knockout (*Parp-1*$^{-/-}$) mice show increased frequency of spontaneous liver tumors compared to wild-type mice after aging. To understand the impact of *Parp-1* deficiency on mutations during aging, in this study, we analyzed spontaneous mutations in *Parp-1*$^{-/-}$ aged mice. *Parp-1*$^{-/-}$ mice showed tendencies of higher mutation frequencies of the *red/gam* genes at 18 months of age, compared to *Parp-1*$^{+/+}$ mice, in the liver and brain. Complex-type deletions, accompanying small insertion were observed only in *Parp-1*$^{-/-}$ mice in the liver and brain, although the difference between the genotypes is not statistically significant. Further analysis in the liver showed that the frequency of single base deletion mutations at non-repeat or short repeat sequences was 5.8-fold higher in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice (p<0.05). A 3.2-fold higher tendency of the deletion frequency of two bases or more was observed in *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice. These results support the model that *Parp-1* is involved in suppressing imprecise repair of endogenous DNA damage leading to the deletion mutation during aging. The mutation frequencies of *gpt* gene in the brain was found to be 3-fold lower in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice at 4 months of age (p<0.01). The frequencies of *gpt* mutation showed an increase at 18 months of age in the *Parp-1*$^{-/-}$ (p<0.05) but not in *Parp-1*$^{+/+}$ brains, suggesting a possibility that *Parp-1* deficiency causes an increase of point mutations in the brain by aging.

Keywords: *Parp-1*, mutation, deletion, *gpt* delta, aging

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Takashima, Y., Sakuraba, M., Koizumi, T., Sakamoto, H., Hayashi, M. and Honma, M.: **Dependence of DNA double strand break repair pathways on cell cycle phase in human lymphoblastoid cells**

Environ Mol Mutagen, **50**, 815-822 (2009)

DNA double-strand breaks (DSBs) are usually repaired by nonhomologous end-joining (NHEJ) or homologous recombination (HR). We previously developed a system to trace the fate of DSBs in the human genome by introducing the homing endonuclease I-SceI site into the thymidine kinase (TK) gene of human lymphoblastoid TK6 cells. Here, we use this system to investigate the relative contribution of HR and NHEJ for repairing I-SceI-induced DSBs under various conditions. The relative contribution of NHEJ and HR for repairing the DSB was 100:1 and did not change with transfection efficiency. Cotransfection with KU80-siRNA significantly diminished KU80 protein levels and decreased NHEJ activity, but did not increase HR. We also investigated HR and NHEJ in synchronized cells. The HR frequency was 2−3 times higher in late-S/G2 phases than in G1, whereas NHEJ was unaffected. Even in late-S/G2 phases, NHEJ remained elevated relative to HR. Therefore, NHEJ is the major pathway for repairing endonuclease-induced DSBs in mammalian cells even in late-S/G2 phase, and does not compete with HR.

Keywords: DNA double-strand breaks (DSBs), nonhomologous end-joining (NHEJ), homologous

recombination（HR）

Wang, J.[*1], Sawyer, J.R.[*2], Chen, L.[*3], Chen, T., Honma, M., Mei, N.[*1] and Moore, M.M.[*1]: **The mouse lymphoma assay detects recombination, deletion, and aneuploidy**

Toxicol Sci, **109**, 96-105（2009）

The mouse lymphoma assay（MLA）uses the thymidine kinase（Tk）gene of the L5178Y/Tk1/2-3.7.2C mouse lymphoma cell line as a reporter gene to evaluate the mutagenicity of chemical and physical agents. Three chemicals, including two clastogens and an aneugen（3#-azido-3#- deoxythymidine, mitomycin C, and taxol）, were used to induce Tk mutants. Loss of heterozygosity（LOH）analysis was used to select mutants that could be informative as to whether they resulted from deletion, mitotic recombination, or aneuploidy. A combination of additional methods, G-banding analysis, chromosome painting, and a real-time PCR method to detect the copy number（CN）of the Tk gene was then used to provide a detailed analysis. LOH involving at least 25% of chromosome 11, a normal karyotype, and a Tk CN of 2 would indicate that the mutant resulted from recombination, whereas LOH combined with a karyotypically visible deletion of chromosome 11 and a Tk CN of 1 would indicate a deletion. Aneuploidy was confirmed using G-banding combined with chromosome painting analysis for mutants showing LOH at every microsatellite marker on chromosome 11. From this analysis, it is clear that mouse lymphoma Tk mutants can result from recombination, deletion, and aneuploidy.

Keywords: mouse lymphoma assay（MLA）, loss of heterozygosity（LOH）, Tk mutants

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Yatagai, F.[*1], Sugasawa, K.[*2], Enomoto, S.[*1] and Honma, M.: **An approach to estimation from DSB Repair Efficiency**

J Radiat Res, **50**, 407-413（2009）

Recently, we proposed a new methodology for evaluating the repair efficiency of DNA double-strand breaks（DSB）using a model system. The model system can trace the fate of a single DSB, which is introduced within intron 4 of the TK gene on chromosome 17 in human lymphoblastoid TK6 cells by the expression of restriction enzyme I-SceI. This methodology was first applied to examine whether repair of the DSB（at the I-SceI site）can be influenced by low-dose, low-dose rate gamma-ray irradiation. We found that such low-dose IR exposure could enhance the activity of DSB repair through homologous recombination（HR）. HR activity was also enhanced due to the pre-IR irradiation under the established conditions for radioadaptation（50 mGy X-ray-6 h-I-SceI treatment）. Therefore, radioadaptation might account for the reduced frequency of homozygous loss of heterozygosity（LOH）events observed in our previous experiment（50 mGy X-ray-6 h-2 Gy X-ray）. We suggest that the present evaluation of DSB repair using this I-SceI system, may contribute to our overall understanding of radioadaptation.

