Tanabe S, Aoyagi K[*1], Yokozaki H[*2], Sasaki H[*1]: Gene expression signatures for identifying diffuse-type gastric cancer associated with epithelial-mesenchymal transition.
Int J Oncol. 2014;44:1955-70.

Epithelial-mesenchymal transition（EMT）is associated with tumor malignancy. The hedgehog-EMT pathway is preferentially activated in diffuse-type gastric cancer（GC）compared with intestinal-type GC; however, histological typing is currently the only method for distinguishing these two major types of GC. We compared the gene expression profiles of 12 bone marrow-derived mesenchymal stem cell cultures and 5 diffuse-type GC tissue samples. Numerous upregulated or downregulated genes were identified in diffuse-type GC, including *CDH1*, *CDH2*, *VIM*, *WNT4* and *WNT5*. Among these genes, the mRNA ratio of *CDH2* to *CDH1* could distinguish the 15 diffuse-type GC samples from the 17 intestinal-type GC samples. Our results suggested that the mesenchymal features were more prominent in diffuse-type GC than in intestinal-type GC, but were weaker in diffuse-type GC than in mesenchymal stem cells. Diffuse-type GC that has undergone extensive EMT, which has a poor prognosis, can be identified by quantitative PCR analysis of only two genes.
Keywords: Epithelial-mesenchymal transition, Gastric cancer, Mesenchymal stem cell

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Amakura Y[*1], Yoshimura M[*1], Yamakami S[*1], Yoshida T[*1], Wakana D, Hyuga M, Hyuga S[*2], Hanawa T[*2], Goda Y: Characterization of Phenolic Constituents from Ephedra Herb Extract.
Molecules. 2013;18:5326-34.

Nine known compounds: *trans*-cinnamic acid, catechin, syringin, epicatechin, symplocoside, kaempferol 3-*O*-rhamnoside 7-*O*-glucoside, isovitexin 2-*O*-rhamnoside, herbacetin 7-*O*-glucoside, and pollenitin B and a new flavonoid glycoside, characterized as herbacetin 7-*O*-neohesperidoside（**1**）on the basis of spectroscopic analysis and chemical evidence, were isolated from a traditional crude drug, “Ephedra herb extract”. Compound **1** had no effects on HGF-induced motility, whereas herbacetin, which is an aglycone of **1**, significantly inhibited it.
Keywords: Ephedra herb, herbacetin 7-*O*-neohesperidoside, Mao

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Fuchino H[*1], Daikonya A[*1], Kumagai T[*1], Goda Y, Takahashi Y[*2], Kawahara N[*1]: Two new labdane diterpenes from fresh leaves of *Leonurus japonicus* and their degradation during drying.
Chem Pharm Bull. 2013;61:497-503.

Degradation of the components of Leonurus Herb was examined during drying. Compounds **1** and **2** were detected on TLC at lower temperature, but not at higher temperature. Their chemical structures were determined by spectral methods. They immediately decomposed even at 40°C in chloroform solution. They are believed to be transformed through a retro-aldol reaction. Compounds **1** and **2** have not been previously reported.
Keywords: *Leonurus japonicus*, labdane diterpene, TLC-MS

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大根谷章浩[*1]，渕野裕之[*1]，新井玲子[*1]，高橋豊[*2]，和田浩志[*3]，合田幸広，川原信夫[*1]：生薬「オウゴン」国内市場品の一酸化窒素産生抑制活性とLC/MSメタボローム解析.
生薬学雑誌 2013;67:35-40.

During the course of our studies on the profiling analysis for the crude drug, we collected various samples of scutellariae radix（*Scutellaria baicalensis* Georgi）from the Japanese market. This crude drug is one of the most widespread herbs used for Kampo medicine. The profiling data of the hot water extract of scutellariae radix were developed by LC/MS-based metabolomics. We examined its inhibitory activity on nitric oxide（NO）production by LPS/IFN-γ activated macrophages. The hot water extracts showed varied

inhibitory activity against NO production from LPS/IFN-γ activated macropharges. Therefore, we performed PCA, OPLS and OPLS-DA with the SIMCA method to search for important markers which contributed anti-inflammatory effect. As a result, the hot water extracts were clearly divided into two groups according to the strength of activity. We quickly found out that wogonoside, a main flavonoid, was a contributor to the distinction between the two groups by S plot. Wogonoside showed the inhibitory effect against NO production in a dose-dependent manner. These results suggested that wogonoside might be a useful biomarker to estimate the anti-inflammatory effect of scutellariae radix.

Keywords: *Scutellaria baicalensis*, LC-MS metabolomics, nitric oxide

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下村裕子，徳本廣子，関田節子*1，佐竹元吉*2，徳川斉正*3，徳川眞木*3，合田幸広：水戸徳川家の宝物「烏〇圓」の内容物の解明.
生薬学雑誌 2013;57:41-58.

The gallipot found as the heirloom of the Mito-Tokugawa family has the unclear label “Usaien” and contains a small amount of dry black preparation. It is historically clear that Ieyasu Tokugawa, who was the founder of the Edo Shogunate, used it. Fortunately, the “Korean Wazaikyokuho”, which was a formulary of natural medicines, has been found as one of the valuable possessions of Kunozan Toshogu, where Ieyasu Tokugawa is enshrined and the formulary contains the “Usaien” formula. It is of interest to reveal the components of the preparation from the viewpoints of historiography and pharmacognosy. Therefore, by utilizing the “Usaien” formula as a clue, we started microscopic analyses to reveal the crude drug components of the historical dry black preparation.

First we found this preparation contained a lot of pollens which were thought to be of multiple origins. This indicated the preparation was a kind of honey paste. Furthermore, the successive analyses on the basis of the morphological characteristics of elements of the crude drugs led to the identification of 52 crude drugs（herbal origins: 35, animal origins: 14 and minerals: 3）as the components. The reference formula in the “Korean Wazaikyokuho” consisted of 58 crude drugs and of them 2 volatile ones, 2 sarcous ones, mercury and calomel have remained unidentified, because of difficulty of confirmation by microscopic analyses or insufficient information the origin of their crude drugs. Since most of the crude drug components of the “Usaien” formula were identified in the dry black preparation, we thought the shogun, Ieyasu Tokugawa, used the formula for his health care.

Keywords: Usaien preparation, Mito-Tokugawa heirloom gallipot, microscopic analyses

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Wakana D, Kawahara N*, Goda Y: Two new pyrrolidine alkaloids, codonopsinol C and codonopiloside A, isolated from *Codonopsis pilosula*.
Chem Pharm Bull. 2013;61:1315-7.

A new pyrrolidine alkaloid codonopsinol C (**1**), and pyrrolidine alkaloidal glycoside, codonopiloside A (**2**), were isolated from the roots of *Codonopsis pilosula*, along with four known pyrrolidine alkaloids, codonopsinol A (**3**), codonopsinol B (**4**), codonopyrrolidium B (**5**), and radicamine A (**6**). The structures of the new compounds were established by acid hydrolysis and spectroscopic methods. We describe those structures in this paper.

Keywords: *Codonopsis pilosula*, pyrrolidine alkaloid, Campanulaceae

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Tsunematsu Y*1, Ishikawa N*1, Wakana D, Goda Y, Noguchi H*1, Moriya H*2, Hotta K*3, Watanabe K*1: Distinct mechanisms for spiro-carbon formation reveal biosynthetic pathway crosstalk.
Nature Chemical Biology. 2013;9:818-25.

Spirotryprostatins, an indole alkaloid class of nonribosomal peptides isolated from *Aspergillus fumigatus*, are known for their antimitotic activity in tumor cells. Because spirotryprostatins and many other chemically complex spiro-carbon-bearing natural products exhibit useful biological activities, identifying

and understanding the mechanism of spiro-carbon biosynthesis is of great interest. Here we report a detailed study of spiro-ring formation in spirotryprostatins from tryprostatins derived from the fumitremorgin biosynthetic pathway, using reactants and products prepared with engineered yeast and fungal strains. Unexpectedly, FqzB, an FAD-dependent monooxygenase from the unrelated fumiquinazoline biosynthetic pathway, catalyzed spiro-carbon formation in spirotryprostatin A via an epoxidation route. Furthermore, FtmG, a cytochrome P450 from the fumitremorgin biosynthetic pathway, was determined to catalyze the spiro-ring formation in spirotryprostatin B. Our results high-light the versatile role of oxygenating enzymes in the biosynthesis of structurally complex natural products and indicate that cross-talk of different biosynthetic pathways allows product diversification in natural product biosynthesis.
Keywords: spirotyprostatin, spiro-carbon-bearing natural product, product diversification

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He JY*1, Zhu S*1, Komatsu K*1, Goda Y, Cai SQ*2: Genetic polymorphism of medicinally-used Codonopsis species in internal transcribed spacer sequence of nuclear ribosomal DNA and its application to authenticate Codonopsis Radix.
J Nat Med. 2014; 68:112-24.

Codonopsis Radix has been prescribed as the roots of *Codonopsis pilosula, C. pilosula* var. *modesta* and *C. tangshen* in Chinese Pharmacopoeia. In order to find out genetic markers for identifying the 3 taxa and to authenticate Codonopsis Radix, the molecular analysis of the internal transcribed spacer sequence of nuclear ribosomal DNA was conducted on Codonopsis plants collected widely from Gansu Prov. and Chongqing city of China, the main producing areas of Codonopsis Radix. Significant genetic polymorphism was observed, represented by 11 types of ITS sequences in C. pilosula, 5 types in *C. pilosula* var. *modesta* and 5 types in *C. tangshen*. Among the determined sequences, 1, 1 and 2 types were thought to be of pure lines of each taxon, respectively, designated as types P0, PM0, T1 and T3, and the rest might be derived from hybridization. Hybrid lines were inferred to be resulting from the combination of these pure lines. The informative sites for discriminating the 3 taxa were detected at the nucleotide positions 122nd, 226th, 441st and 489th from upstream of the ITS sequence. For discrimination of the types of *C. tangshen*, including one type T0 registered in GenBank, the nucleotides at positions 135th, 489th and 500th were informative. Botanical sources of the crude drugs produced in a wide range of the southeast Gansu Prov. were *C. pilosula*, just those from Wenxian of Gansu Prov. were *C. pilosula* var. *modesta*. The crude drugs produced in Chongqing were derived from *C. tangshen*.
Keywords: Codonopsis, genetic polymorphism, molecular identification

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細江潤子，杉本直樹，末松孝子*1，山田裕子*2，三浦亨*2，早川昌子*2，鈴木裕樹*3，勝原孝雄*3，西村浩昭*3，菊地祐一*3，山下忠俊*4，合田幸広：日本薬局方における定量NMR（qNMR）の利用に関する準備研究（第1報）．
医薬品医療機器レギュラトリーサイエンス 2014;45:243-50.

Preliminary studies were performed to establish the quantitative nuclear magnetic resonance（“qNMR）test” in the crude drug test section of the Japanese Pharmacopoeia（JP）. In this report, we examined impurity signals from internal reference substances and targeted marker compounds, chemical shifts of internal reference substances, and the suitability of signal peaks of targeted marker compounds for qNMR. For example, the internal reference substance 1,4-BTMSB-d_4 showed an impurity signal at about 7.3ppm derived from 1,4-$(Me_3Si)_2$-$C_6D_3\underline{H}$ in the highly accumulated NMR spectrum at 400 MHz. The impurity signal increased time-dependently in $CDCl_3$, but not in CD_3OD or CD_3COCD_3. This impurity signal interfered with integration of the signal of geniposide at 7.26ppm.

Therefore, we consider that this signal of geniposide is unsuitable for quantification. Our data also suggest that it is important to measure qNMR immediately after sample preparation when $CDCl_3$ is used as the solvent. Similarly, the highly accumulated NMR spectrum of another internal reference substance, DSS-d_6, showed impurity signals at 0.59, 1.72 and 2.88ppm in D_2O and at 0.48, 1.54 and 2.37ppm in DMSO-d_6, which are derived from $Me_3SiC\underline{H}DCD_2CD_2SO_3Na$, $Me_3SiCD_2C\underline{H}DCD_2SO_3Na$, and $Me_3SiCD_2CD_2C\underline{H}DSO_3Na$, respectively. Therefore, it is considered essential that the spectral regions around these impurity signals be avoided in selecting suitable signals of targeted compounds for integration. Very small, but distinct, impurity signals also appeared in the spectra of several targeted marker compounds when the data were obtained under highly accumulated (about 3800 times) conditions. These observations suggest that prior determination of impurity signals arising from the internal reference substances and the targeted samples would be essential to assure the validity of qNMR.

The present results are expected to be helpful in the process of establishing "the qNMR test" in the JP.

Keywords: Quantitative NMR, Impurity signals, Japanese Pharmacopoeia

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Hoshino T*, Narukawa Y*, Haishima Y, Goda Y, Kiuchi F*: Two new sulfated oleanan saponins from Achyranthes root.

J Nat Med. 2013;67:386-9.

Two new sulfated oleanan saponins, sulfachyranthosides B and D, were isolated from a water extract of Achyranthes root (root of *Achyranthes bidentata*). The structures were determined by analyses of spectroscopic data to be sulfates of achyranthosides B and D, respectively, at the 4'-position of the glucose moiety attached to the C-28 carboxylic acid of oleanolic acid.

Keywords: Achyranthes root, *Achyranthes bidentata*, oleanan sulfated saponin

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Izutsu K, Yomota C, Okuda H, Kawanishi T, Randolph TW*1, Carpenter JF*2: Impact of heat treatment on miscibility of proteins and disaccharides in frozen solutions.

Eur J Pharm Biopharm. 2013;85:177-83.

The purpose of this study was to elucidate the effect of heat treatment (annealing) on the miscibility of concentrated protein and disaccharide mixtures in the freezing segment of lyophilization. Frozen solutions containing a protein (e.g., recombinant human albumin, chicken egg lysozyme, bovine plasma immunoglobulin G, or a humanized IgG1k monoclonal antibody) and a non-reducing disaccharide (e.g., sucrose or trehalose) showed single thermal transitions of the solute mixtures (glass transition temperature of maximally freeze-concentrated solutes: T_g') in their first heating scans. Heat treatment (*e.g., -5* °C, 30 min) of some disaccharide-rich mixture frozen solutions at temperatures far above their T_g' induced two-step T_g' transitions in the subsequent scans, suggesting the separation of the solutes into concentrated protein-disaccharide mixture phase and disaccharide phase. Other frozen solutions showed a single transition of the concentrated solute mixture both before and after heat treatment. The apparent effects of the heat treatment temperature and time on the changes in thermal properties suggest molecular reordering of the concentrated solutes from a kinetically fixed mixture state to a more thermodynamically favorable state as a result of increased mobility. The implications of these phenomena on the quality of protein formulations are discussed.

Keywords: Freeze-drying, Protein formulation, Phase separation

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Shibata H, Yomota C, Okuda H: Simultaneous determination of polyethylene glycol-conjugated liposome components by using reversed-phase high-performance liquid chromatography with UV and evaporative light scattering detection.

Pharm Sci Tech. 2013;14:811-7.

Liposomes incorporating polyethylene glycol (PEG)

-conjugated lipids (PEGylated liposomes) are of great interest as drug delivery carriers. The lipid composition is one of important factors to ensure the quality/safety/efficacy of liposomal products. The lipid hydrolysis is also considered a critical parameter for the chemical stability of liposomal products. Thus, in this study, we attempted to develop a simple reversed-phase HPLC method with an evaporative light scattering detector (ELSD) for simultaneous determination of lipid components and hydrolysis products in PEGylated liposomes.

Keywords: liposome, reversed-phase HPLC, evaporative light scattering

Katori N: Regulated bioanalysis in Japan: where do we come from and where are we going?.
Bioanalysis. 2013;5:1321-3.

Japan had a late start in regulated bioanalysis; fortunately, however, by a great deal of effort by the people in this area (e.g., the Japan Bioanalysis Forum), the first guideline was finalized within a shorter period than expected ... Our next step will be setting a wider area for discussion of regulated bioanalysis in Japan.