Keywords: Double strand break（DSB）, Low-dose effect, Homologous recombination（HR）

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Katafuchi, A., Sassa, A.[*1], Niimi, N., Grúz, P., Fujimoto, H.[*2], Masutani, C.[*3], Hanaoka, F.[*4], Ohta, T.[*1] and Nohmi, T.: **Critical amino acids in human DNA polymerases η and κ involved in erroneous incorporation of oxidized nucleotides**

Nuc. Acids Res., **38**, 859-867（2010）

To gain insight into the mechanisms underlying erroneous nucleotide incorporation, we changed amino acids in human Poleta and Polkappa proteins that might modulate their specificity for incorporating 8-oxo-dGTP into DNA. We found that Arg61 in Poleta was crucial for erroneous nucleotide incorporation. Similarly, Tyr112 in Polkappa was crucial for erroneous nucleotide incorporation. The results suggested that amino acids at distinct positions in the active sites of Poleta and Polkappa might enhance 8-oxo-dGTP to favor the syn conformation, and thus direct its misincorporation into DNA.

Keywords: DNA polymerase, erroneous incorporation, oxidized nucleotides

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Toyoda-Hokaiwado, N., Inoue, T., Masumura, K., Hayashi, H.*1, Kawamura, Y.*1, Kurata, Y.*1, Takamune, M., Yamada, M., Sanada, H.*2, Umemura, T., Nishikawa, A. and Nohmi, T.: **Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers**

Toxicol Sci., **114**, 71-78 (2010)

We examined the genotoxicity and hepatotoxicity of structural isomers of 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT). Male F344 *gpt* delta rats were fed diet containing 2,4-DAT at doses of 125, 250, or 500 ppm for 13 weeks, or 2,6-DAT at a dose of 500 ppm for the same period. The mutation frequencies of base substitutions were significantly increased in the livers of 2,4-DAT-treated rats at all three doses. In contrast, no induction of genotoxicity was identified in the kidneys of 2,4-DAT-treated rats or in the livers of 2,6-DAT-treated rats. GST-P-positive foci were detected in the livers of rats treated with 2,4-DAT at a dose of 500 ppm, but not in those treated with 2,6-DAT. Integrated genotoxicity and short-term carcinogenicity assays may be useful for early identifying genotoxic and nongenotoxic carcinogens in a reduced number of experimental animals.

Keywords: *gpt* delta transgenic rat, diaminotoluenes, genotoxicity

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Okudaira, N.*1, Uehara, Y.*1, Fujikawa, K.*2, Kagawa, N.*2, Ootsuyama, A.*3, Norimura, T.*3, Saeki, K.*4, Nohmi, T., Masumura, K., Matsumoto, T.*5, Oghiso, Y.*5, Tanaka, K.*5, Ichinohe, K.*5, Nakamura, S.*5, Tanaka, S.*5 and Ono, T.*1: **Radiation dose-rate effect on mutation induction in spleen and liver of *gpt* delta mice**

Radiat Res., **173**, 138-147 (2010)

The effect of dose rate on radiation-induced mutations in the spleen and liver was examined in transgenic *gpt* delta mice. The dose rates examined were 920 mGy/min, 1 mGy/min and 12.5 mGy/min. In both tissues, the number of mutations increased with increasing dose at each of the three dose rates examined. The mutation induction rate was higher in the spleen than in the liver at the medium dose rate but was similar in the two tissues at the high and low dose rates. Analysis of the molecular nature of the mutations indicated that 2- to 1,000-bp deletion mutations were specifically induced by radiation in both tissues after high- and low-dose-rate irradiation. The results indicate that the mutagenic effects of radiation in somatic tissues are dependent on dose rate and that there is some variability between tissues.

Keywords: *gpt* delta transgenic mouse, radiation, dose rate, deletion

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Facciotti, M.*1, 2, Pang, L.*1, Lo, F.*1, Whitehead, K.*1, Koide, T.*1, Masumura, K., Pan, M.*1, Kaur, A.*1, Larsen, D.*2, Reiss, D.*1, Hoang, L.*3, Kalisiak, E.*3, Northen, T.*3, Trauger, S.*3, Siuzdak, G.*3 and Baliga, N.*1: **Large scale physiological readjustment during growth enables rapid, comprehensive and inexpensive systems analysis**

BMC Systems Biology, **4**, 64 (2010)

We have discovered in the model organism *Halobacterium salinarum* NRC-1 that batch culturing in complex medium stimulates meaningful changes in the expression of approximately two thirds of all genes. The majority of these changes occur during transition from rapid exponential growth to the stationary phase. In sum, integrated analysis of transcript and metabolite changes has helped uncover growth phase-associated physiologies, operational interrelationships among two thirds of all genes, specialized functions for gene family members, waves of transcription factor activities, and growth phase associated cell morphology control. The integration of such growth and perturbation studies with measurements of associated environmental factor changes is a practical and economical route for the elucidation of comprehensive systems-level models of biological systems.

Keywords: Systems biology, archaea, growth phase

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Whitehead, K.*1, Pan, M.*1, Masumura, K., Bonneau, R.*2 and Baliga, N.*1: **Diurnally entrained anticipatory behavior in archaea**

PLoS One, **4**, e5485（2009）

By sensing changes in one or few environmental factors biological systems can anticipate future changes in multiple factors over a wide range of time scales. We report the first observation of light-dark（LD）-entrained diurnal oscillatory transcription in up to 12% of all genes of a halophilic archaeon *Halobacterium salinarum* NRC-1. Significantly, the diurnally entrained transcription was observed under constant darkness after removal of the LD stimulus（free-running rhythms）. The memory of diurnal entrainment was also associated with the synchronization of oxic and anoxic physiologies to the LD cycle. Our results suggest that under nutrient limited conditions halophilic archaea take advantage of the causal influence of sunlight（via temperature）on O2 diffusivity in a closed hypersaline environment to streamline their physiology and operate oxically during nighttime and anoxically during daytime.

Keywords: Systems Biology, archaea, diurnal cycle archaea, growth phase

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Totsuka, Y.*1, Higuchi, T.*1, Imai, T.*1, Nishikawa, A., Nohmi, T., Kato, T.*2, Masuda, S.*2, Kinae, N.*2, Hiyoshi, K.*2, Ogo, S.*3, Kawanishi, M.*3, Yagi, T.*3, Ichinose, T.*4, Fukumori, N.*5, Watanabe, M.*6, Sugimura, T.*1 and Wakabayashi, K.*1: **Genotoxicity of nano/microparticles in *in vitro* micronuclei, *in vivo* comet and mutation assay systems**

Particle and Fibre Toxicology, **6**, 23（2009）

To examine genotoxic effects by fullerenes（C60）, carbon black（CB）and kaolin, an *in vitro* micronuclei（MN）test was conducted with human lung cancer cell line, A549 cells. In addition, DNA damage and mutations were analyzed by *in vivo* assay systems using male C57BL/6J or *gpt* delta transgenic mice which were intratracheally instilled with single or multiple doses of 0.2 mg per animal of particles. In *in vitro* genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C60, CB and kaolin in a dose-dependent manner. These three nano/microparticles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Single or multiple instillations of C60 and kaolin, increased either or both of *gpt* and Spi^- mutant frequencies in the lungs of gpt delta transgenic mice. Mutation spectra analysis showed transversions were predominant, and more than 60% of the base substitutions occurred at G:C base pairs in the *gpt* genes. The G:C to C:G transversion was commonly increased by these particle instillations.

Keywords: nanoparticles, *in vivo* genotoxicity assay, fullerenes

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Salem, A.M.*, Nakano, T.*, Takuwa, M.*, Matoba, N.*, Tsuboi, T.*, Terato, H.*, Yamamoto, K.*, Yamada, M., Nohmi, T. and Ide, H.*: **Genetic analysis of repair and damage tolerance mechanisms for DNA-protein cross-links in *Escherichia coli***

J. Bacteriol., **191**, 5657-5568（2009）

DNA-protein cross-links（DPCs）are unique among DNA lesions in their unusually bulky nature. We have recently shown that nucleotide excision repair（NER）and RecBCD-dependent homologous recombination（HR）collaboratively alleviate the lethal effect of DPCs in *Escherichia coli*. In this study, to gain further insight into the damage-processing mechanism for DPCs, we assessed the sensitivities of a panel of repair-deficient *E. coli* mutants to DPC-inducing agents, including formaldehyde（FA）and 5-azacytidine（azaC）. We show here that the damage tolerance mechanism involving HR and subsequent replication restart（RR）provides the most effective means of cell survival against DPCs. Elimination of DPCs from the genome relies primarily on NER, which provides a second and moderately effective means of cell survival against DPCs. Interestingly, Cho rather than UvrC seems to be an effective nuclease for the NER of DPCs. DNA glycosylases mitigate azaC toxicity, independently of the repair of DPCs,

presumably by removing 5-azacytosine or its degradation product from the chromosome.
Keywords: DNA-protein crosslink, homologous recombination, replication restart

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Yamada, M., Matsui, K., Katafuchi, A., Takamune, M. and Nohmi, T.: **Development of tester strains deficient in Nth/Nei DNA glycosylases to selectively detect the mutagenicity of oxidized DNA pyirimidines**

Genes & Environ., **31**, 69-79 (2009)