Keywords: regulated bioanalysis, guideline on Bioanalytical Method Validation (BMV), Japan Bioanalysis Forum (JBF)

Stevenson L*[1], Garofolo F*[2], DeSilva B*[3], Dumont I*[2], Martinez S*[2], Rocci M*[4], Amaravadi L*[1], Kloeppel MB*[5], Musuku A*[6], Booth B*[7], Dicaire C*[2], Wright L*[8], Provencher LM*[2], Losauro M*[8], Gouty D*[8], Arnold M*[3], Bansal S*[9], Dudal S*[9], Dufield D*[10], Duggan J*[11], Evans C*[12], Fluhler E*[10], Fraser S*[10], Gorovits B*[10], Haidar S*[7], Hayes R*[13], Ho S*[14], Houghton R*[15], Islam R*[16], Jenkins R*[17], Katori N, Kaur S*[18], Kelley M*[19], Knutsson M*[20], Lee MJ*[21], Liu H*[14], Lowes S*[22], Ma M*[21], Mikulskis A*[1], Myler H*[3], Nicholson B*[17], Olah T*[3], Ormsby E*[23], Patel S*[24], Pucci V*[25], Ray C*[10], Schultz G*[22], Shih J*[21], Shoup R*[26], Simon C*[23], Song A*[18], Neto JT*[27], Theobald V*[28], Thway T*[21], Smith JW*[29], Wang J*[3], Wang L*[30], Welink J*[31], Whale E*[29], Woolf E*[32] & Xu R*[33]: 2013 White Paper on Recent Issues in Bioanalysis: "Hybrid" - the best of LBA & LCMS.
Bioanalysis. 2013;5:2903-18.

The 2013 7th Workshop on Recent Issues in Bioanalysis was held in Long Beach, California, USA, where close to 500 professionals from pharmaceutical and biopharmaceutical companies, CROs and regulatory agencies convened to discuss current topics of interest in bioanalysis. These 'hot' topics, which covered both small and large molecules, were the starting point for fruitful exchanges of knowledge, and sharing of ideas among speakers, panelists and attendees. The discussions led to specific recommendations pertinent to bioanalytical science. Such as the previous editions, this 2013 White Paper addresses important bioanalytical issues and provides practical answers to the topics presented, discussed and agreed upon by the global bioanalytical community attending the 7th Workshop on Recent Issues in Bioanalysis.

Keywords: regulated bioanalysis, guideline on Bioanalytical Method Validation (BMV), Japan Bioanalysis Forum (JBF)

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Yamamoto Y*1, Fukami T*2, Koide T, Onuki Y*3, Suzuki T*2, Metori K*2, Katori N, Hiyama Y, Tomono K*2: Comparative pharmaceutical evaluation of brand and generic clobetasone butyrate ointments.
Int J Pharm. 2014;463:62-7.

In the present study, we performed comprehensive pharmaceutical evaluation among an original clobetasone butyrate（CLB）ointment product and three generic products. Although spherocrystal images were observed under a polarizing microscope for only Kindavate, the original product, distribution of active and inactive ingredients was chemically equivalent between the original and generic medicine by the attenuated total reflection infrared spectroscopy. These results suggest that the spherocrystals observed in Kindavate are composed of hydrocarbon. On GC/MS, it was revealed that linear alkanes having 25-27carbon atoms are densely present in Sun White®, the base used in Kindavate. On the other hand, linear alkanes having 22-31 carbon atoms were broadly distributed in most other white petrolatums. In the CLB ointment products, the distribution equivalent of linear alkane to Sun White was observed only in Kindavate. Thus, the GC/MS method is extremely useful for identification of white petrolatum used in the ointment. A similar amount of CLB among the pharmaceutical products was detected in the skin tissue by skin accumulation test, although there were the differences in rheological properties and the quality of white petrolatum. The present results will be very useful for pharmacists in selecting medicine products that match the needs of the patient. Such pharmaceutical information will help spread objective knowledge about products in the future, and will contribute to the appropriate selection of medication.

Keywords: Image analysis, Rheological property, Skin accumulation

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Un K, Sakai-Kato K, Kawanishi T, Okuda H, Goda Y: Effects of liposomal phospholipids and lipid transport-related protein on the intracellular fate of encapsulated doxorubicin.
Mol Pharm. 2014;11:560-7.

We have previously reported the intracellular trafficking mechanism of liposomal phospholipids. In the present study, we investigated the intracellular trafficking mechanism of polyethylene glycol（PEG）-modified phospholipids, and the effects of liposomal phospholipids on the intracellular trafficking and cytotoxicity of doxorubicin（DXR）. Following endocytosis of liposomes, although phospholipids were localized to the endoplasmic reticulum（ER）and Golgi apparatus, PEG-modified phospholipids was only localized to the ER. Moreover, differed from phospholipids, the intracellular concentrations of PEG-modified phospholipids were not affected by suppressing CERT and sec31A expression, suggesting that ER-Golgi transport is not involved in the intracellular trafficking of PEG-modified phospholipids. Although DSPC was transported to the cell membrane by PITP and extracellular effluxed via ABCG1, PEG-modified phospholipids was transported to the cell membrane by ORP2 and extracellular effluxed via ABCB1. Thus, PEG modification affects the intracellular trafficking of other phospholipid components. In experiments to determine the effects of liposomal DXR on phospholipid trafficking, the intracellular concentrations of liposomal phospholipids derived from DXR-loaded liposomes were increased by the suppression of PITP expression. These results suggest that DXR enhances the extracellular efflux of liposomal phospholipids by enhancing PITP expression. We also indicated that the effects of liposomal phospholipids on the intracellular trafficking and cytotoxicity of DXR. DXR forms a complex with PITP and phospholipids, and that DXR was subject to intracellular trafficking as a complex in cells exposed to DXR-encapsulated liposomes. These findings provide valuable information to help improve the therapeutic effects of DXR by encapsulating DXR in PEG-modified liposomes.

Keywords: polyethylene glycol-modified liposome,

doxorubicin, intracellular trafficking

Sakai-Kato K, Hidaka M, Un K, Kawanishi T, Okuda H: Physicochemical properties and in vitro intestinal permeability properties and intestinal cell toxicity of silica particles, performed in simulated gastrointestinal fluids.
Biochim Biophys Acta. 2014;1840:1171-80.

Amorphous silica particles with the primary dimensions of a few tens of nm, have been widely applied as additives in various fields including medicine and food. Especially, they have been widely applied in powders for making tablets and to coat tablets. However, their behavior and biological effects in the gastrointestinal tracts associated with the oral administration remains unknown. Our study indicated the effect of diet on the agglomeration of silica particles. The sizes of silica particles affected the particles' absorption into or transport through the Caco-2 cells, and cytotoxicity in vitro, depending on the various biological fluids. The findings obtained from our study may offer valuable information to evaluate the behavior of silica particles in the gastrointestinal tracts or safety of medicines or foods containing these materials as additives.
Keywords: nanomaterials, silica particles, in vitro model

Sakai-Kato K, Un K, Nanjo K, Nishiyama N[*1], Kusuhara, H[*2], Kataoka K[*3,4], Kawanishi T, Goda Y, Okuda H: Elucidating the molecular mechanism for the intracellular trafficking and fate of block copolymer micelles and their components.
Biomaterials. 2014;35:1347-58.

Block copolymer micelles have shown promise for the intracellular delivery of chemotherapeutic agents, proteins, and nucleic acids. Understanding the mechanism of their intracellular trafficking and fate, including the extracellular efflux of the polymers, will help improve their efficacy and minimize their safety risks. In this Leading Opinion paper, we discuss the molecular mechanism of block copolymer micelle trafficking, from intracellular uptake to extracellular efflux, on the basis of studies with HeLa cells. By using FRET (fluorescence resonance energy transfer) with confocal microscopy, we found that, following their intracellular transport via endocytosis, the micelles dissociated into their polymeric components in late endosomes and/or lysosomes. Furthermore, we confirmed that the intrinsic proteins NPC1 and ORP2 are involved in the intermembrane transfer of polymers from the endosome to the plasma membrane via the ER (endoplasmic reticulum) by using knockdown experiments with siRNAs. After the polymers were transported to the plasma membrane with the aid of ORP2, they were extruded into the cell medium via ABC transporter, ABCB1. Experiments with ABCB1-expressing vesicles indicated that the polymer itself, and not the fluorescent compounds, was recognized by the transporter. These findings, and the analysis of related mechanisms, provide valuable information that should help minimize the potential risks associated with the intracellular accumulation of block copolymer micelles and to improve their therapeutic efficacy.
Keywords: block copolymer micelles, intracellular traffickingm, intermembrane transport

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Sakai-Kato K, Nanjo K, Yamaguchi T, Okuda H, Kawanishi, T: High performance liquid chromatography separation of monoclonal IgG2 isoforms on a column packed with nonporous particles.
Analytical Methods. 2013;5:5899-902.

The IgG2 subclass of antibodies has emerged as an attractive therapeutic framework. However, a method for sufficiently separating the three IgG2 disulfide isoforms has not been developed. Here, we report a method for efficient separation of monoclonal IgG2 isoforms by means of high-performance liquid chromatography on a column packed with 2-μm nonporous ODS silica particles. Under optimized conditions, the isoforms were separated with high resolution because mass transfer resistance in the

stationary phase was reduced by the use of the small, nonporous particles. The number of separated peaks was more than twice that reported previously. The **run-to-run** repeatability of the IgG2 separation pattern was satisfactory, and the day-to-day repeatability of the retention time of the main peak was good (relative standard deviation 0.9%). The separation pattern can be expected to be useful for monitoring quality consistency of therapeutic antibodies.

Keywords: IgG2, monoclonal IgG2 isoforms, a column packed with nonporous particles

Ehmann F[*1], Sakai-Kato K, Duncan R[*1], Perez de la Ossa D H[*1], Pita R[*1], Vidal J-M[*1], Kohli A[*2], Tothfalusi L[*1], Sanh A[*3], Tinton S[*1], Robert J.-L[*4], Lima B.S.[*5], Amati M.P[*6]: Next-generation nanomedicines and nanosimilars: EU regulators' initiatives relating to the development and evaluation of nanomedicines.

Nanomedicine. 2013;8:849-56.

Over the last three decades many first-generation nanomedicines have successfully entered routine clinical use and it is now important for medicines regulatory agencies to consider the mechanisms needed to ensure safe introduction of 'follow-on' nanomedicine products, 'nanosimilars'. Moreover, drug regulators need to ensure that 'next'-generation nanomedicines enter clinical development and consequently the market in a safe and timely way for the benefit of public health. Here we review recent European Medicines Agency activities that relate to the effective development and evaluation of nanomedicine products while keeping patient and consumer safety at the forefront.

Keywords: Block copolymer micelles, liposomal formulations, nano-similars

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原園景，橋井則貴，栗林亮佑，高久明美，柳原繁弘[*1]，西基宏[*1]，岡本寿美子[*2]，津田祐理子[*2]，中島和幸[*3]，森啓太郎[*4]，筑紫周子[*4]，佐藤貴之[*5]，四方靖[*6]，村上弘次[*7]，掛樋一晃[*8]，木下充弘[*8]，神末和哉[*8]，阪口－阿部碧[*9]，川崎ナナ：抗体医薬品の標準的の標準的糖鎖試験法：2-アミノベンザミド誘導体化及び親水性相互作用クロマトグラフィー／蛍光検出.

医薬品医療機器レギュラトリーサイエンス 2013;44(4):357-61.

抗体のFc領域に結合した糖鎖は比較的限られており，主要な糖鎖は，末端にガラクトースが０～２個結合したフコシル化２本複合型糖鎖（FG0-2）であり，また，微量糖鎖としてシアル酸が結合した糖鎖が存在する．抗体医薬品において糖鎖試験は，糖鎖不均一性の恒常性の確認，安全性に影響を及ぼす可能性がある非ヒト型糖鎖の含量の評価，有効性に影響を及ぼす可能性があるフコシル化の程度や高マンノース型及びハイブリッド型糖鎖の含量の確認に用いられる．また，単に製造工程の恒常性の確認のために利用されることもある．糖鎖試験の必要性並びに規格については，その抗体において糖鎖が有効性及び安全性にどのように影響するか，並びに，実際の製造ロットにおけるばらつきを勘案して設定する．抗体医薬品の糖鎖の標準的糖鎖試験法の作成を行ったので，資料としてここに報告する．10機関で本分析条件にてNS0細胞産生抗体を分析したとき，同様の糖鎖プロファイルが得られることを確認している．

Keywords: 抗体医薬品，糖鎖試験法，2-アミノベンザミド誘導体化

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Harazono A, Hashii N, Kuribayashi R, Nakazawa S, Kawasaki N: Mass spectrometric glycoform profiling of the innovator and biosimilar erythropoietin and darbepoetin by LC/ESI-MS.

J Pharm Biomed Anal. 2013;83:65-74.

The recent patent expirations of erythropoietin

（EPO）have promoted the development of biosimilars. Two and one biosimilar EPO products were approved in 2007 in Europe and in 2010 in Japan, respectively. Glycosylation heterogeneity of EPO is very complex, and its pattern has a large impact on its in vivo activity. In this study, glycoform profilings of biosimilar and innovator EPO products were performed using LC/ESI-MS. Glycoforms of EPO were detected within the range of m/z 1,700-3,600 at the 10+ to 16+ charge states. The charge-deconvolved spectra showed complex glycoform mass profiles at 28,000-32,000 Da, and most of the observed peaks were assigned to the peptide（18,236 Da）+ glycans with the compositions of NeuAc10-14Hexn+3HexNAcnFuc3（n = 16 - 26）with or without some O-acetylations（+42 Da）and attachment of NeuGc for NeuAc or oxidation（+16 Da）. Analysis of de-N-glycosylated EPO showed the distributions of O-glycans of NeuAc1-2Hex1HexNAc1 and site occupancy. Each EPO product showed a characteristic glycoform profile with respect to sialylation, glycan size, O-acetylation of sialic acids and O-glycosylation. Analysis of darbepoetin suggested that glycans of darbepoetin were highly sialylated and O-acetylated. LC/ESI-MS was shown to be useful to evaluate the similarity of the glycoform profiles of EPO.

Keywords: erythropoietin, glycoform analysis, LC/ESI-MS

Nakazawa S[*1], Ahn J[*2], Hashii N, Hirose K[*3], Kawasaki N: Analysis of the local dynamics of human insulin and a rapid-acting insulin analog by hydrogen/deuterium exchange mass spectrometry.
Biochim Biophys Acta. 2013;1834:1210-4.

Human insulin and insulin lispro（lispro）, a rapid-acting insulin analog, have identical primary structures, except for the transposition of a pair of amino acids. This mutation results in alterations in their higher order structures, with lispro dissociating more easily than human insulin. In our previous study performed using hydrogen/deuterium exchange mass spectrometry（HDX/MS）, differences were observed in the rates and levels of deuteration among insulin analog products, which were found to be related to their self-association stability. In this study, we carried out peptide mapping of deuterated human insulin and lispro to determine the regions responsible for these deuteration differences and to elucidate the type of structural changes that affect their HDX reactivity. We identified A3-6 and B22-24 as the 2 regions that showed distinct differences in the number of deuteriumatoms incorporated between human insulin and lispro. These regions contain residues that are thought to participate in hexamerization and dimerization, respectively. We also determined that over time, the differences in deuteration levels decreased in A3-6, whereas they increased in B22-24, suggesting a difference in the dynamics between these 2 regions. This article is part of a Special Issue entitled: Mass spectrometry in structural biology.

Keywords: HDX/MS, Insulin, Insulin lispro

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Yuan Y, Yokoyama M[*1], Maeda Y[*2], Terasawa H[*2], Harada S[*2], Sato H[*1], Yusa K: Structure and dynamics of the gp120 V3 loop that confers noncompetitive resistance in R5 HIV-1JR-FL to maraviroc.
PLOS One. 2013;8(6):e65115.