Oxidative DNA damage is a major cause of mutation and cell death in aerobic organisms. In addition to 8-hydroxyguanine, oxidized DNA pyrimidines play important roles in mutagenesis. To detect oxidative mutagens that selectively modify pyrimidines, we constructed a derivative of strain TA1535, termed YG3206, which lacks the Nei and Nth DNA glycosylases that excise oxidized pyrimidines from DNA. This novel strain easily detected the mutagenicity of L-cysteine, L-penicillamine, dopamine-HCl, and phenazine methosulfate, which are non-mutagenic or only weakly mutagenic in the TA1535. A second strain that is equivalent to YG3206 but harbors the plasmid pKM101 which carries mucAB encoding DNA polymerase R1, termed YG3216, was significantly sensitive to phenazine ethosulfate. The number of spontaneous His^+ revertants suggested a significant contribution to spontaneous mutagenesis by endogenous pyrimidine oxidation. In the absence of exogenous chemical treatment, exposure to fluorescent light enhanced the spontaneous mutation frequency by approximately two-fold (YG3206), 13-fold (YG3001), and 10-fold (TA1535). These results suggest that certain environmental chemicals may selectively introduce mutagenic damage at DNA pyrimidines.
Keywords: Ames tester strain, oxidized pyrimidine, glycosylase

Fukuda, H.[*1], Takamura-Enya, T.[*2], Masuda, Y.[*3], Nohmi, T., Seki, C.[*1], Kamiya, K.[*3], Sugimura, T.[*1], Masutani, C.[*4], Hanaoka, F.[*4] and Nakagama, H.[*1]: **Translesional DNA synthesis through a C8-guanyl adduct of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP) *in Vitro*: REV1 inserts dC opposite the lesion, and DNA polymerase kappa potentially catalyzes extension reaction from the 3'-dC terminus**

J. Biol. Chem., **284**, 25585-25592 (2009)

To shed further light on the molecular mechanisms underlying the induction of mutations by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), *in vitro* DNA synthesis analyses were carried out using a dG-C8-PhIP-modified oligonucleotide template. This represents one of the mutation hot spots in the rat Apc gene that is targeted by PhIP. DNA synthesis with A- or B-family DNA polymerases was completely blocked at the adducted guanine base. Translesional synthesis polymerases of the Y-family, pol eta, pol iota, pol kappa, and REV1, were also used with the same templates. REV1, pol eta, and pol kappa were able to insert dCTP opposite dG-C8-PhIP, although the efficiencies for pol eta and pol kappa were low. pol kappa was also able to catalyze the extension reaction from the dC opposite dG-C8-PhIP, during which it often skipped over one dG of the triple dG sequence on the template. This slippage probably leads to the single dG base deletion in colon tumors.
Keywords: PhIP, DNA adduct, Y-family DNA polymerase

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Niimi, N., Sassa, A., Katafuchi, A., Grúz, P., Fujimoto, H.[*1], Bonala, R.R.[*2], Johnson, F.[*2], Ohta, T.[*3] and Nohmi, T.: **The steric gate amino acid tyrosine 112 is required for efficient mismatched-primer extension by human DNA polymerase kappa**

Biochemistry., **48**, 4239-4246 (2009)

We report that tyrosine 112 (Y112), the steric gate amino acid of hPol kappa, which distinguishes dNTPs from rNTPs by sensing the 2'-hydroxy group of incoming nucleotides, plays a crucial role in extension reactions with mismatched primer termini. When Y112 was replaced with alanine, the amino acid change severely reduced the catalytic constant of the extending mismatched primers and lowered the efficiency of this process by approximately 400-fold compared with that of the wild-type enzyme. In contrast, the amino acid replacement did not reduce the insertion efficiency of

dCMP opposite BPDE-N^2-dG in template DNA, nor did it affect the ability of hPolkappa to bind strongly to template-primer DNA with BPDE-N^2-dG/dCMP. We conclude that the steric gate of hPolkappa is a major fidelity factor that regulates extension reactions from mismatched primer termini.

Keywords: human DNA polymerase kappa, steric gate, amino acid substitution

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Grúz, P. and Shimizu, M.*: **Origins of age-related DNA damage and dietary strategies for its reduction**

Rejuvenation Res., **13**(2-3), 285-287（2010）

The polyunsaturated fatty acids in biological membranes serve as both the target and source of oxidative damage and can be regarded as the most unstable class of biomolecules in the body. Lipid peroxides arising from both spontaneous and enzymatic oxidation of polyunsaturated fatty acids are the major source of endogenous DNA damage linked to various age-related pathologies and initiating carcinogenesis. Here we describe the major types of lipid peroxide-derived DNA adducts and propose a simple dietary strategy to reduce their formation. This may be particularly beneficial to the aging organism, which has progressively impaired natural protective systems.

Keywords: aging, genotoxicity, lipid peroxide

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Hirata-Koizumi, M., Matsuno, K.*, Kawabata, M.*, Yajima, K.*, Matsuyama, T.*, Hirose, A., Kamata, E. and Ema, M.: **Gender-related difference in the toxicity of 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl) benzotriazole in rats: relationship to the plasma concentration, in vitro hepatic metabolism, and effects on hepatic metabolizing enzyme activity**

Drug Chem. Toxicol., **32**, 204-214（2009）

Previously, we showed that the toxic susceptibility of male rats to an ultraviolet absorber, 2-(2'-hydroxy- 3',5'-di-*tert*-butylphenyl) benzotriazole (HDBB), was nearly 25 times higher than that of females. The present study aimed to clarify the mechanism of gender-related differences in HDBB toxicity. Male and female rats were given HDBB by gavage at 0.5, 2.5, or 12.5 mg/kg/day for 28 days, and plasma HDBB levels were measured at various time points by using liquid chromatography-tandem mass spectrometry. HDBB was rapidly absorbed and eliminated from the plasma in both sexes, and no sexual variations were found in the plasma levels. In the plasma, HDBB metabolites were not detected at any dose by the liquid chromatography-photodiode array detector. In an in vitro metabolic study using hepatic microsomes from male and female rats, HDBB was slightly metabolized, but no sexual differences were found in the residual HDBB ratio after a 60-minute incubation with an NADPH-generation system. Following 28-day HDBB administration, sexually different changes were found in cytochrome P450-dependent microsomal mixed-function oxidase activities in the liver. In males, 7-ethoxyresorufin O-deethylase activity decreased and lauric acid 12-hydroxylase activity increased at all doses. Decreases in aminopyrine N-demethylase activity and testosterone 2alpha- and 16alpha-hydroxylase activity were also found at 2.5 mg/kg and above in males. In females, the only significant change was increased lauric acid 12-hydroxylase activity at 12.5 mg/kg. These findings indicate that HDBB would have hepatic peroxisome proliferative activity, and the difference in susceptibility of male and female rats to this effect might lead to marked gender-related differences in HDBB toxicity.

Keywords: benzotriazole UV absorber, gender-related difference

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Ema, M., Arima, A.*, Fukunishi, K.*, Matsumoto, M., Hirata-Koizumi, M., Hirose, A. and Ihara, T.*: **Developmental toxicity of dibutyltin dichloride given on three consecutive days during organogenesis in cynomolgus monkeys**

Drug Chem. Toxicol., **32**, 150-157（2009）

We previously reported that the administration of dibutyltin dichloride (DBTCl) by nasogastric intubation during the entire period of organogenesis, days 20-50 of pregnancy, was embryolethal, but not teratogenic, in cynomolgus monkeys. The present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days

during organogenesis. Cynomolgus monkeys were given DBTCl at 7.5 mg/kg body weight/day by nasogastric intubation on days 19-21, 21-23, 24-26, 26-28, 29-31, 31-33, or 34-36 of pregnancy, and the pregnancy outcome was determined on day 100 of pregnancy. Embryonic/fetal loss was observed in 1 female given DBTCl on days 19-21, 2 females given DBTCl on days 24-26, and 1 female given DBTCl on days 34-36. There were no effects of DBTCl on developmental parameters in surviving fetuses, including fetal body weight, crown-rump length, tail length, or placental weight. No external, internal, or skeletal malformations were detected in fetuses in any group. DBTCl did not affect the incidence of fetuses with skeletal variation or skeletal ossification of fetuses. These data confirm our previous findings that DBTCl was embryolethal, but not teratogenic, in cynomolgus monkeys.

Keywords: developmental toxicity, dibutyltin, monkey

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Ema, M., Ise, R.[*1], Kato, H.[*1], Oneda, S.[*2], Hirose, A., Hirata-Koizumi, M., Singh, A. V.[*3], Knudsen, T. B.[*4] and Ihara, T.[*2]: **Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide**

Reprod. Toxicol., **29**, 49-56 (2009)

The present study was performed to determine experimental conditions for thalidomide induction of fetal malformations and to understand the molecular mechanisms underlying thalidomide teratogenicity in cynomolgus monkeys. Cynomolgus monkeys were orally administered thalidomide at 15 or 20mg/kg-d on days 26-28 of gestation, and fetuses were examined on day 100-102 of gestation. Limb defects such as micromelia/amelia, paw/foot hyperflexion, polydactyly, syndactyly, and brachydactyly were observed in seven of eight fetuses. Cynomolgus monkeys were orally administered thalidomide at 20mg/kg on day 26 of gestation, and whole embryos were removed from the dams 6h after administration. Three embryos each were obtained from the thalidomide-treated and control groups. Total RNA was isolated from individual embryos, amplified to biotinylated cRNA and hybridized to a custom Non-Human Primate (NHP) GeneChip ((R)) Array. Altered genes were clustered into genes that were up-regulated (1281 genes) and down-regulated (1081 genes) in thalidomide-exposed embryos. Functional annotation by Gene Ontology (GO) categories revealed up-regulation of actin cytoskeletal remodeling and insulin signaling, and down-regulation of pathways for vasculature development and the inflammatory response. These findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the monkey embryo. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure has now identified many key pathways implicated in thalidomide embryopathy, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype.