Maraviroc, an（HIV-1）entry inhibitor, binds to CCR5 and efficiently prevents R5 human immunodeficiency virus type 1（HIV-1）from using CCR5 as a coreceptor for entry into CD4(+) cells. However, HIV-1 can elude maraviroc by using the drug-bound form of CCR5 as a coreceptor. This property is known as noncompetitive resistance. HIV-1V3-M5 derived from HIV-1JR-FLan is a noncompetitive-resistant virus that contains five mutations（I304V/F312W/T314A/E317D/I318V）in the gp120 V3 loop alone. To obtain genetic and structural insights into maraviroc resistance in HIV-1, we performed here mutagenesis and computer-assisted structural study. A series of site-directed mutagenesis experiments demonstrated that combinations of V3 mutations are required for HIV-1JR-FLan to replicate in the presence of 1 μM maraviroc, and that a T199K mutation in the C2 region increases viral fitness in combination with V3 mutations. Molecular dynamic（MD）simulations of the gp120 outer domain V3 loop with or without the five mutations showed that the V3 mutations induced（i）changes in V3 configuration on the gp120 outer domain,（ii）reduction of an anti-

parallel β-sheet in the V3 stem region, (iii) reduction in fluctuations of the V3 tip and stem regions, and (iv) a shift of the fluctuation site at the V3 base region. These results suggest that the HIV-1 gp120 V3 mutations that confer maraviroc resistance alter structure and dynamics of the V3 loop on the gp120 outer domain, and enable interactions between gp120 and the drug-bound form of CCR5.

Keywords: drug resistant virus, entry inhibitor, CCR5

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Hyuga S*1, Hyuga M, Yoshimura M*2, Amakura Y*2, Goda Y, Hanawa T*1: Herbacetin, A Constituent of Ephedrae herba, Suppresses the HGF-Induced Motility of Human Breast Cancer MDAMB231 Cells by Inhibiting c-Met and Akt Phosphorylation.
Planta Medica. 2013;79:1525-30.

Ephedrae herba suppresses hepatocyte growth factor-induced cancer cell motility by inhibiting tyrosine phosphorylation of the hepatocyte growth factor receptor, c-Met, and the PI3K/Akt pathway. Moreover, Ephedrae herba directly inhibits the tyrosine-kinase activity of c-Met. Ephedrine-type alkaloids, which are the active component of Ephedrae herba, do not affect hepatocyte growth factor-c-Met-Akt signalling, prompting us to study other active molecules in the herb. We recently discovered herbacetin glycosides and found that their aglycon, herbacetin, inhibits hepatocyte growth factor-c-Met-Akt signalling. This study revealed a novel biological activity of herbacetin. Herbacetin suppressed hepatocyte growth factor-induced motility in human breast cancer MDA-MB-231 cells by inhibiting c-Met and Akt phosphorylation and directly inhibiting c-Met tyrosine kinase activity. The effects of herbacetin were compared to those of kaempferol, apigenin, and isoscutellarein, all of which have similar structures. Herbacetin inhibition of hepatocyte growth factor-induced motility was the strongest of those for the tested flavonols, and only herbacetin inhibited the hepatocyte growth factor-induced phosphorylation of c-Met. These data suggest that herbacetin is a novel Met inhibitor with a potential utility in cancer therapeutics.

Keywords: Ephedrae herba, Met inhibitor, herbacetin

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Suzuki T, Ishii-Watabe A, Hashii N, Nakagawa Y*1, Takahashi T*1, Ebisawa A*1, Nishi S*2, Fujita N*2, Bando A*3, Sekimoto Y*3, Miyata K*3, Endo T*4, Otsu T*4, Sugimoto S*5, Kondou T*5, Fujita Y*6, Miyanaga N*7, Mashimo M*7, Shimada N*7, Yoden H*8, Shimamura H*8, Kurata Y*8, Koyama S*9, Kawasaki N: The establishment and validation of efficient assays for anti-IIa and anti-Xa activities of heparin sodium and heparin calcium.
Biologicals. 2013;41(6):415-23.

Heparin is used as an anticoagulant drug. The anticoagulation process is mainly caused by the interaction of heparin with antithrombin followed by inhibition of anticoagulant factor IIa and factor Xa. The anti-factor IIa and anti-factor Xa activities of heparin are critical for its anticoagulant effect. however, physicochemical methods that can reflect these activities have not been established. Thus, the measurements of anti-IIa and anti-Xa activities by biological assay are critical for the quality control of heparin products. Currently in the Japanese Pharmacopoeia (JP), the activities of heparin sodium and heparin calcium are measured by an anti-Xa activity assay (anti-Xa assay), but anti-IIa activity is not measured. Here, we established an anti-IIa activity assay (anti-IIa assay) and an anti-Xa assay having good accuracy and precision. When samples having a relative activity of 0.8, 1.0 and 1.2 were measured by the established anti-IIa and anti-Xa assays in nine laboratories, good accuracy (100.0–102.8% and 101.6–102.8%, respectively), good intermediate precision (1.9–2.1% and 2.4–4.2%, respectively) and good reproducibility (4.0–4.8% and 3.6–6.4%, respectively) were obtained.The established anti-IIa and anti-Xa assays have similar protocols, and could be performed by single person without a special machine. The established assays would be useful for quality control of heparin.

Keywords: Heparin, Anti-IIa assay, Anti-Xa assay

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Itoh S, Hiruta Y, Hashii N, Fujita N*1, Natsuga T*1, Hattori T*1, Bando A*2, Sekimoto Y*2, Miyata K*2, Namekawa H*3, Mabuchi K*3, Sakai T*4, Shimahashi H*5, Kawai K*6, Yoden H*6, Koyama S*7, Odgaard S*8, Natsuka S*9, Yamaguchi T, Kawasaki N: Determination of galactosamine impurities in heparin sodium using fluorescent labeling and conventional high-performance liquid chromatography.
Biologicals. 2013;41(6):355-63.

Heparin is a sulfated glycosaminoglycan (GAG), which contains N-acetylated or N-sulfated glucosamine (GlcN). Heparin, which is generally obtained from the healthy porcine intestines, is widely used as an anticoagulant during dialysis and treatments of thrombosis such as disseminated intravascular coagulation. Dermatan sulfate (DS) and chondroitin sulfate (CS), which are galactosamine (GalN)-containing GAGs, are major process-related impurities of heparin products. The varying DS and CS contents between heparin products can be responsible for the different anticoagulant activities of heparin. Therefore, a test to determine the concentrations of GalN-containing GAG is essential to ensure the quality and safety of heparin products. In this study, we developed a method for determination of relative content of GalN from GalN-containing GAG in heparin active pharmaceutical ingredients (APIs). The method validation and collaborative study with heparin manufacturers and suppliers showed that our method has enough specificity, sensitivity, linearity, repeatability, reproducibility, and recovery as the limiting test for GalN from GalN-containing GAGs. We believe that our method will be useful for ensuring quality, efficacy, and safety of pharmaceutical heparins. On July 30, 2010, the GalN limiting test based on our method was adopted in the heparin sodium monograph in the Japanese Pharmacopoeia.

Keywords: Heparin sodium, limiting test, galactosamine

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Morise J*1, Kizuka Y*2, Yabuno K*1, Tonoyama Y*1, Hashii N, Kawasaki N, Manya H*3, Miyagoe-Suzuki Y*4, Takeda S*4, Endo T*3, Maeda N*5, Takematsu H*1, Oka S*1: Structural and biochemical characterization of O-mannose-linked human natural killer-1 glycan expressed on phosphacan in developing mouse brains.
Glycobiology. 2014;24(3):314-24.

The human natural killer-1 (HNK-1) carbohydrate comprising a sulfated trisaccharide (HSO3-3GlcAβ1-3Galβ1-4GlcNAc-) is expressed on N-linked and O-mannose-linked glycans in the nervous system and involved in learning and memory functions. Although whole/core glycan structures and carrier glycoproteins for the N-linked HNK-1 epitope have been studied, carrier glycoproteins and the biosynthetic pathway of the O-mannose-linked HNK-1 epitope have not been fully characterized. Here, using mass spectrometric analyses, we identified the major carrier glycoprotein of the O-linked HNK-1 as phosphacan in developing mouse brains and determined the major O-glycan structures having the terminal HNK-1 epitope from partially purified phosphacan. The O-linked HNK-1 epitope on phosphacan almost disappeared due to the knockout of protein O-mannose β1,2-N-acetylglucosaminyltransferase 1, an N-acetylglucosaminyltransferase essential for O-mannose-linked glycan synthesis, indicating that the reducing terminal of the O-linked HNK-1 is mannose. We also showed that glucuronyltransferase-P (GlcAT-P) was involved in the biosynthesis of O-mannose-linked HNK-1 using the gene-deficient mice of GlcAT-P, one of the glucuronyltransferases for HNK-1 synthesis. Consistent with this result, we revealed that GlcAT-P specifically synthesized O-linked HNK-1 onto phosphacan using cultured cells. Furthermore, we characterized the as-yet-unknown epitope of the 6B4 monoclonal antibody (mAb), which was thought to recognize a

unique phosphacan glycoform. The reactivity of the 6B4 mAb almost completely disappeared in GlcAT-P-deficient mice, and exogenously expressed phosphacan was selectively recognized by the 6B4 mAb when co-expressed with GlcAT-P, suggesting that the 6B4 mAb preferentially recognizes O-mannose-linked HNK-1 on phosphacan. This is the first study to show that 6B4 mAb-reactive O-mannose-linked HNK-1 in the brain is mainly carried by phosphacan.

Keywords: HNK-1, O-mannose, glucuronyltransferase-P

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Takakura D, Hashii N, Kawasaki N: An improved in-gel digestion method for efficient identification of protein and glycosylation analysis of glycoproteins using guanidine hydrochloride.
Proteomics. 2014;14(2-3):196-201.

In-gel digestion followed by LC/MS/MS is widely used for the identification of trace amounts of proteins and for the site-specific glycosylation analysis of glycoproteins in cells and tissues. A major limitation of this technique is the difficulty in acquiring reliable mass spectra for peptides present in minute quantities and glycopeptides with high heterogeneity and poor hydrophobicity. It is considered that the SDS used in electrophoresis can interact with proteins noncovalently and impede the ionization of peptides/glycopeptides. In this study, we report an improved in-gel digestion method to acquire reliable mass spectra of a trace amount of peptides/glycopeptides. A key innovation of our improved method is the use of guanidine hydrochloride, which forms complexes with the residual SDS molecules in the sample. The precipitation and removal of SDS by addition of the guanidine hydrochloride was successful in improving the S/N of peptides/glycopeptides in mass spectra and acquiring a more comprehensive MS/MS data set for the various glycoforms of each glycopeptide.

Keywords: Glycoproteomics, Glycosylation analysis, Guanidine hydrochloride

在間一将，最所和宏，丸山卓郎，合田幸広：DapoxetineおよびFlibanserinのLC–PDA–MS分析.
日本食品化学学会誌 2013;20:119-23.

Dapoxetine（**1**）and flibanserin（**2**）have pharmaceutical effects against premature ejaculation（PE）and hypoactive sexual desire disorder（HSDD）, respectively. In international markets, these compounds have been reported as illegal additives in health foods for which tonic effects are implicitly indicated. Therefore, we performed LC-PDA-MS analysis in preparation for the distribution of illegal health foods containing these compounds in Japan. Compounds **1** and **2** were completely separated at r.t. 11.3 and 10.7 min, respectively, under the conditions described as the analytical method for udenafil by the Ministry of Health, Labour and Welfare, Japan. Their spectroscopic data（UV and MS）corresponded to the literature data. Subsequently, we added authentic dapoxetine and flibanserin to an extract of the food supplements with or without an ED treatment agent including sildenafil and tadarafil etc., and then these sample solutions were analyzed using the proposal method. As a result, each compound was completely separated on the UV and mass chromatograms. This study provides useful data for the surveillance of unapproved/unlicensed drugs in health food products.

Keywords: dapoxetine, flibanserin, LC-PDA-MS analysis

若菜大悟，富澤裕一郎*1，丸山卓郎，神谷洋*1，川崎武志*1，横倉胤夫*2，山本豊*3，近藤誠三*4，小松かつ子*5，合田幸広：シンギの確認試験法について.
医薬品医療機器レギュラトリーサイエンス 2013;44:672-8.

Hedysari Radix（HR）is a crude drug derived from the roots of *Hedysarum polybotrys*（Leguminosae）. It is often used in various Kampo formulae as a substitute for Astragali Radix（AR）to avoid the side effects attributed to AR. In this study, we developed an identification test for HR by TLC in preparation for the listing of HR in the Japanese Pharmacopoeia（JP）. First, medicarpin（**1**）was isolated and examined as a candidate marker compound for TLC test. However, it proved unsuitable because wide content variation was observed in inter and intra-HR sample comparisons.

Next, 9-*O*-methylcoumestrol（**2**）was isolated as a candidate marker compound, and was detected consistently in all cuttings of HR samples. Therefore, we selected an identification test based on the detection of compound **2** by TLC. The test could clearly distinguish HR from AR which is similar crude drug listed in JP.

The established TLC conditions were as follows: chromatographic support, silica gel; developing solvent, hexane/2-butanone/formic acid（60/40/1）; developing length, 10 cm; visualization, UV（365 nm）; R_f value of compound **2**, 0.4.

Keywords: Hedysari Radix, identification test, 9-*O*-methylcoumestrol

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Maruyama T, Kawamura M, Kikura-Hanajiri R, Goda Y: Botanical origin of dietary supplements labeled as “kwao keur”, a folk medicine from Thailand.

J Nat Med. 2014;68:220-4.

In the course of our study on the quality of dietary supplements in Japan, both the internal transcribed spacer（ITS）sequence of nrDNA and the *rps*16 intron sequence of cpDNA of products labeled as “Kwao Keur” were investigated. As a result, the DNA sequence of *Pueraria candollei* var. *mirifica*, which is the source plant of Kwao Keur, was observed in only about half of the products. Inferred from the determined sequences, source plants in the other products included *Medicago sativa*, *Glycyrrhiza uralensis*, *Pachyrhizus erosus* and *Ipomoea batatas*, etc. These inferior products are estimated to lack the efficacy implied by their labeling. In order to guarantee the quality of dietary supplements, it is important to identify the source materials exactly; in addition, an infrastructure that can exclude these inferior products from the market is needed for the protection of consumers from potential damage to their health and finances. The DNA analysis performed in this study is useful for this purpose.

Keywords: *Pueraria candollei* var. *mirifica*, dietary supplement, DNA sequencing analysis

Kumeta Y, Maruyama T, Asama H*1, Yamamoto Y*2, Hakamatsuka T Goda Y: Species identification of *Asini Corii Collas*（donkey glue）by PCR amplification of cytochrome *b* gene.

J Nat Med. 2014;68:181-5.

Asini Corii Collas（*ACC*; donkey glue）is a crude drug used to promote hematopoiesis and arrest bleeding. Because adulteration of the drug with substances from other animals such as horses, cattle, and pigs has been found, we examined PCR methods based on the sequence of cytochrome *b* gene for source species identification. Two strategies for extracting DNA from *ACC* were compared, and the ion-exchange resin procedure was revealed to be more suitable than the silica-based one. Using DNA extracted from *ACC* by the ion-exchange resin procedure, PCR methods for species-specific detection of donkey, horse, cattle, and pig substances were established. When these species-specific PCR methods were applied to *ACC*, amplicons were obtained only by the donkey-specific PCR. Cattle-specific PCR detected as little as 0.1% admixture of cattle glue in the *ACC*. These results suggest that the species-specific PCR methods established in this study would be useful for simple and easy detection of adulteration of *ACC*.

Keywords: *Asini Corii Collas*, species identification, cytochrome *b*

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堀井周文*，小此木明*，大窪俊樹*，鎌倉浩之，合田幸広：葛根湯エキス製剤および湯剤の同等性に関する研究（I）.

生薬学雑誌 2014;68:9-12.

In order to obtain basic information of the bio-equivalence between the Kakkonto decoction and its extract product, a crossover study was performed involving 6 healthy adult males as study participants randomly divided into 2 groups. The change of concentrations of the two marker compounds, ephedrine（E）and pseudoephedrine（PE）, in human blood plasma was observed after their oral administration. As the results, no significant differences in the plasma levels between the decoction and the product were noted at any sampling times. Variance analysis of the maximum plasma concentration（C_{max}）

and the area under the plasma concentration-time curve (AUC) on both E and PE revealed that no significant differences were observed between the decoction and the product and also between the administration days. The statistical power (1-β) is determined to be sufficient (more than 80%) for both C_{max} and AUC on PE, but not on E. However, assuming that the standard deviation is the same as our result of E, when the number of the study participants is 14 it is revealed that its statistical power become sufficient (more than 80%) for both C_{max} and AUC on E. Since E and PE are known to be important biologically active components in Kakkonto formula, these results suggest that E and PE may use as the marker compounds for the bio-equivalence judgment between preparations, although further study are needed to discuss this issue.
Keywords: bio-equivalence, Kakkonto, ephedrine

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Masada-Atsumi S, Kumeta Y, Takahashi Y*, Hakamatsuka T, Goda Y: Evaluation of the botanical origin of black cohosh products by genetic and chemical analyses.
Biol Pharm Bull. 2014;37:454-60.

We genetically identified the botanical sources of 10 black cohosh products and 5 Cimicifuga Rhizome crude drugs of Japanese Pharmacopoeia grade, and analyzed the metabolic profiling of 25 black cohosh products using liquid chromatography-tandem mass spectrometry. Consequently, we found that *C. dahurica* and possibly *C. foetida* are misused as sources of the black cohosh products and in some cases, the extracts of black cohosh were adulterated with the plant materials of *C. dahurica*. We demonstrated that these three species can be distinguished by three marker compounds in a specific mass range.
Keywords: Black cohosh, DNA identification, Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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渥美さやか，大沼美貴*1，末永恵美，丸山卓郎，菱田敦之*2，木内文之*2，小林進*1，合田幸広，袴塚高志：DNA配列情報を利用したブラックコホシュ国内市場品の基原鑑別．
日本食品化学学会誌 2013;20:178-88.

国内で健康食品として流通する西洋ハーブ，ブラックコホシュ（*Cimicifuga racemosa*）の基原鑑別法の確立を目的として，特異的プライマーを用いたPCRによりブラックコホシュと近縁植物を区別するARMS法を確立した．同法により国内市場で流通するブラックコホシュ製品の基原鑑別を行った結果，８製品中２製品には近縁種が使用され，１製品にはCimicifuga属植物は含まれないことが明らかになった．この結果は指標成分の化学分析結果ともよく一致し，組織を含むブラックコホシュ製品の基原鑑別にARMS法が有用であることが示された．
Keywords: Black cohosh, *Cimicifuga racemosa*, Amplification refractory mutation system (ARMS) analysis

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Uchiyama N, Kawamura M, Kikura-Hanajiri R, Goda Y: URB-754: A new class of designer drug and 12 synthetic cannabinoids detected in illegal products.
Forensic Sci Int. 2013;227:21-32.

URB-754 (6-methyl-2-[(4-methylphenyl)amino]-1-benzoxazin-4-one) was identified as a new type of designer drug in illegal products. Though many of the synthetic cannabinoids detected in illegal products are known to have affinities for cannabinoid CB_1/CB_2 receptors, URB-754 was reported to inhibit an endocannabinoid deactivating enzyme. Furthermore, an unknown compound (*N*,5-dimethyl-*N*-(1-oxo-1-(p-tolyl)butan-2-yl)-2-(*N*'-(*p*-tolyl)ureido)benzamide), which is deduced to be the product of a reaction between URB-754 and a cathinone derivative 4-methylbuphedrone (4-Me-MABP), was identified along with URB-754 and 4-Me-MABP in the same product. It is of interest that the product of a reaction between two different types of designer drugs, namely, a cannabinoid-related designer drug and a cathinone-type designer drug, was found in one illegal product. In addition, 12 cannabimimetic compounds, 5-fluoropentyl-3-pyridinoylindole, JWH-307, JWH-030, UR-144, 5FUR-144 (synonym: XLR11), (4-methylnaphtyl)-JWH-022 [synonym: *N*-(5-fluoropentyl)-JWH-122], AM-2232, (4-methylnaphtyl)-AM-2201 (MAM-2201), *N*-(4-pentenyl)-JWH-122, JWH-213, (4-ethylnaphtyl)-AM-2201 (EAM-2201) and AB-001, were also detected herein as newly distributed designer drugs in Japan.

Furthermore, a tryptamine derivative, 4-hydroxy-diethyltryptamine (4-OH-DET), was detected together with a synthetic cannabinoid, APINACA, in the same product.

Keywords: URB-754, (*N*,5-dimethyl-*N*-(1-oxo-1-(*p*-tolyl)butan-2-yl)-2-(*N*'-(*p*-tolyl)ureido)benzamide), 4-Methylbuphedrone

Uchiyama N, Matsuda S, Kawamura M, Kikura-Hanajiri R, Goda Y: Two new-type cannabimimetic quinolinyl carboxylates, QUPIC and QUCHIC, two new cannabimimetic carboxamide derivatives, ADB-FUBINACA and ADBICA, and five synthetic cannabinoids detected with a thiophene derivative α-PVT and an opioid receptor agonist AH-7921 identified in illegal products.

Forensic Toxicol. 2013;31:223-40.

We identified two new-type cannabimimetic quinolinyl carboxylates, quinolin-8-yl 1-pentyl-(1*H*-indole)-3-carboxylate (QUPIC, **1**) and quinolin-8-yl 1-(cyclohexylmethyl)-1*H*-indole-3-carboxylate (QUCHIC, **2**), two new cannabimimetic carboxamide derivatives, *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (ADB-FUBINACA, **3**) and *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indole-3-carboxamide (ADBICA, **4**), as designer drugs in illegal products. Compound **3** was reported to have a potent affinity for cannabinoid CB_1 receptor by Pfizer in 2009, but this is the first report of its detection in illegal products. There have been no chemical or pharmacological informations about compounds **1**, **2** and **4** until now, making this the first report on these compounds. We also detected synthetic cannabinoids, *N*-(5-fluoropentyl)-APICA (**5**), *N*-(5-fluoropentyl)-APINACA (**6**), *N*-(5-chloropentyl)-UR-144 (**7**), *N*-(5-chloropentyl)-JWH-122 (**8**) and 4-methoxynaphtyl-AM-2201 (4-MeO-AM-2201, **9**) herein as newly distributed designer drugs in Japan. It is of interest that compounds **1** and **2** were detected with their synthetic component, 8-qunolinol (**10**). A stimulant thiophene analog, α-pyrrolidinovalerothiophenone (α-PVT, **11**), and an opioid receptor agonist, 3,4-dichloro-*N*-((1-(dimethylamino)cyclohexyl)methyl)benzamide (AH-7921, **12**) were also detected as new types of designer drugs coexisting with several synthetic cannabinoids and cathinone derivatives in illegal products.

Keywords: Quinolin-8-yl 1-pentyl-(1*H*-indole)-3-carboxylate (QUPIC), Quinolin-8-yl 1-(cyclohexylmethyl)-1*H*-indole-3-carboxylate (QUCHIC), *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (ADB-FUBINACA)

Tan K*, Wakimoto T*, Takada K*, Ohtsuki T, Uchiyama N, Goda Y, Abe I*: Cycloforskamide, a cytotoxic macrocyclic peptide from the sea slug *Pleurobranchus forskalii*.

J Nat Prod. 2013;76:1388-91.

A macrocylic dodecapeptide, cycloforskamide, was isolated from the sea slug *Pleurobranchus forskalii*, collected off Ishigaki Island, Japan. Its planar structure was deduced by extensive NMR analyses and was further confirmed by MS/MS fragmentation analyses. Finally, the absolute configuration was determined by total hydrolysis and chiral-phase gas chromatographic analysis. This novel dodecapeptide contains three d-amino acids and three thiazoline heterocycles and exhibits cytotoxicity against murine leukemia P388 cells, with an IC_{50} of 5.8 μM.

Keywords: Cycloforskamide, macrocyclic peptide, *Pleurobranchus forskalii*

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Uchiyama N, Matsuda S, Kawamura M, Kikura-Hanajiri R, Goda Y: Identification of two new-type designer drugs, a piperazine derivative MT-45 (I-C6) and a synthetic peptide Noopept (GVS-111), with a synthetic cannabinoid A-834735, a cathinone derivative 4-methoxy-α-PVP and a phenethylamine derivative 4-methylbuphedrine from illegal products.

Forensic Toxicol. 2014;32:9-18.

We identified two new-type designer drugs, a piperazine derivative MT-45 [1-cyclohexyl-4-(1,2-diphenylethyl)piperazine, synonym: I-C6, **1**] and a synthetic peptide Noopept [ethyl 2-(1-(2-phenylacetyl)pyrrolidine-2-carboxamido)acetate, synonym: GVS-111, **2**], in chemical and herbal products. MT-45 (**1**) was previously reported as an opiate-like analgesic substance, and Noopept (**2**) was previously reported to have a nootropic (cognitive enhancer) activity. We also detected two synthetic cannabinoids, A-834735 (**3**) and

QUPIC *N*-(5-fluoropentyl) analog (synonym: 5-fluoro-PB-22, **4**), in illegal products. A-834735 (**3**) was previously reported to act as an agonist at both cannabinoid CB_1 and CB_2 receptors. Additionally, a cathinone derivative 4-methoxy-α-pyrrolidinovalerophenone (4-methoxy-α-PVP, **5**) and a phenethylamine derivative 4-methylbuphedrine (**6**) were newly detected with a known cathinone derivative 4-methylbuphedrone (**7**) in illegal products.
Keywords: MT-45 [1-cyclohexyl-4-(1,2-diphenylethyl) piperazine, synonym: I-C6], Noopept [ethyl 2-(1-(2-phenylacetyl)pyrrolidine-2-carboxamido)acetate, synonym: GVS-111], A-834735

Uchiyama N, Shimokawa Y, Matsuda S, Kawamura M, Kikura-Hanajiri R, Goda Y: Two new synthetic cannabinoids, AM-2201 benzimidazole analog (FUBIMINA) and (4-methylpiperazin-1-yl)(1-pentyl-1H-indol-3-yl)methanone (MEPIRAPIM), and three phenethylamine derivatives, 25H-NBOMe 3,4,5-trimethoxybenzyl analog, 25B-NBOMe, and 2C-N-NBOMe, identified in illegal products.
Forensic Toxicol. 2014;32:105-17.

Two new types of synthetic cannabinoids an AM-2201 benzimidazole analog (FUBIMINA, **1**) and (4-methylpiperazin-1-yl)(1-pentyl-1*H*-indol-3-yl) methanone (MEPIRAPIM, **2**), and three newly-emerged phenethylamine derivatives 25B-NBOMe (**3**), 2C-N-NBOMe (**4**) and a 25H-NBOMe 3,4,5-trimethoxybenzyl analog (**5**), were detected in illegal products distributed in Japan. The identification was based on liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), high-resolution MS and nuclear magnetic resonance (NMR) analyses. Different from the representative synthetic cannabinoids, such as JWH-018, which have a naphthoylindole moiety, compounds **1** and **2** were completely new types of synthetic cannabinoids; compound **1** had a benzimidazole group in place of an indole group, and compound **2** had a 4-methylpiperazine group in place of the naphthyl group. Compounds **3** and **4** were *N-o*-methoxybenzyl derivatives of 2,5-dimethoxyphenethylamines (25-NBOMe series), which had been previously detected in European countries, but have newly emerged in Japan. Compound **5** had an *N*-trimethoxybenzyl group in place of an *N-o*-methoxybenzyl group. Data on chemistry and pharmacology of compounds **1**, **2** and **5** have never been reported to our knowledge.
Keywords: AM-2201 benzimidazole analog (FUBIMINA), (4-Methylpiperazin-1-yl)(1-pentyl-1*H*-indol-3-yl)methanone (MEPIRAPIM), 25H-NBOMe 3,4,5-trimethoxybenzyl analog

Hirasawa Y[*1], Kato Y[*1], Wong CP[*1], Uchiyama N, Goda Y, Hadi HA[*2], Ali HM[*2], Morita H[*1]: Hupermine A, a novel $C_{16}N_2$-type *Lycopodium* alkaloid from *Huperzia phlegmaria*.
Tetrahedron Lett. 2014;55:1902-4.

A novel $C_{16}N_2$-type *Lycopodium* alkaloid consisting of a quinolizidine with a 6-dimethylaminohexyl side chain, hupermine A (**1**), was isolated from the club moss of *Huperzia phlegmaria*, and the structure and relative stereochemistry were elucidated on the basis of spectroscopic data.
Keywords: Hupermine A, *Huperzia phlegmaria*, *Lycopodium phlegmaria*

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Ogata J, Uchiyama N, Kikura-Hanajiri R, Goda Y: DNA sequence analyses of blended herbal products including synthetic cannabinoids as designer drugs.
Forensic Sci Int. 2013;227:33-41.

In recent years, various herbal products adulterated with synthetic cannabinoids have been distributed worldwide via the Internet. Although their labels indicate that they contain mixtures of several potentially psychoactive plants, and numerous studies have reported that they contain a variety of synthetic cannabinoids, their exact botanical contents are not always clear. In this study, we investigated the origins of botanical materials in 62 Spice-like herbal products distributed on the illegal drug market in Japan, by DNA sequence analyses and BLAST searches. The sequences of "Damiana" (*Turnera diffusa*) and Lamiaceae herbs (*Mellissa*, *Mentha* and *Thymus*) were frequently detected in a number of products.
Keywords: BLAST, DNA barcode, herbal product

Hirata Y[*], Yamamori N[*], Kono N[*], Lee HC[*], Inoue T, Arai H[*]: Identification of small subunit of serine

palmitoyltransferase as a lysophosphatidylinositol acyltransferase 1-interacting protein.
Genes Cells. 2013;18:397-409.

Lysophosphatidylinositol acyltransferase 1 (LPIAT1) is a phospholipid acyltransferase that selectively incorporates arachidonic acid (AA) into phosphatidylinositol (PI). We previously demonstrated that LPIAT1 plays a crucial role in brain development in mice. However, how LPIAT1 is regulated and which proteins function cooperatively with LPIAT1 are unknown. In this study, we identified ssSPTad as an LPIAT1-interacting protein. ssSPTa co-immunoprecipitated and colocalized with LPIAT1 in cultured mammalian cells. Knockdown of ssSPTa decreased the LPIAT1-dependent incorporation of exogenous AA into PI. Interestingly, knockdown of ssSPTa decreased the protein level of LPIAT1 in the crude mitochondrial fraction. LPIAT1 was localized to the mitochondria-associated membrane (MAM), where AA-selective acyl-CoA synthetase is enriched. These results suggest that ssSPTa plays a role in fatty acid remodeling of PI, probably by facilitating the MAM localization of LPIAT1.
Keywords: LPIAT1, phosphatidylinositol

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Ohba Y*[1], Sakuragi T*[1], Kage-Nakadai E*[2], Tomioka NH*[1], Kono N*[1], Imae R*[1], Inoue A*[3], Aoki J*[3], Ishihara N*[4], Inoue T, Mitani S*[2], Arai H*[1]: Mitochondria-type GPAT is required for mitochondrial fusion.
EMBO J. 2013;3:1265-79.

GPAT is involved in the first step in glycerolipid synthesis and is localized in both the ER and mitochondria. To clarify the functional differences between ER-GPAT and mitochondrial (Mt)-GPAT, we generated C. elegans GPAT mutants and demonstrated that mutation of Mt-GPAT caused excessive mitochondrial fragmentation. The defect was rescued by injection of lysophosphatidic acid (LPA) and by inhibition of LPA acyltransferase, both of which lead to accumulation of LPA in the cells. Mitochondrial fragmentation in Mt-GPAT mutants was also rescued by inhibition of mitochondrial fission protein DRP-1, suggesting that the fusion/fission balance is affected by Mt-GPAT depletion. Mitochondrial fragmentation was also observed in Mt-GPAT-depleted HeLa cells. We postulate from these results that LPA produced by Mt-GPAT functions not only as a precursor for glycerolipid synthesis but also as an essential factor of mitochondrial fusion.
Keywords: mitochondria, LPA, acyltransferase

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Nishimura T*[1], Uchida Y*[1], Yachi R*[1], Kudlyk T*[2], Lupashin V*[2], Inoue T, Taguchi T*[1], Arai H*[1]: Oxysterol-binding protein (OSBP) is required for the perinuclear localization of intra-Golgi v-SNAREs.
Mol Biol Cell. 2013;24:13534-44.