Keywords: thalidomide, teratogenicity, gene expression profile

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Hirode, M.[*1], Horinouchi, A.[*1], Uehara, T.[*2], Ono, A., Miyagishima, T.[*2], Yamada, H.[*2], Nagao, T.[*3], Ohno, Y. and Urushidani, T.[*4] : **Gene expression profiling in rat liver treated with compounds inducing elevation of bilirubin**

Hum. Exp. Toxicol., **28**, 231-244 (2009)

We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for the diagnosis of elevated total bilirubin (TBIL) and direct bilirubin (DBIL), we extracted 59 probe sets of rat hepatic genes from the data for seven typical drugs, gemfibrozil, phalloidin, colchicine, bendazac, rifampicin, cyclosporine A, and chlorpromazine, which induced this phenotype from 3 to 28 days of repeated administration in the present study. Principal component analysis (PCA) using these probes clearly separated dose- and time-dependent clusters in the treated groups from their controls. Eighteen more drugs in the database, reported to elevate TBIL and DBIL, were estimated by PCA using these probe sets. Of these, 12 drugs, that is methapyrilene, thioacetamide, ticlopidine, ethinyl estradiol, alpha-naphthylisothiocyanate, indomethacin, methyltestosterone, penicillamine, allyl alcohol, aspirin,

iproniazid, and isoniazid were also separated from the control clusters, as were the seven typical drugs causing elevation of TBIL and DBIL. The principal component 1 (PC1) value showed high correlation with TBIL and DBIL. In the cases of colchicine, bendazac, chlorpromazine, gemfibrozil, and phalloidin, the possible elevation of TBIL and DBIL could be predicted by expression of these genes 24 h after single administration. We conclude that these identified 59 probe sets could be useful to diagnose the cause of elevation of TBIL and DBIL, and that toxicogenomics would be a promising approach for prediction of this type of toxicity.

Keywords: toxicogenomics, bilirubin, biological markers

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Hirode, M.[*1], Omura, K.[*1], Kiyosawa, N.[*1], Uehara, T.[*1], Shimuzu, T.[*1], Ono, A., Miyagishima, T.[*1], Nagao, T.[*2], Ohno, Y. and Urushidani, T.[*3]: **Gene expression profiling in rat liver treated with various hepatotoxic-compounds inducing coagulopathy**

J. Toxicol. Sci., **34**, 281-293 (2009)

A large-scale transcriptome database of rat liver (TG-GATEs) has been established by the Toxicogenomics Project in Japan. In the present study, we focused on 8 hepatotoxic compounds within TG-GATEs, i.e., clofibrate, omeprazole, ethionine, thioacetamide, benzbromarone, propylthiouracil, Wy-14,643 and amiodarone, which induced coagulation abnormalities. Aspirin was selected as a reference compound that directly causes coagulation abnormality, but not through liver toxicity. In blood chemical examinations, for all the coagulopathic compounds there was little elevation of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT), suggesting no severe cell death by treatment with the compounds. We extracted 344 probe sets from the data for these 8 typical drugs, which induced this phenotype at any time from 3 to 28 days of repeated administration. Principal component analysis using these probe sets clearly separated dose- and time-dependent clusters of the treated groups from their controls, except aspirin and propylthiouracil, both of which were considered to cause coagulopathy not due to their hepatotoxicity but due to their direct effects on the blood coagulation system. Reviewing the extracted genes, changes in lipid metabolism were found to be dominant. Genes related to blood coagulation were generally down-regulated by these drugs except that vitamin K epoxide reductase complex subunit 1 (Vkorc1) like 1, a paralogous gene of Vkorc1, was up-regulated. As expected, expression changes of these genes were least prominent in aspirin or propylthiouracil-treated liver. We concluded that these probe sets could be a good starting point in developing mechanism-based biomarkers for diagnosis or prognosis of hepatotoxicity-related coagulation abnormalities in the early stage of drug development.

Keywords: coagulopathy, toxicogenomics, hepatotoxicity

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Kobayashi, K.[*1], Sakuratani, Y.[*1], Abe, T.[*1], Nishikawa, S.[*1], Yamada, J.[*1], Hirose, A., Kamata, E. and Hayashi, M.[*1, 2]: **Relation between statistics and treatment-related changes obtained from toxicity studies in rats: if detected a sgnificant difference in low or middle dose for quantitative values, this change is considered as incidental change?**

J. Toxicol. Sci., **35**, 79-85 (2010)

The purpose of a toxicity test is to determine the no-observed-effect level (NOEL) of test substance through biological and pharmacological techniques. If the low dose not does show statistically significant and biologically relevant changes in the data evaluated in a study, the usual practice is to consider this dose as the NOEL. To overcome this, 6 types of techniques that seemed to be appropriate are presented in this paper by investigating the results of several domestic and foreign theses on toxicology. The most appropriate techniques appear to be the trend test, comparison between treatment group and historical control by t-test, and confirmation that all individual values lie within the 95% confidence interval (2 SD) of the historical control value, if a significant difference is admitted in the low dose.

Keywords: toxicity, statistics, historical control data

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Kondo, C.[*1], Minowa, Y.[*2], Uehara, T.[*1], Okuno, Y.[*3], Nakatsu, N.[*1], Ono, A., Maruyama, T.[*1], Kato, I.[*1], Yamate, J.[*4], Yamada, H.[*2], Ohno, Y. and Urushidani, T.[*5]: **Identification of genomic biomarkers for concurrent diagnosis of drug-induced renal tubular injury using a large-scale toxicogenomics database** *Toxicology*, **265**, 15-26 (2009)