OSBP have been implicated in the distribution of sterols among intracellular organelles. OSBP regulates the Golgi cholesterol level, but how it relates to Golgi function is elusive. Here we report that OSBP is essential for the localization of intra-Golgi v-SNAREs. Depletion of OSBP causes mislocalization of intra-Golgi v-SNAREs GS28 and GS15 throughout the cytoplasm. GS28 mislocalization is also induced by cellular cholesterol depletion. Finally, GS28 mislocalization in OSBP-depleted cells is largely restored by depletion of ArfGAP1, a regulator of the budding of COP-I vesicles. From these results, we postulate that Golgi cholesterol level, which is controlled by OSBP, is essential for Golgi localization of intra-Golgi v-SNAREs by ensuring proper COP-I vesicle transport.
Keywords: cholesterol, Golgi, SNARE

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Udagawa O*[1], Ito C*[2], Ogonuki N*[3], Sato H*[4], Lee S*[1], Tripvanuntakul P*[1], Ichi I*[1], Uchida Y*[1], Nishimura T*[1], Murakami M*[4], Ogura A*[3], Inoue T, Toshimori K*[2], Arai H*[1]: Oligo-astheno-teratozoospermia in mice lacking ORP4, a sterol-binding protein in the OSBP-related protein family.
Genes to Cells. 2014;19:13-27.

Oligo-astheno-teratozoospermia (OAT), a condition that includes low sperm number, low sperm motility and abnormal sperm morphology, is the commonest

cause of male infertility. Here, we show that deficiency of a sterol-binding protein ORP4 causes male infertility due to severe OAT in mice. In ORP4-deficient mice, spermatogonia proliferation and subsequent meiosis occurred normally, but the morphology of elongating and elongated spermatids was severely distorted. Spermatozoa derived from ORP4-deficient mice had little or no motility and no fertilizing ability in vitro. In ORP4-deficient testis, postmeiotic spermatids underwent extensive apoptosis, leading to a severely reduced number of spermatozoa. These results suggest that ORP4 is essential for the postmeiotic differentiation of germ cells.

Keywords: cholesterol, Oligo-astheno-teratozoospermia, ORP4

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Kanemura H*1, Go MJ*1, Nishishita N*1, Sakai N*2, Kamao H*2, Sato Y, Takahashi M*2, Kawamata S*1: Pigment Epithelium-Derived Factor Secreted from Retinal Pigment Epithelium Facilitates Apoptotic Cell Death of iPSC.

Sci Rep. 2013;3:2334.

We show that pigment epithelium-derived factor (PEDF), which is secreted from primary or iPSC-derived retinal pigment epithelium (RPE), dramatically inhibits the growth of iPSCs. PEDF is detected abundantly in culture supernatants of primary or iPSC-derived RPE. Apoptotic cell death is induced in iPSC when co-cultured with RPE, a process that is significantly blocked by addition of antibody against PEDF. Indeed, addition of recombinant PEDF to the iPSC cell culture induces apoptotic cell death in iPSCs, but the expression of pluripotency related-genes is maintained, suggesting that PEDF causes cell death, not differentiation, of iPSCs. To recapitulate this event in vivo, we examined tumor formation in NOG mice after subcutaneous injection of iPSCs with or without an iPSC-derived RPE sheet (2.5 × 10(5) RPE cells). We observed that the tumor forming potential of iPSCs was significantly suppressed by simultaneous transplantation with an iPSC-derived RPE sheet.

Keywords: intracellular signaling peptides and proteins, apoptosis, induced pluripotent stem cells

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Suzuki T: Unconscious Exposure to Radiation.

Genes and Environment. 2013;35:63-8.

We are internally exposed to 40K radiation through the foods we eat on a daily basis, and we have already been exposed to the 1,000-10,000 times higher background of the nuclear fallout that occurred during the 1960s because of world-wide nuclear bomb experiments. It is important to know these facts to consider the excess risk derived from the Fukushima accident. Obtaining a proper answer scientifically about the health effects of low-level radiation exposure is very difficult when using available data. Increasing risk awareness and communication is also important together with proving the real risk of low-level radiation. Radiation risk should be considered in a relative manner by comparing it with other confounding factors. The increased risk posed by radiation exposure can be traded-off by reducing other risk factors affecting our lifestyle. The most important task for us is to transfer available scientific knowledge to the public such that the information is more understandable to help people make their own decisions on how to face radiation risk.

Keywords: radiation risk, Fukushima nuclear accident, risk communication

Harashima M*, Seki T*, Ariga T*, Niimi S: Role of p16(INK4a) in the inhibition of DNA synthesis stimulated by HGF or EGF in primary cultured rat hepatocytes.

Biomed Res. 2013;34:269-73.

In the present study, we investigated the role of p16 (INK4a) in the inhibition of DNA synthesis stimulated by hepatocyte growth factor (HGF) or epidermal growth factor (EGF) using RNA interference in primary cultured rat hepatocytes. The transfection of small interfering RNAs targeting p16(INK4a) reduced the corresponding mRNA and protein expression by more than approximately 90% and 50%, respectively, at 24 h after transfection. In the cells transfected with p16 (INK4a) small interfering RNA, control, HGF, and EGF-stimulated DNA synthesis as assessed by (3)

H-thymidine incorporation increased by approximately 1.5-fold, 1.6-fold, and 1.7-fold, respectively, compared with that in the control small interfering RNA-transfected cells. These findings indicate that p16 (INK4a) plays a significant role in the inhibition of DNA synthesis.

Keywords: p16(INK4a), DNA synthesis, primary cultured rat hepatocytes

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Arakaki N*, Yamashita A*, Niimi S, Yamazaki T*: Involvement of reactive oxygen species in osteoblastic differentiation of MC3T3-E1 cells accompanied by mitochondrial morphological dynamics.

Biomed Res. 2013;34:161-6.

Bone remodeling is regulated by local factors that regulate bone-forming osteoblasts and bone-resorbing osteoclasts, in addition to hormonal activity. Recent studies have shown that reactive oxygen species (ROS) act as an intracellular signal mediator for osteoclast differentiation. However the role of ROS on osteoblast differentiation is poorly understood. Here, we investigated the impact of ROS on osteoblastic differentiation of MC3T3-E1 cells. Osteogenic induction resulted in notable enhancement of mineralization and expression of osteogenic marker gene alkaline phosphatase, which were accompanied by an increase in ROS production. Additionally, we found that mitochondrial morphology dynamically changed from tubular reticulum to fragmented structures during the differentiation, suggesting that mitochondrial morphological transition is a novel osteoblast differentiation index. The antioxidant N-acetyl cysteine prevented not only ROS production but also mineralization and mitochondrial fragmentation. It is therefore suggested that the ROS-dependent signaling pathways play a role in osteoblast differentiation accompanied by mitochondrial morphological transition.

Keywords: reactive oxygen species, osteoblastic differentiation, mitochondrial morphological dynamics

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Haishima Y, Kawakami T, Hasegawa C, Tanoue A*[1], Yuba T*[2], Isama K, Matsuoka A, Niimi S: Screening study on hemolysis suppression effect of an alternative plasticizer for the development of a novel blood container made of polyvinyl chloride.

J Biomed Mater Res Part B. 2014;102B:721-8.

The aim of this study is to identify a plasticizer that is effective in the suppression of the autohemolysis of the stored blood and can be used to replace di (2-ethylhexyl) phthalate (DEHP) in blood containers. The results of hemolysis test using mannitol-adenine-phosphate/red cell concentrates (MAP/RCC) spiked with plasticizers included phthalate, phthalate-like, trimeliate, citrate, and adipate derivatives revealed that di-isononyl-cyclohexane-1,2-dicarboxylate (Hexamoll® DINCH), di(2-ethylhexyl)-1,2,3,6-tetrahydro-phthalate (DOTP), and diisodecyl phthalate (DIDP) exhibited a hemolysis suppression effect almost equal to that of DEHP, but not other plasticizers. This finding suggested that the presence of 2 carboxy-ester groups at the *ortho* position on a 6-membered ring of carbon atoms may be required to exhibit such an effect. The hemolytic ratios of MAP/RCC-soaked polyvinyl chloride (PVC) sheets containing DEHP or different amounts of DINCH or DOTP were reduced to 10.9%, 9.2-12.4%, and 5.2-7.8%, respectively (MAP/RCC alone, 28.2%) after 10 weeks of incubation. The amount of plasticizer eluted from the PVC sheet was 53.1, 26.1-36.5, and 78.4-150 μg/mL for DEHP, DINCH, and DOTP, respectively. PVC sheets spiked with DIDP did not suppress the hemolysis induced by MAP/RCC because of low leachability (4.8-6.0 μg/mL). These results suggested that a specific structure of the plasticizer and the concentrations of least more than approximately 10 μg/mL were required to suppress hemolysis due to MAP/RCC.

Keywords: DEHP, alternative plasticizer, PVC medical device

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Haishima Y, Isama K, Hasegawa C, Yuba T*, Matsuoka A: A development and biological safety evaluation of novel PVC medical devices with surface structures modified by UV irradiation to suppress plasticizer migration.

J Biomed Mater Res Part A. 2013;101A:2630-43.

This study examines the chemical, physicochemical, and biological properties of PVC sheets treated with UV irradiation on their surfaces to suppress the elution of a plasticizer, di-(2-ethylhexyl) phthalate (DEHP), for developing novel polyvinyl chloride (PVC) medical devices. The PVC sheets irradiated under conditions 1 (52.5 μW/cm^2, 136 J/cm^2) and 2 (0.45 mW/cm^2, 972 J/cm^2) exhibited considerable toxicity in cytotoxicity tests and chromosome aberration tests due to the generation of DEHP oxidants, but no toxicity was detected in the PVC sheet irradiated under condition 3 (8.3 mW/cm^2, 134 J/cm^2). The release of DEHP from the surface irradiated under condition 3 was significantly suppressed, and mono-(2-ethylhexyl) phthalate (MEHP) converted from a portion of DEHP could be easily removed from the surface by washing with methanol. The physicochemical properties of the surface regarding the suppression of DEHP elution remained stable through all sterilizations tested, but MEHP elution was partially recrudesced by the sterilizations except for gamma irradiation. These results indicated that UV irradiation using a strong UV-source over a short time (condition 3) followed by methanol washing and gamma sterilization may be useful for preparing novel PVC products that did not elute plasticizers and do not exhibit toxicity originating from UV irradiation.

Keywords: DEHP, surface modification, PVC medical device

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Nomura Y, Tanaka Y*[1,2], Fukunaga J*[1,2], Fujiwara K*[1,3], Chiba M*[3], Iibuchi1 H*[3], Tanaka T*[1,3], Nakamura Y*[1,4], Kawai G*[3], Kozu T*[1,2], Sakamoto T*[1,3]: Solution structure of a DNA mimicking motif of an RNA aptamer against transcription factor AML1 Runt domain.

J Biochem. 2013;154:513-9.

AML1/RUNX1 is an essential transcription factor involved in the differentiation of hematopoietic cells. AML1 binds to the Runt-binding double-stranded DNA element (RDE) of target genes through its N-terminal Runt domain. In a previous study, we obtained RNA aptamers against the AML1 Runt domain by systematic evolution of ligands by exponential enrichment (SELEX) and revealed that RNA aptamers exhibit higher affinity for the Runt domain than that for RDE and possess the 5′-GCGMGNN-3′ and 5′-N′N′CCAC-3′ conserved motif (M: A or C; N and N′ form Watson-Crick base pairs) that is important for Runt domain binding. In the present study, to understand the structural basis of recognition of the Runt domain by the aptamer motif, the solution structure of a 22-mer RNA was determined using NMR. The motif contains the AH+-C mismatch and base triple and adopts an unusual backbone structure. Structural analysis of the aptamer motif indicated that the aptamer binds to the Runt domain by mimicking the RDE sequence and structure. Our data should enhance the understanding of the structural basis of DNA mimicry by RNA molecules.

Keywords: AML1, NMR structure, RNA aptamer

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Fukunaga J*[1,2], Nomura Y, Tanaka Y*[1,2,3], Amano R*[4], Nakamura Y*[1,5], Kawai G*[4], Sakamoto T*[1,4], Kozu T*[1,2]: The Runt domain of AML1 (RUNX1) binds a sequence-conserved RNA motif that mimics a DNA element.

RNA. 2013;19:927-36.

AML1 (RUNX1) is a key transcription factor for hematopoiesis that binds to the Runt-binding double-stranded DNA element (RDE) of target genes through its N-terminal Runt domain. Aberrations in the AML1 gene are frequently found in human leukemia. To better understand AML1 and its potential utility for diagnosis and therapy, we obtained RNA aptamers that bind specifically to the AML1 Runt domain. Enzymatic probing and NMR analyses revealed that Apt1-S, which is a truncated variant of one of the aptamers, has a CACG tetraloop and two stem regions separated by an internal loop. All the isolated aptamers were found to contain the conserved sequence motif 5′-NNCCAC-3′ and 5′-GCGMGN′N′-3′ (M:A or C; N and N′ form Watson-Crick base pairs). The motif contains one AC mismatch and one base bulged out. Mutational analysis of Apt1-S showed that three guanines of the motif are important for Runt binding as are the three guanines of RDE, which are directly

recognized by three arginine residues of the Runt domain. Mutational analyses of the Runt domain revealed that the amino acid residues used for Apt1-S binding were similar to those used for RDE binding. Furthermore, the aptamer competed with RDE for binding to the Runt domain in vitro. These results demonstrated that the Runt domain of the AML1 protein binds to the motif of the aptamer that mimics DNA. Our findings should provide new insights into RNA function and utility in both basic and applied sciences.

Keywords: AML1, NMR, RNA aptamer

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Shida T*, Koseki H*, Yoda I*, Horiuchi H*, Sakoda H, Osaki M*: Adherence ability of *Staphylococcus epidermidis* on prosthetic biomaterials: an in vitro study.
International Journal of Nanomedicine. 2013;8:3955-61.

Bacterial adhesion to the surface of biomaterials is an essential step in the pathogenesis of implant-related infections. In this in vitro research, we evaluated the ability of Staphylococcus epidermidis to adhere to the surface of solid biomaterials, including oxidized zirconium-niobium alloy (Oxinium), cobalt-chromium-molybdenum alloy, titanium alloy, commercially pure titanium, and stainless steel, and performed a biomaterial-to-biomaterial comparison. The test specimens were physically analyzed to quantitatively determine the viable adherent density of the S. epidermidis strain RP62A (American Type Culture Collection [ATCC] 35984). Field emission scanning electron microscope and laser microscope examination revealed a featureless, smooth surface in all specimens (average roughness<10 nm). The amounts of S. epidermidis that adhered to the biomaterial were significantly lower for Oxinium and the cobalt-chromium-molybdenum alloy than for commercially pure titanium. These results suggest that Oxinium and cobalt-chromium-molybdenum alloy are less susceptible to bacterial adherence and are less inclined to infection than other materials of a similar degree of smoothness.

Keywords: biofilm, zirconium oxide, cobalt-chromium alloy

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迫田秀行，松岡厚子，菅野伸彦*：人工股関節における内部クラックとデラミネーション破壊.
臨床バイオメカニクス 2013;34:191-6.

人工関節摺動面に使用される超高分子量ポリエチレン（UHMWPE）のデラミネーション破壊は，人工膝関節の摺動面だけでなく，股関節インプラントのリムにも発生する．本研究では，抜去されたインプラントを解析し，知見に乏しい股関節インプラントにおけるデラミネーション破壊発生の原因と機構の解明を目指した．15例のインプラントを対象とし，デラミネーションと内部クラックの有無を光学顕微鏡とレーザー顕微鏡を用いて調べた．酸化度やガンマ線照射の有無も調べた．内部クラックはインピンジした部分でのみ観察された．酸化が進行した群でデラミネーションは高率に見られ，酸化が認められなかった群では，デラミネーションは少なかった．酸化した群ではガンマ線照射の痕跡があった．股関節インプラントでは，インピンジにより内部クラックが発生し，デラミネーション破壊に至ると示唆された．また，ガンマ線照射に起因する酸化劣化により，この機構は加速するものと思われた.

Keywords: artificial hip joint, delamination, UHMWPE

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迫田秀行，植月啓太*，松岡厚子：超高分子量ポリエチレンのデラミネーション破壊特性へのビタミンEの影響.
日本人工関節学会誌 2013;43:353-4.

人工関節摺動面に使用される超高分子量ポリエチレン（UHMWPE）に起因する不具合は多いため，改良を加えた新材料が多く開発されている．しかし，これら新材料のデラミネーション特性に関する報告はない．本研究では，新材料の一つである，ビタミンEを混合したUHMWPEのデラミネーション特性を評価した.

ビタミンEを混合すると，デラミネーション特性が向上すると同時に，長期の酸化抑制効果もあることがわかった．また，ビタミンEを混合し，さらに電子線照射により架橋を施した材料では，ビタミンEによる酸化抑制効果と，ビタミンEと架橋によるデラミネーション特性向上効果が見られ，長期にわたりデラミネーション発生

が抑制される可能性が示された.
Keywords: artificial joint, delamination, vitamin E

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小関弘展*，志田崇之*，依田周*，堀内英彦*，尾崎誠*，迫田秀行：生体人工材料表面への表皮ブドウ球菌付着性の比較.
日本関節病学会誌 2014;33:79-83.

生体材料表面への細菌の付着は，インプラント周囲感染の発病における重要な過程である．本研究では，表皮ブドウ球菌の酸化ジルコニウム合金，コバルトクロム合金，チタン合金，純チタン，ステンレス鋼表面におけるバイオフィルム形成能を評価するため，表面粗さ，接触角，細菌付着密度を測定した．全ての表面は表面粗さが10 nm以下の平滑面だった．コバルトクロム合金への細菌の付着は，チタン合金，純チタン，ステンレス鋼への付着より有意に少なかった．より平滑で疎水性を示すコバルトクロム合金の表面は，細菌が付着しにくいことが示唆され，他の材料より感染を生じる傾向が低いと思われた.
Keywords: *Staphylococcus epidermidis*, adhesion, biomaterial

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志田崇之*，小関弘展*，依田周*，尾崎誠*，迫田秀行：チタン系人工材料の表面粗さと表皮ブドウ菌付着量の関係.
日本骨・関節感染症学会誌 2013;27:91-4.

チタン合金，純チタン材料を平滑群（算術平均粗さ：Ra < 10 nm）と不整群（Ra < 30 nm）の2群に分類し，表皮ブドウ球菌の付着量を計測した．各群の付着菌数の平均値（CFU x 10^5/ml）は，チタン合金の平滑群：15.8，不整群：18.8，純チタンの平滑群：16.3，不整群：17.5であり，Ra < 30 nmの範囲内での表面粗さや両材料間の化学組成の違いによる菌付着量の統計学的有意差は認めなかった.
Keywords: *Staphylococcus epidermidis*, adhesion, biomaterial

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Sawada R, Kono K, Isama K, Haishima Y, Matsuoka A: Calcium-incorporated titanium surfaces influence the osteogenic differentiation of human mesenchymal stem cells.
J Biomed Mater Res A. 2013;101(9):2573-85.

In this study, a titanium surface was chemically modified with calcium ions and assessed for its influence on osteogenic differentiation and molecular responses of human mesenchymal stem cells (hMSCs). Titanium disks were treated with NaOH (NaOH treatment), NaOH + $CaCl_2$ ($CaCl_2$ treatment), or NaOH + $Ca(OH)_2$ ($Ca(OH)_2$ treatment). $Ca(OH)_2$ treatment caused significantly greater calcium incorporation onto the titanium surface and apatite formation than $CaCl_2$ treatment. The morphology of hMSCs differed on $CaCl_2$- and $Ca(OH)_2$-treated disks. The osteopontin (OPN) expression in hMSCs cultured on $CaCl_2$-treated titanium was significantly higher than that in cells cultured on NaOH-treated disks; OPN expression was significantly higher in cells cultured on $Ca(OH)_2$-treated disks than on un-, NaOH-, and $CaCl_2$-treated disks. Osteocalcin (OCN) protein expression in hMSCs cultured on $Ca(OH)_2$-treated disks was significantly higher than that on all the other disks. Comparative expression profiling by DNA microarray and pathway analyses revealed that calcium modification of the titanium surface induced integrin $\beta 3$ after OPN upregulation and promoted Wnt/β-catenin signaling in hMSCs. In addition, $Ca(OH)_2$ treatment upregulated the expression of bone morphogenetic protein 2, cyclooxygenase2, and parathyroid hormone-like hormone in comparison to $CaCl_2$ treatment. These observations suggest that calciummodified titanium surfaces affect osteogenic differentiation in hMSCs and that $Ca(OH)_2$ treatment induced osteogenic differentiation in hMSCs, whereas $CaCl_2$ treatment had a limited effect.
Keywords: surface modification, stem cell, osteogenesis

Ito-Nagahata T*[1,2], Kurihara C*[1], Hasebe M*[1], Ishii A*[1], Yamashita K*[1], Iwabuchi M*[1], Sonoda M*[2], Fukuhara K*[3], Sawada R, Matsuoka A*[4], Fujiwara Y*[1]: Stilbene Analogs of Resveratrol Improve Insulin Resistance through Activation of AMPK.
Biosci Biotechnol Biochem. 2013;77(6):1229-35.

Resveratrol (RSV), 3,5,4'-trihydroxy-*trans*-stilbene, is known to have many beneficial physiological activities. We have synthesized several stilbene analogues and have reported that the hydroxyl group in the 4' position of RSV exhibited strong radical scavenging action. Using stilbene analogs, we investigated the

structure of RSV to explain its protective effect against obesity and type 2 diabetes. All six analogs used in this study inhibited the differentiation of 3T3-L1 adipocyes. 3-Hydroxy-*trans* stilbene (3(OH)ST), and 3,4'-dihydroxy-*trans* stilbene (3,4'(OH)$_2$ST) increased glucose uptake and induced adenosine monophosphate kinase (AMPK) phosphorylation in C2C12 myotubes independently of inslin. An *in vivo* study using mice fed high-fat diets indicated that 3(OH)ST was more effective than RSV in improving insulin resistance. In conclusion, RSV and its derivatives, particularly 3(OH)ST, inhibited adipocyte differentiation and enhanced glucose uptake in the myotubes, resulting in a reduction of obesity and an improvement in glucose tolerance *in vivo*.

Keywords: resveratrol, stiblene analog, adenosine monophosphate kinase (AMPK)

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Saito A*1, Nomaguchi M*2, Kono K, Iwatani Y*3, Yokoyama M*4, Yasutomi Y*5, Sato H*4, Shioda T*6, Sugiura W*3, Matano T*7, Adachi A*2, Nakayama EE*6, Akari H*1: *TRIM5* genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceotibility to monkey-tropic human immunodeficiency virus type 1.
Journal of General Virology. 2013;94(Pt 6):1318-24.

TRIM5α restricts human immunodeficiency virus type 1 (HIV-1) infection in cynomolgus monkey (CM) cells. We previously reported that a TRIMCyp allele expressing TRIM5-cyclophilin A fusion protein was frequently found in CMs. Here, we examined the influence of TRIM5 gene variation on the susceptibility of CMs to a monkey-tropic HIV-1 derivative (HIV-1mt) and found that TRIMCyp homozygotes were highly susceptible to HIV-1mt not only in vitro but also in vivo. These results provide important insights into the inter-individual differences in susceptibility of macaques to HIV-1mt.

Keywords: HIV-1, Cynomolgus monkey, TRIM5

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Kono K, Takeda E*1, Tsutsui H*1, Kuroishi A*1, Hulme AH*2, Hope TJ*2, Nakayama EE*1, Shioda T*1: Slower uncoating is associated with impaired replicative capability of simian-tropic HIV-1.
PLOS ONE. 2013;8(8):e72531.

Human immunodeficiency virus type 1 (HIV-1) productively infects only humans and chimpanzees, but not Old World monkeys, such as rhesus and cynomolgus (CM) monkeys. To establish a monkey model of HIV-1/AIDS, several HIV-1 derivatives have been constructed. We previously generated a simian-tropic HIV-1 that replicates efficiently in CM cells. This virus encodes a capsid protein (CA) with SIVmac239-derived loops between α-helices 4 and 5 (L4/5) and between α-helices 6 and 7 (L6/7), along with the entire *vif* from SIVmac239 (NL-4/5S6/7SvifS). These SIVmac239-derived sequences were expected to protect the virus from HIV-1 restriction factors in monkey cells. However, the replicative capability of NL-4/5S6/7SvifS in human cells was severely impaired. By long-term cultivation of human CEM-SS cells infected with NL-4/5S6/7SvifS, we succeeded in partially rescuing the impaired replicative capability of the virus in human cells. This adapted virus encoded a G-to-E substitution at the 116th position of the CA (NL-4/5SG116E6/7SvifS). In the work described here, we explored the mechanism by which the replicative capability of NL-4/5S6/7SvifS was impaired in human cells. Quantitative analysis (by real-time PCR) of viral DNA synthesis from infected cells revealed that NL-4/5S6/7SvifS had a major defect in nuclear entry. Mutations in CA are known to affect viral core stability and result in deleterious effects in HIV-1 infection; therefore, we measured the kinetics of uncoating of these viruses. The uncoating of NL-4/5S6/7SvifS was

significantly slower than that of wild type HIV-1 (WT), whereas the uncoating of NL-4/5SG116E6/7SvifS was similar to that of WT. Our results suggested that the lower replicative capability of NL-4/5S6/7SvifS in human cells was, at least in part, due to the slower uncoating of this virus.

Keywords: HIV-1, Uncoating

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Nomaguchi M*1, Yokoyama M*2, Kono K, Nakayama EE*3, Shioda T*3, Doi N*1,4, Fujiwara S*1, Saito A*5, Akari H*5, Miyakawa K*4,6, Ryo A*6, Ode H*4,7, Iwatani Y*7, Miura T*8, Igarashi T*8, Sato H*2, Adachi A*1: Generation of Rhesus Macaque-Tropic HIV-1 Clones That Are Resistant to Major Anti-HIV-1 Restriction Factors.

Journal of Virology. 2013;87(21):11447-61.

Human immunodeficiency virus type 1 (HIV-1) replication in macaque cells is restricted mainly by antiviral cellular APOBEC3, TRIM5α/TRIM5CypA, and tetherin proteins. For basic and clinical HIV-1/AIDS studies, efforts to construct macaque-tropic HIV-1 (HIV-1mt) have been made by us and others. Although rhesus macaques are commonly and successfully used as infection models, no HIV-1 derivatives suitable for in vivo rhesus research are available to date. In this study, to obtain novel HIV-1mt clones that are resistant to major restriction factors, we altered Gag and Vpu of our best HIV-1mt clone described previously. First, by sequence- and structure-guided mutagenesis, three amino acid residues in Gag-capsid (CA) (M94L/R98S/G114Q) were found to be responsible for viral growth enhancement in a macaque cell line. Results of in vitro TRIM5α susceptibility testing of HIV-1mt carrying these substitutions correlated well with the increased viral replication potential in macaque peripheral blood mononuclear cells (PBMCs) with different TRIM5 alleles, suggesting that the three amino acids in HIV-1mt CA are involved in the interaction with TRIM5α. Second, we replaced the transmembrane domain of Vpu of this clone with the corresponding region of simian immunodeficiency virus SIVgsn166 Vpu. The resultant clone, MN4/LSDQgtu, was able to antagonize macaque but not human tetherin, and its Vpu effectively functioned during viral replication in a macaque cell line. Notably, MN4/LSDQgtu grew comparably to SIVmac239 and much better than any of our other HIV-1mt clones in rhesus macaque PBMCs. In sum, MN4/LSDQgtu is the first HIV-1 derivative that exhibits resistance to the major restriction factors in rhesus macaque cells.

Keywords: macaque-tropic HIV-1, TRIM5, Vpu

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Nakaoka R, Hirano Y*1, Mooney DJ*2, Tsuchiya T, Matsuoka A: Study on the potential of RGD- and PHSRN-modified alginates as artificial extracellular matrices for engineering bone.

J Artif Organs. 2013;16:284-93.

Alginate is a polysaccharide that can be crosslinked by divalent cations, such as calcium ions, to form a gel. Chemical modification is typically used to improve its cell adhesive properties for tissue engineering applications. In this study, alginates were modified with peptides containing RGD (arginine-glycine-aspartic acid) or PHSRN (proline-histidine-serine-arginine-asparagine) sequences from fibronectin to study possible additive and synergistic effects on adherent cells. Alginates modified with each peptide were mixed at different ratios to form gels containing various concentrations and spacing between the RGD and PHSRN sequences. When normal human osteoblasts (NHOsts) were cultured on or in the gels, the ratio of RGD to PHSRN was found to influence cell behaviors, especially differentiation. NHOsts cultured on gels composed of RGD- and PHSRN-modified alginates showed enhanced differentiation when the gels

contained [33 % RGD-alginate, suggesting the relative distribution of the peptides and the presentation to cells are important parameters in this regulation. NHOsts cultured in gels containing both RGD- and PHSRN-alginates also demonstrated a similar enhancement tendency of calcium deposition that was dependent on the peptide ratio in the gel. However, calcium deposition was greater when cells were cultured in the gels, as compared to on the gels. These results suggest that modifying this biomaterial to more closely mimic the chemistry of natural cell adhesive proteins, (e.g., fibronectin) may be useful in developing scaffolds for bone tissue engineering and provide three-dimensional cell culture systems which more closely mimic the environment of the human body.
Keywords: Bone tissue engineering, Peptide modification, 3D Cell culture

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Muragaki, Y*1, Uematsu M, Isek, H*1, Umezu M*2: Analysis of Benefit-risk Balance in Decision-making of the Food and Drug Administration for Premarket Approval of Therapeutic Medical Devices.
Advanced Biomedical Engineering. 2013;2:101-6.

Compared to the evaluation of new pharmaceutical drugs, the assessments of the design and results of clinical trials for medical devices are not well established. For medical devices, the definition of the benefit-risk balance assessed during approval by regulatory agencies is not clear, which may result in subjectivity of the decision-making process. It is possible to hypothesize that the newly approved medical device should be superior in both risk and efficacy to the already existing device, which is used as control. To test this hypothesis, we performed an independent analysis of the premarket approvals (PMA) of therapeutic medical devices based on assessment review of reports of a regulatory agency, the Food and Drug Administration (FDA). A total of 74 studies that tested various medical devices for PMA were selected. For each clinical trial, the study design was evaluated with particular emphasis on its nature (retrospective or prospective), presence of a control arm, randomization, and masking. We performed an objective analysis of the benefit-risk balance between effectiveness and safety in the test arm compared to that in the control arm, using an original method for data evaluation. Of the 74 studies, 56 (76%) were prospective, 1 was purely retrospective (1%). 15 were mixed (20%), and 2 (3%) did not specify the nature of study. Only 46 studies (62%) included a comparative control group, 26 of which (57%) demonstrated "equivalence" but not "superiority" of the primary effectiveness measure. Depending on the evaluation criteria (mortality, complications, adverse effects, others) the results of safety assessment revealed advantage of the test arm in only 16-38% of comparative studies. The designs of the protocols for testing therapeutic medical devices and the criteria of objective evaluation during approval for broad clinical practice are not standardized. For PMA approval, FDA does not ultimately require better effectiveness and/or safety of the new device compared to the existing control device.
Keywords: premarket approval, benefit-risk balance, regulatory science

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小林憲弘，久保田領志，田原麻衣子，杉本直樹，木村謙治*1，林広宣*2，山田義隆*3，小林利男*4，舟洞健二*4，三枝慎一郎*5，古谷智仁*6，杉本智美*7，五十嵐良明：固相抽出－GC/MSによる水道水中の未規制農薬の一斉分析法の妥当性評価.
水道協会雑誌 2013;82(7):2-12.

固相抽出－GC/MSによる水道水中の未規制農薬を対象とした新たな一斉分析法の妥当性を評価した．今回の評価では，分析対象物質のうち９物質（メトラクロール，プロポキスル（PHC），エトベンザニド，パクロブトラゾール，シンメチリン，ボスカリド，アセタミプリド，オリサストロビン，およびブロマシル）を選択し，水道事業体７機関において，各農薬につき２段階の濃度で水道水への添加回収試験を行った．実験結果から，各農薬の定量下限，真度（回収率），併行精度，および室間精度を評価したところ，概ね良好な結果が得られた．よって，

本法は水道水中の未規制農薬の新たな一斉分析法として妥当であると結論した.