Drug-induced renal tubular injury is one of the major concerns in preclinical safety evaluations. Toxicogenomics is becoming a generally accepted approach for identifying chemicals with potential safety problems. In the present study, we analyzed 33 nephrotoxicants and 8 non-nephrotoxic hepatotoxicants to elucidate time- and dose-dependent global gene expression changes associated with proximal tubular toxicity. The compounds were administered orally or intravenously once daily to male Sprague-Dawley rats. The animals were exposed to four different doses of the compounds, and kidney tissues were collected on days 4, 8, 15, and 29. Gene expression profiles were generated from kidney RNA by using Affymetrix GeneChips and analyzed in conjunction with the histopathological changes. We used the filter-type gene selection algorithm based on t-statistics conjugated with the SVM classifier, and achieved a sensitivity of 90% with a selectivity of 90%. Then, 92 genes were extracted as the genomic biomarker candidates that were used to construct the classifier. The gene list contains well-known biomarkers, such as Kidney injury molecule 1, Ceruloplasmin, Clusterin, Tissue inhibitor of metallopeptidase 1, and also novel biomarker candidates. Most of the genes involved in tissue remodeling, the immune/inflammatory response, cell adhesion/proliferation/migration, and metabolism were predominantly up-regulated. Down-regulated genes participated in cell adhesion/proliferation/migration, membrane transport, and signal transduction. Our classifier has better prediction accuracy than any of the well-known biomarkers. Therefore, the toxicogenomics approach would be useful for concurrent diagnosis of renal tubular injury.

Keywords: biological markers, toxicogenomics, kidney diseases

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Sakamoto, Y.[*1], Dai, N.[*1, 2], Hagiwara, Y.[*3, 4], Satoh, K.[*1], Ohashi, N.[*1], Fukamachi, K.[*5], Tsuda, H.[*5], Hirose, A., Nishimura, T., Hino, O.[*3] and Ogata, A.[*1]: **Serum level of expressed in renal carcinoma (ERC)/ mesothelin in rats with mesothelial proliferative lesions induced by multi-wall carbon nanotube (MWCNT)** *J. Toxicol. Sci.*, **35**, 265-270 (2010)

Expressed in renal carcinoma (ERC) /mesothelin is a good biomarker for human mesothelioma and has been investigated for its mechanistic rationale during the mesothelioma development. Studies are thus ongoing in our laboratories to assess expression of ERC/mesothelin in sera and normal/proliferative/neoplastic mesothelial tissues of animals untreated or given potentially mesothelioma-inducible xenobiotics, by an enzyme-linked immunosorbent assay (ELISA) for N- and C-(terminal fragments of) ERC/mesothelin and immunohistochemistry for C-ERC/mesothelin. In the present paper, we intend to communicate our preliminary data, because this is the first report to show how and from what stage the ERC/mesothelin expression changes during the chemical induction of mesothelial proliferative/neoplatic lesions. Serum N-ERC/mesothelin levels were 51.4 ± 5.6 ng/ml in control male Fischer 344 rats, increased to 83.6 ± 11.2 ng/ml in rats given a single intrascrotal administration of 1 mg/kg body weight of multi-wall carbon nanotube (MWCNT) and bearing mesothelial hyperplasia 52 weeks thereafter, and further elevated to 180 ± 77 ng/ml in rats similarly treated and becoming moribund 40 weeks thereafter, or killed as scheduled at the end of week 52, bearing mesothelioma. While C-ERC/mesothelin was expressed in normal and hyperplastic mesothelia, the protein was detected only in epithelioid mesothelioma cells at the most superficial layer. It is thus suggested that ERC/mesothelin can be used as a biomarker of mesothelial proliferative lesions also in animals, and that the increase of levels may start from the early stage and be enhanced by the progression of the mesothelioma development.

Keywords: serum mesothelin, MWCNT, mesothelial proliferative lesions

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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)

Background: Perfluoroalkanoates, [e.g., perfluorooctanoate (PFOA)], are known peroxisome proliferators that induce hepatomegaly and hepatocarcinogenesis in rodents, and are classic nongenotoxic 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. Objectives: 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. Methods: We used an incision load/dye transfer technique to assess GJIC in livers of rats exposed to PFOA and PFPeA. We used in vitro assays with inhibitors of cell signaling enzymes and antioxidants known to regulate GJIC to identify which enzymes regulated PFOA-induced inhibition of GJIC. Results: 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. Conclusions: 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: gap-junctional intercellular communication, perfluorooctanoate, tumor promotion

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Watanabe, W.*, Shimizu, T.*, Sawamura, R.*, Hino, A.*, Konno, K.*, Hirose, A. and Kurokawa, M.*: **Effects of tetrabromobisphenol A, a brominated flame retardant, on the immune response to respiratory syncytial virus infection in mice**
Int. Immunopharmacol., **10**, 393-397 (2010)