Keywords: 水道水，農薬，ガスクロマトグラフ質量分析計

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Shimizu K, Kubota R, Kobayashi N, Tahara M, Sugimoto N, Nishimura T*, Ikarashi Y: Cytotoxic effects of hydroxylated fullerenes in three types of liver cells.

Materials. 2013;6:2713-22.

Fullerenes C_{60} have attracted considerable attention in the biomedical field due to their interesting properties. Although there has been a concern that C_{60} could be metabolized to hydroxylated fullerenes ($C_{60}(OH)_x$) *in vivo*, there is little information on the effect of hydroxylated C_{60} on liver cells. In the present study, we evaluated the cytotoxic effects of fullerene C_{60} and various hydroxylated C_{60} derivatives, $C_{60}(OH)_2$, $C_{60}(OH)_{6\text{-}12}$, $C_{60}(OH)_{12}$ and $C_{60}(OH)_{36}$, with three different types of liver cells, dRLh-84, HepG2 and primary cultured rat hepatocytes. C_{60}, $C_{60}(OH)_2$ and $C_{60}(OH)_{36}$ exhibited little or no cytotoxicity in all of the cell types, while $C_{60}(OH)_{6\text{-}12}$ and $C_{60}(OH)_{12}$ induced cytotoxic effects in dRLh-84 cells, accompanied by the appearance of numerous vacuoles around the nucleus. Moreover, mitochondrial activity in liver cells was significantly inhibited by $C_{60}(OH)_{6\text{-}12}$ and $C_{60}(OH)_{12}$. These results indicate that the number of hydroxyl groups on $C_{60}(OH)_x$ contribute to the difference of their cytotoxic potential and mitochondrial damage in liver cells.

Keywords: hydroxylated fullerene, C_{60}, cytotoxic activity

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達晃一*1，星野邦広*2，岩崎貴普*3，曽根孝*4，何佳*5，神野透人，加藤信介*5：プレート吸着によるSVOCs評価法の基礎検討：DEHPの評価方法.

空気調和・衛生工学会論文集 2013;197:19-26.

車室内VOCの低減対策のために，日本自動車工業会は車室内VOC濃度の自主規制に取り組んでいる．自主規制対象成分の中にはフタル酸エステル類などの高沸点成分が含まれているが，これら高沸点成分は従来のTenaxによる捕集では精度良く測定することが困難である．そこで，我々は高沸点成分の定量的な評価手法としてガラスプレートを高沸点成分の吸着媒体とする評価手法を検討した．本論文では高沸点成分の定量的評価手法のためのプレートの選定方法および保管方法の結果を報告する.

Keywords: SVOCs，DEHP，プレート吸着

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久保田領志，小林憲弘，田原麻衣子，今村悠佑*1，木村謙治*1，小林利男*2，齋藤信裕*3，杉本智美*4，林広宣*5，古谷智仁*6，舟洞健二*2，三枝慎一郎*7，山田義隆*8，杉本直樹，西村哲治*9，五十嵐良明：固相抽出－誘導体化GC/MS 法を用いたEDTA検査法の妥当性評価.

水道協会雑誌 2013;82(8):2-11.

固相抽出－誘導体化GC/MS 法を用いたEDTA 検査法が，公的な標準検査法として適用可能であるか判定するため，水道事業体8機関を対象に妥当性評価試験を行った．水道水を用いた2設定値（10 μg/L 及び50 μg/L）における添加回収試験の報告値を基に，真度（回収率），併行精度（RSD_r (%)），室間精度（RSD_R (%)）を評価した．その結果，真度(回収率)は，2設定値で84.6%, 86.8%であった．また，併行精度は，2設定値ともに2.0～19.0%の範囲であり，室間精度は，2設定値でそれぞれ30.0%および21.8%であった．これらの結果は，妥当性評価の判定基準を満たすことから，本検査法がEDTA の標準検査法として適用可能であると判断された.

Keywords: EDTA，固相抽出，妥当性評価

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Tahara M, Obama T, Ikarashi Y: Development of analytical method for determination of 1,4-dioxane in cleansing products.

Int J Cosmet Sci. 2013;35:575-80.

OBJECTIVE: 1,4-Dioxane is a toxic by-product formed during the synthesis of surfactants used in finished cosmetic products. There are no set permissible levels of toxic impurities in finished cosmetic products in Japan. In this study, we have established a simple and sufficiently precise analytical method to determine the activity of 1,4-dioxane in finished cosmetic cleansing products.

METHODS: This method involves the standard addition approach and headspace-gas chromatography/mass spectrometry without pre-conditioning.

RESULTS: Fifteen cleansing products that are sold in the Japanese market, such as shampoo, hand soap, and dishwashing liquid, were analyzed, and 1,4-dioxane was detected at a concentration of a few micrograms per gram of the product in almost all of them. The concentration of 1,4-dioxane in two dishwashing liquid products was high. The maximum concentration of 1,4-dioxane in all of the cleansing products was below 10 mg g-1, which is a limit that is thought to be safe and technically achievable through the application of good manufacturing practices. Since 1,4-dioxane is formed during the synthesis of polyoxyethylene ether sulfate, it was detected at high concentrations in cleansing products that contained a lot of polyoxyethylene ether sulfate.

CONCLUSION: Therefore, control of the synthesis of polyoxyethylene ether sulfate can be effective in reducing the concentration of 1,4-dioxane in cleansing products.

Keywords: 1,4-dioxane, finished cosmetic product, impurity

Iwasawa K*, Tanaka G*, Aoyama T*, Chowdhury M M*, Komori K*, Tanaka-Kagawa T, Jinno H, Sakai Y*: Prediction of phthalate permeation through pulmonary alveoli using a cultured A549 cell-based in vitro alveolus model and a numerical simulation.

AATEX. 2013;18:19-31.

The animal-free prediction of inhalation toxicities in the lungs is very important concerning various low-volatile organic carbons such as phthalate. Phthalate are contained in plastics as plastisizer, easily released into environment as plastic ages, and ingested through dust. We therefore investigated benzylbutyl phthalate (BBP) permeation using an A549 cell-based lung alveolus model, in which the cell monolayers were formed on semipermeable membranes between two chambers filled with cell culture medium. With kinetic parameters obtained via these experiments, the model largely described the concentration changes in the three compartments (the apical, A549 cell, and basolateral layers) but revealed very high BBP accumulation in the alveolus cell layer at equilibrium, which did not likely reflect the in vivo situation. We therefore changed the parameter of thickness of the cell layer from 10 (cultured A549 cells) to 0.5μm (alveoli) and the parameter of the concentration in basolateral compartment to be always zero because of the continuous perfusion of blood in vivo. After changing these parameters, the accumulation of BBP remarkably decreased, and the total permeated amount significantly increased. These results indicated that various parameters and assumptions should be changed to overcome the limitations and/or properties of existing culture models to improve the predictive accuracy of the system when using in vitro cell-based tissue models and numerical simulations to predict health hazards in humans.

Keywords: phthalate, alveolus, numerical model

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Akiyama T, Sekiguchi W, Sugimoto N, Tada A, Ito Y, Yamazaki T, Akiyama H: Revised method for analyzing 2-acetyl-4-tetrahydroxybutylimidazole in caramel III.

Jpn J Food Chem Safety. 2013;20:190-5.

Caramel III, a food-coloring additive, is tested in Japan for the presence of the impurity, 2-acetyl-4-tetrahydroxybutylimidazole (THI), using an official HPLC method. In this HPLC method, THI is derivatized with 2,4-dinitrophenylhydrazine and then separated using octyl column. Improvement of the analytical conditions was attempted because contaminants can often compromise this test. Isolation of the analyte was improved when 0.1 mol/L phosphoric acid /methanol mixed solution (70:30) was

used as the mobile phase. The revised method gave higher analyte concentrations compared to the standard method. The quantitative values obtained by LC/MS were equivalent to those obtained using the revised method, demonstrating the superiority of the revised method to the standard method.

Keywords: caramel, 2-acetyl-4-tetrahydroxybutylimidazole, HPLC

小林憲弘，久保田領志，田原麻衣子，杉本直樹，塚本多矩*，五十嵐良明：水道水中の農薬類のLC/MS/MS一斉分析法の開発.

環境科学会誌 2014;27:3-19.

水質管理目標設定項目に設定されているものの標準検査法が定められていない農薬類のうち，76物質を対象にLC/MS/MSを用いた一斉分析法の分析条件を確立した．さらに，水道水を用いた添加回収試験を行い，これらの物質の検出感度や分析精度を「水道水質検査方法の妥当性評価ガイドライン」に基づいて評価した．その結果，39物質については各農薬の目標値の1/100以下の濃度まで定量でき，かつガイドラインの回収率および併行精度の目標を満たした．また，13物質については目標値の1/100の濃度まで定量できなかったが，1/10以下の濃度まで定量でき，ガイドラインの回収率および併行精度の目標を満たした．以上のことから，確立した一斉分析法は，上記の52物質を対象とした水道水質検査に適用できると考えられた.

Keywords: 水道水，農薬，LC/MS/MS

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Shimizu K, Sano T*[1], Kubota R, Kobayashi N, Tahara M, Obama T, Sugimoto N, Nishimura T*[2], Ikarashi Y: Effects of the amino acid constituents of microcystin variants on cytotoxicity to primary cultured rat hepatocytes.

Toxins. 2014;6:168-79.

Microcystins, which are cyclic heptapeptides produced by some cyanobacterial species from algal blooms, strongly inhibit serine/threonine protein phosphatase and are known as hepatotoxins. Microcystins have many structural variations, yet insufficient information is available on the differences in the cytotoxic potentials among the structural variants. In this study, the cytotoxicities of 16 microcystin variants at concentrations of 0.03-10 μg/mL to primary cultured rat hepatocytes were determined by measuring cellular ATP content, and subsequently determined by their 50% inhibitory concentration (IC_{50}). Differences in the amino acid constituents were associated with differences in cytotoxic potential. [D-Asp^3, *Z*-Dhb^7] microcystin-LR exhibited the strongest cytotoxicity at IC_{50} of 0.053 μg/mL among the microcystin variants tested. Furthermore, [D-Asp^3, *Z*-Dhb^7] microcystin-HtyR was also highly cytotoxic. These results suggest that both D-Asp and *Z*-Dhb residues are important in determining the cytotoxic potential of microcystin variants.

Keywords: microcystin, variants, cytotoxicity

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味村真弓*，中島晴信*，吉田仁*，吉田俊明*，河上強志，伊佐間和郎：有害物質含有家庭用品規制法で規制されている繊維製品中のトリス（2,3-ジブロムプロピル）ホスフェイト分析法の改定に向けた検討.

薬学雑誌 2014;134:259-68.

The official analytical method for tris(2,3-dibromopropyl)phosphate (TDBPP), which is banned from use in textile products by the "Act on Control of Household Products Containing Harmful Substances", requires revision. This study examined an analytical method for TDBPP by GC/MS using a capillary column. Thermal decomposition of TDBPP was observed by GC/MS measurement using capillary column, unlike in the case of gas chromatography/flame photometric detector (GC/FPD) measurement based on a direct injection method using a capillary megabore column. A quadratic curve, $Y=2572X^{1.416}$, was obtained for the calibration curve of GC/FPD in the concentration range 2.0-100 μg/mL. The detection limit was 1.0 μg/mL under S/N=3. The reproducibility for repetitive injections was satisfactory. A pretreatment method was established using methanol extraction, followed by liquid-liquid partition and purification with a florisil cartridge column. The recovery rate of this method was ～100%. TDBPP was not detected in any of the five commercial products that this study analyzed. To understand the cause of TDBPP decomposition during GC/MS (electron ionization; EI) measurement using capillary column, GC/MS (chemical ionization; CI), GC/FPD, and gas

chromatography/flame ionization detector（GC/FID）measurements were conducted. It was suggested that TDBPP might thermally decompose both during GC injection, especially through a splitless injection method, and in the column or ion sources. To attempt GC/MS measurement, an injection part comprising quartz liner was used and the column length was halved（15 m）; thus, only one peak could be obtained.
Keywords: tris（2,3-dibromopropyl）phosphate, textile, gas chromatography

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Kitamura K*, Maruyama K*, Hamano S*, Kishi T*, Kawakami T, Takahashi Y*, Onodera S*：Effect of hypochlorite oxidation on cholinesterase-inhibition assay of acetonitrile extracts from fruits and vegetables for monitoring traces of organophosphate pesticides.

J Toxicol Sci. 2014;39:71-81.

A reproducible method for monitoring traces of cholinesterase（ChE）inhibitors in acetonitrile extracts from fruits and vegetables is described. The method is based on hypochlorite oxidation and ChE inhibition assay. Four common representative samples of produce were selected from a supermarket to investigate the effect of different matrices on pesticides recoveries and assay precision. The samples were extracted with acetonitrile to prepare them for ChE inhibition assays: if necessary, clean-up was performed using dispersive solid-phase extraction for gas chromatography-mass spectrometry（GC/MS）analyses. Chlorine was tested as an oxidising reagent for the conversion of thiophosphorus pesticides（P=S compounds）into their P=O analogues, which have high ChE-inhibiting activity. Chlorine consumption of individual acetonitrile extracts was determined and was strongly dependent on the individual types of fruits and vegetables. After treating the acetonitrile extracts with an excess hypochlorite at 25°C for 15 min, the ChE-inhibiting activities and detection limits for each chlorine-treated pesticide solution were determined. Matrix composition did not interfere significantly with the determination of the pesticides. Enhanced anti-ChE activities leading to low detection limits（ppb levels）were observed for the chlorine-treated extracts that were spiked with chlorpyrifos, diazinon, fenitrothion, and isoxathion. This combination of oxidative derivatisation and ChE inhibition assays was used successfully to monitor and perform semi-quantitative determination of ChE inhibitors in apple, tomato, cucumber, and strawberry samples.
Keywords: ChE assay, hypochlorite oxidation, organophosphate pesticides

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田原麻衣子，杉本直樹，小林憲弘，穐山浩，五十嵐良明：追加農薬の標準品の供給調査および定量核磁気共鳴法を用いた純度測定.

水道協会雑誌 2014;83(3):9-16.

In order to set the official analytical methods of thirty one agricultural chemicals to Complementary Items for Japanese Water Quality Management, we surveyed the availability of the commercial reagents and reference material products. There are only four certified reference materials on reagent market. The purities of the twenty two commercial agricultural chemical reagent products were determined with the traceability to International System of Units（SI）by using quantitative nuclear magnetic resonance method（qNMR）, and the distribution range was from 89.2 ± 0.3 to 100.1 ± 0.6 %（n=3 average ± relative standard deviation, RSD）. The purities of all products except methyl isothiocyanate were almost same as their labeled purities by the manufactures. Other compounds are not available on reagent market as analytical standard materials, and the purities of the reagent grade products are low or not evidenced. So the traceability and certification of the purities of analytical standard materials are necessary to secure the accuracy and reliability of quantitative analytical value of agricultural chemicals for Water Quality Management, our survey represented that it was difficult to set official analytical methods for some agricultural chemicals at present.
Keywords: 水質試験，精度管理，純度

鍋師裕美，堤智昭，五十嵐敦子，蜂須賀暁子，松田りえ子：流通食品中の放射性セシウム調査.

食品衛生学雑誌 2013;54(2):131-50.

放射性物質汚染が予想される地域産食品の流通段階での買い上げ調査を実施した．NaI（Tl）シンチレーションスペクトロメータによるスクリーニング検査と，ゲルマ

ニウム半導体検出器付γ線スペクトロメータによる確定検査を行った．1,427試料中，暫定規制値であった500 Bq/kgを超過した試料数は６であり，全調査数に対する割合は0.4％であった．食品群ごとの放射性セシウム検出率から，今後も監視を継続すべき食品群は，栗・ギンナンのような果実，原木シイタケを中心としたきのこ類，山菜類，海水魚と考えられた．
Keywords: surveillance of radioactive cesium, foods on the market, screening method

鍋師裕美，堤智昭，蜂須賀暁子，松田りえ子：調味液への浸漬による牛肉中放射性セシウム量の変化に関する検討．
食品衛生学雑誌 2013;54(4):298-302.