Effects of the brominated flame retardants (BFRs), decabrominated diphenyl ether (DBDE), hexabromocyclododecane (HBCD), and tetrabromobisphenol A (TBBPA), on host immunity of mice were evaluated using respiratory syncytial virus (RSV) infection. Five-week-old female mice were fed a diet containing 1% BFRs for 28 days, and subsequently infected with RSV. No toxicological sign was observed in BFR-treated mice before infection. TBBPA significantly increased the pulmonary viral titer in the infected mice on day 5 post-infection, but DBDE and HBCD did not. Slight histological changes were observed in lung tissues of TBBPA-treated mice with mock infection. These changes due to TBBPA were much exacerbated by RSV infection. Cytokine analysis of bronchoalveolar lavage fluid (BALF) from RSV-infected mice treated with or without TBBPA revealed that TBBPA significantly increased the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-γ at each time point after virus infection, but no change was observed for IL-1β and IL- 12. The levels of IL-4 and IL-10, Th2 cytokines, significantly decreased. Thus, TBBPA caused unusual production of the various cytokines in RSV-infected mice. Flow cytometry revealed that the percentage of double-positive CD4+CD8+ cells, immature T lymphocytes, in the cell populations in BALF from RSVinfected mice increased due to TBBPA treatment. The change was not observed in spleen cells of TBBPAtreated mice. The response to RSV infection verified that TBBPA treatment affected the host immunity of mice. Irregular changes in cytokine production and immune cell populations due to TBBPA treatment were suggested to cause exacerbation of pneumonia in RSV-infected mice.
Keywords: respiratory syncytial virus, tetrabromobisphenol A, pneumonia

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S.[*2], Furukawa, F.[*2], Uchino, T., Tokunaga, H.[*3], Nishimura, T., Hirose, A., Kanno, J. and Tsuda, H.[*1]:

Involvement of macrophage inflammation protein 1{alpha} (MIP1{alpha}) in promotion of rat lung and mammary carcinogenic activity of nano-scale titanium dioxide particles administered by intrapulmonary spraying

Carcinogenesis, **31**, 927-935 (2010)

Titanium dioxide (TiO_2) is evaluated by World Health Organization/International Agency for Research on Cancer as a Group 2B carcinogen. The present study was conducted to detect carcinogenic activity of nanoscale TiO_2 administered by a novel intrapulmonary spraying (IPS)-initiation-promotion protocol in the rat lung. Female human c-Ha-ras proto-oncogene transgenic rat (Hras128) transgenic rats were treated first with N-nitrosobis(2-hydroxypropyl)amine (DHPN) in the drinking water and then with TiO_2 (rutile type, mean diameter 20 nm, without coating) by IPS. TiO_2 treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas, confirming the effectiveness of the IPS-initiation-promotion protocol. TiO_2 aggregates were localized exclusively in alveolar macrophages and had a mean diameter of 107.4 nm. To investigate the underlying mechanism of its carcinogenic effects, TiO_2 was administered to wild-type rats by IPS five times over 9 days. TiO_2 treatment significantly increased 8-hydroxydeoxy guanosine level, superoxide dismutase activity and macrophage inflammatory protein 1a (MIP1a) expression in the lung. MIP1a, detected in the cytoplasm of TiO2-laden alveolar macrophages in vivo and in the media of rat primary alveolar macrophages treated with TiO_2 in vitro, enhanced proliferation of human lung cancer cells. Furthermore, MIP1a, also detected in the sera and mammary adenocarcinomas of TiO_2-treated Hras128 rats, enhanced proliferation of rat mammary carcinoma cells. These data indicate that secreted MIP1a from TiO_2-laden alveolar macrophages can cause cell proliferation in the alveoli and mammary gland and suggest that TiO_2 tumor promotion is mediated by MIP1a acting locally in the alveoli and distantly in the mammary gland after transport via the circulation.

Keywords: titanium dioxide, nanomaterials, carcinogenicity

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Yamazaki, T.[*1], Hirose, A., Sakamoto, T.[*1], Okazaki, M.[*1], Mitsumoto, A.[*2], Kudo, N.[*1] and Kawashima, Y.[*1]:

Peroxisome proliferators attenuate free arachidonic acid pool in the kidney through inducing lysophospholipid acyltransferases

J. Pharmacol. Sci., **111**, 201-210 (2009)

Attenuating effects of peroxisome proliferators on the concentration of free arachidonic acid by inducing 1-acyl-2-lysophospholipid acyltransferases in the kidney were studied. The administration of the three structurally dissimilar peroxisome proliferators, 2-(4-chlorophenoxy)-2-methylpropionic acid (clofibric acid), di (2-ethylhexyl) phthalate, and 2,2'-(decamethylenedithio) diethanol, to rats or mice considerably increased the activities of microsomal 1-acylglycerophosphoethanolamine acyltransferase (LPEAT), 1-acylglycerophosphoinositol acyltransferase (LPIAT), 1-acylglycerophosphoserine acyltransferase (LPSAT), and 1-acylglycerophosphocholine acyltransferase (LPCAT), and the mRNA level of LPCAT3, but not the mRNA level of LPCAT1, LPCAT4, or LPEAT1, in the kidney and the liver. The proportions of arachidonic acid in phospholipids in renal microsomes are rather high for the low proportion of arachidonic acid in free fatty acids in renal microsomes of control rats. The treatment of rats with clofibric acid attenuated the concentration and the proportion of free arachidonic acid to about a half; nevertheless the treatment lowered slightly the proportions of arachidonic acid in phospholipids other than phosphatidylcholine. These results indicate that peroxisome proliferators upregulate the four 1-acyl-2-lysophospholipid acyltransferases of the kidney and, and the induced 1-acyl-2-lysophospholipid acyltransferases seem to play a physiologically crucial contribution in attenuating the pool of free arachidonic acid in the kidney.

Keywords: peroxisome proliferatore, free arachidonic acid, kidney

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