食品中の放射性物質を低減させる調理・加工に関する情報の収集は，放射性物質の内部被ばく量を低減させ，より安全で安心な食品摂取を実現するために重要である．そこで，本研究では牛肉を用いて調味液への浸漬の際に生じる放射性セシウム（Cs）量の変化を検討した．その結果，牛肉中の放射性Csは，塩分濃度８～10％の調味液中に24時間浸漬することで浸漬前の約20％が，塩分濃度約９％の味噌調味液に７日間浸漬することで浸漬前の約55％が除去された．また，10％食塩水を交換しながら７日後まで浸漬することにより，牛肉中の放射性Csを約75％除去することが可能であった．浸漬後の調味液は廃棄されることが多く，調味液への浸漬は牛肉中の放射性Csの除去に有効であるといえる．
Keywords: radioactive material contaminated food, radioactive cesium, beef

鍋師裕美，堤智昭，蜂須賀暁子，松田りえ子：わかさぎ中の放射性セシウムの調理による除去効果に関する検討．
食品衛生学雑誌 2013;54(4):303-8.

わが国は海に囲まれ，魚介類が豊富な食環境にあるため，さまざまな魚介類を多種多様な調理法によって調理し，日常的に摂取している．平成23年３月の原発事故以降，放射性物質による魚介類の汚染が懸念されるため，魚介類の汚染状況を把握するとともに魚介類を介した放射性物質の内部被ばくを回避することが必要不可欠となった．そこで，本研究ではわかさぎを４種類の方法（素焼き，甘露煮，から揚げ，南蛮漬け）で調理し，わかさぎ中の放射性セシウム量の調理前後の変化を検討した．その結果，素焼き，甘露煮，から揚げでは，放射性セシウムの除去率は10％以下であり，除去効果は少なかった．一方で南蛮漬けは，今回の検討の中で最も高い約30％の除去率を示し，加熱後に調味液へ浸漬する調理法でも放射性セシウムの除去に効果があることが明らかとなった．
Keywords: radioactive material contaminated food, radioactive cesium, pond smelt

堤智昭，石井利華，松田りえ子：生あん中のシアン化合物分析法の性能評価と生あん中のシアン化合物の実態調査．
食品衛生学雑誌 2013;54(4):345-50.

生あん中のシアン化合物の規格への適合を判定する試験法として，水蒸気蒸留－ピリジンカルボン酸・ピラゾロン法を検討した．生あんでは豆に含まれていたシアン配糖体分解酵素が失活している恐れがあるため，本法ではリナマラーゼによるシアン配糖体分解操作を加えた．シアン化物イオンとして５ mg/kgおよび10 mg/kgに相当するシアン配糖体（リナマリン）を生あん２種に添加した分析結果から推定された真度は86～90％，併行精度は1.0～2.4％，室内精度は2.6～4.9％であった．本法は５～10 mg/kgのシアン化合物を分析する方法として妥当であることが確認された．評価した分析法を用いて，国内で製造された生あん28試料中のシアン化合物量を測定した．27試料については５ mg/kg未満であったが，１試料において15 mg/kgのシアン化合物が認められた．
Keywords: cyanogen, pyridine carbonate-pyrazolone method, raw bean paste

堤智昭，足立利華，高附巧，根井大介*，亀谷宏美*，等々力節子*，松田りえ子，手島玲子：振とう抽出法による放射線照射した食肉およびサーモンにおける2-アルキルシクロブタノン類の検知．
食品照射 2013;48(1):31-7.

2-dodecylcyclobutanone（DCB）and 2-tetradecylcyclobutanone（TCB）are specific radiolytic products in irradiated lipid-containing food and can be used to detect irradiation of foodstuffs. Here, we evaluated a rapid shaking extraction method to detect irradiation in beef, pork, chicken and salmon. The amounts of DCB and TCB extracted by the shaking extraction method were 77- 121% of those by the conventional Soxhlet extraction method in irradiated meats and salmon. The selected ion-mode chromatograms of DCB and TCB obtained from both extractions were visually inspected, but showed no significant differences. These results suggest that the shaking extraction method achieved similar extraction efficiencies for DCB and TCB to the Soxhlet extraction method. Finally, we used the shaking extraction method to detect irradiation in beef, pork, chicken and

salmon irradiated at 0.5 kGy or 1 kGy. All of the non-irradiated samples were judged negative and all of the irradiated samples were judged positive. Overall, our results indicate that the shaking extraction is a useful method for extracting DCB and TCB from meats and salmon. The main advantage of this method is the short extraction time (approximately 1 h), thereby allowing rapid detection of irradiated meats and salmon.

Keywords: irradiated food, cyclobutanones, shaking extraction method

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齊藤静夏，根本了，松田りえ子：LC-MS/MSを用いた茶熱湯浸出液中の残留農薬一斉分析法.
日本食品化学学会誌 2013;20(3):221-5.

An LC-MS/MS multiresidue method for the determination of pesticides in tea infusion was developed. An aliquot of tea infusion was cleaned up by macroporous diatomaceous earth column prior to LC-MS/MS determination. The recoveries for the tested pesticides (43 compounds) from infusion of green tea, oolong tea, and black tea after spiking at 0.05 ppm (0.1 ppm for lufenuron and triflumizole) were within the range 71-108%, with the relative standard deviations <15%, except for acrinathrin in oolong tea and black tea. No interfering peak was observed in the chromatograms of the blank extracts, indicating high selectivity of the method. The developed method is an efficient and reliable tool for the determination of pesticide residues in tea infusion.

Keywords: multiresidue method, pesticide, tea

鍋師裕美，菊地博之，堤智昭，蜂須賀暁子，松田りえ子：牛肉部位間の放射性セシウム濃度の差について.
食品衛生学雑誌 2013;54(6):415-8.

平成23年３月の福島第一原子力発電所事故後，牛肉から高濃度の放射性セシウムが検出されたことから，暫定規制値を上回る牛肉が市場に流通しないよう全頭検査が実施された．しかし，検査の過程で同一個体の部位間で放射性セシウム濃度が異なる例が明らかとなり，検査結果の信頼性に疑問が生じる事態となった．そこでわれわれは放射性セシウムを含む同一個体由来の５部位の肉を用いて測定部位間の放射性セシウム濃度の違いについて原因の解明を試みた．その結果，検討した３個体すべてにおいて，脂肪含量が高い部位ほど放射性セシウム濃度が低下することが判明し，部位間の放射性セシウムの濃度差が脂肪含量に起因することが明らかとなった．さらに，筋肉組織は平均して脂肪組織の７倍以上の放射性セシウムを含んでいたことから，ウシの個体検査で放射性セシウム濃度を測定する場合には，脂肪の少ない筋肉部を用いた検査が適当であると考えられた.

Keywords: radioactive cesium, concentration by part, beef

Yoshida T*1, Yoshioka Y*1, Tochigi S*1, Hirai T*1, Uji M*1, Ichihashi K*1, Nagano K*2, Abe Y*2, Kamada H*2, Tsunoda S*2, Nabeshi H, Higashisaka K*1, Yoshikawa T*1, Tsutsumi Y*1: Intranasal exposure to amorphous nanosilica particles could activate intrinsic coagulation cascade and platelets in mice.
Part Fibre Toxicol. 2013;10:41.

To ensuring the safety of nanomaterials which have been already applied in various applications, we examined the localization and biological responses of intranasally administered amorphous nanosilica particles in mice, focusing on the coagulation system. The results of the in vivo transmission electron microscopy analysis after intranasally exposure of mice to various size of nanosilica, it was shown that nanosilica were absorbed through the nasal cavity and were distributed into liver and brain. The results of hematological examination and coagulation tests suggest that intranasally administered nanosilica particles with diameters of 30 and 70 nm could induce abnormal activation of the coagulation system through the activation of an intrinsic coagulation cascade. This study provides information to advance the development of safe and effective nanosilica particles.

Keywords: amorphous nanosilica particles, Intranasal exposure, intrinsic coagulation

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Nagano T*1, Higashisaka K*1, Kunieda A*1, Iwahara Y*1, Tanaka K*1, Nagano K*2, Abe Y*2, Kamada H*2, Tsunoda S*2, Nabeshi H, Yoshikawa T*1, Yoshioka Y*1, Tsutsumi Y*1: Liver-specific microRNAs as biomarkers of nanomaterial-induced liver damage.
Nanotechnology. 2013;24(40):405102.

Although nanomaterials are being used in various

fields, their safety is not yet sufficiently understood. We have been attempting to establish a nanomaterials safety-assessment system by using biomarkers to predict nanomaterial-induced adverse biological effects. Here, we focused on microRNAs (miRNAs) because of their tissue-specific expression and high degree of stability in the blood. We previously showed that high intravenous doses of silica nanoparticles of 70 nm diameter (nSP70) induced liver damage in mice. In this study, we compared the effectiveness of serum levels of liver-specific or -enriched miRNAs (miR-122, miR-192, and miR-194) with that of conventional hepatic biomarkers (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) as biomarkers for nSP70. After mice had been treated with nSP70, their serum miRNAs levels were measured by using quantitative RT-PCR. Serum levels of miR-122 in nSP70-treated mice were the highest among the three miRNAs. The sensitivity of miR-122 for liver damage was at least as good as those of ALT and AST. Like ALT and AST, miR-122 may be a useful biomarker of nSP70. We believe that these findings will help in the establishment of a nanomaterials safety-assessment system.

Keywords: nanomaterials, microRNA, biomarkers

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Yamashita K*1, Yoshioka Y*1, Pan H*1, Taira M*1, Ogura T*1, Nagano T*1, Aoyama M*1, Nagano K*2, Abe Y*2, Kamada H*2, Tsunoda SI*2, Aoshima H*3, Nabeshi H, Yoshikawa T*1, Tsutsumi Y*1: Biochemical and hematologic effects of polyvinylpyrrolidone-wrapped fullerene C60 after oral administration.

Pharmazie. 2013;68(1):54-7.

The fullerene C60 is used in consumer products such as cosmetics owing to its antioxidative effects and is being developed for nanomedical applications. However, knowledge regarding the safety of fullerene C60, especially after oral administration, is sparse. Here, we examined the safety of fullerene C60 in mice after 7 d of exposure to orally administered polyvinylpyrrolidone (PVP)-wrapped fullerene C60 (PVP-fullerene C60). Mice treated with PVP-fullerene C60 showed few changes in the plasma levels of various markers of kidney and liver injury and experienced no significant hematologic effects. Furthermore, the histology of the colon of PVP-fullerene C60-treated mice was indistinguishable from that of control mice. These results suggest that PVP-fullerene C60 lacks toxicity after high-dose oral administration and indicate that PVP-fullerene C60 can be considered safe for oral medication. These data provide basic information that likely will facilitate the production of safe and effective forms of fullerene C60.

Keywords: polyvinylpyrrolidone (PVP)-wrapped fullerene C60, oral administration, Biochemical and hematologic effects

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Yoshioka Y*, Yoshikawa T*, Nabeshi H, Tsutsumi Y*: Recent topics about nano-safety science and its future.

薬学雑誌 2013;133(2):169-74.

Recently, it is concerned that nanomaterials induce undesirable biological responses (NanoTox) which is different from conventional materials attributed to their unique physicochemical properties in the world. Therefore, the movements to regulate the development and practical use of nanomaterials are accelerated in North America and Europe in corporation with Organisation for Economic Co-operation and Development (OECD). However, for our enjoying the benefits of nanomateirals, it is most important not to regulate nanomaterials in the blind way but to assure the security of nanomaterials and support the development of nanomaterial industries. These are duty of our country to be advanced country, technology-oriented nation and intellectual property nation. From these viewpoints, we are engaged on not NanoTox study but Nano-Safety Science study. That is, we try to research the relationship between physicochemical properties, biodistribution, intracellular localization, kinetics and biological responses (safety) of nanomaterials for the purpose of the collection and the transmission of safety information of nanomaterials based on scientific evidence lead to a support of nanomaterials' development. In this review, we would like to

introduce our Nano-safety science study using mainly amorphous silica nanoparticles.

Keywords: nanomaterials, Nano-Safety Science

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Takabatake R*[1], Takashima K*[1], Kurashima T*[1], Mano J*[1], Furui S*[1], Kitta K*[1], Koiwa T*[1,2], Akiyama H, Teshima R, Futo S*[3], Minegishi Y*[4]: Interlaboratory study of qualitative PCR methods for genetically modified maize events MON810, Bt11 and GA21, and CaMV P35S.

J AOAC Int. 2013;96:1-7.

Qualitative PCR methods for the genetically modified (GM) maize events MON810, Bt11, and GA21, and the 35S promoter (P35S) region of the cauliflower mosaic virus were evaluated in an interlaboratory study. Real-time PCR-based quantitative methods for these GM events using the same primer pairs have already been validated in previous studies. Fifteen laboratories in Japan participated in this interlaboratory study. Each participant extracted DNA from blind samples, performed qualitative PCR assays, and then detected the PCR products with agarose gel electrophoresis. The specificity, sensitivity, and false-negative and false-positive rates of these methods were determined with different concentrations of GM mixing samples. The limit of detections of MON810, Bt11, GA21, and the P35S segment calculated as the amount of MON810 of these methods were 0.2, 0.2, 0.1 and 0.2% or less, respectively. The current study demonstrated that the qualitative methods would fit for the detection and identification of these GM maize events and P35S segment.

Keywords: genetically modified maize, qualitative PCR, interlaboratory study

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Minegishi Y*[1], Mano J*[2], Kato Y*[3], Kitta K*[2], Akiyama H, Teshima R: Development and evaluation of a novel DNA extraction method suitable for processed foods.

Jpn J Food Chem Safety. 2013;20:114-8.

For easy and rapid DNA extraction from processed foods, we developed a new silica membrane-based DNA extraction method. DNA extraction conditions suitable for processed foods were examined based on an existing DNA extraction kit for raw grain materials, GM quicker 2. Twentymicroliters of proteinase K solution (20 mg/ml) was used for cell lysis and the digestion was carried out at 65°C for 30 min. In addition, 200 µl for wet processed foods or 500 µl for dry processed foods of 2.0 M potassium acetate (pH 3.7) and 600 µl of 8.0 M guanidine hydrochloride were adopted as buffers to achieve good DNA recovery from cell lysates. The novel method was compared to four conventionalmethods using six kinds of processed foods as analytical samples, i.e., roasted soybean flour, soy milk, miso, canned whole kernel sweet corn, corn snack and dried soup mix. The developed method showed wide applicability to variousprocess foods and it gave sufficient amounts of DNA withhigh purity. Also, the method was highly user-friendly because of the short handling time, the small number ofpipette operations and non-use of toxic organic solvents. The method would be practically used for food testing to detect genetically modified organisms, allergens, pathogenic microorganisms and so on.

Keywords: processed foods, DNA extraction, genetically modified organism

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笠間菊子*，小熊恭代*，穐山浩，鈴木達也*，渡辺卓穂*，小島幸一*：ダイズおよびトウモロコシ抽出DNAの精製度の検討.

日本食品化学学会誌 2013;20:203-8.

通知法に従い，ダイズおよびトウモロコシからDNeasy Plant Mini kit（Miniキット）およびGM quicker（GM quickerキット）でDNAを抽出し，抽出DNAの精製度および収量を検討した．各抽出液のDNA含量を，UV法と蛍光法のそれぞれで定量した結果，ダイズのMiniキットDNA（Mini-DNA）のUV法による収量は蛍光法の約3倍であったが，GM quickerキットDNA（Quicker-DNA）ではUV法による収量が僅かに上回るに留まった．また，トウモロコシでは，Mini-DNAのUV法による収量は蛍光法の1.77倍，Quicker-DNAでは1.52倍で，定量法間の差が

小さかった．ダイズのMini-DNAでは定量法によって収量が大きく異なったことから，各抽出DNAの精製度をアガロースゲル電気泳動およびリアルタイムPCRを用いて内在性遺伝子のコピー数を測定することにより検討した．その結果ダイズでは，UV法で濃度調製したDNA溶液でアガロースゲル電気泳動におけるゲノミックDNAのバンド，内在性遺伝子のコピー数ともにMini-DNAがQuicker-DNAに比べて少なく測定され，UV法によるMini-DNAの定量値は正の誤差を含んでいることが示唆された．一方，トウモロコシDNAでは，UV法で濃度調製したDNA溶液の内在性遺伝子のコピー数，ゲノミックDNAのバンドともに抽出キット間で差はほとんど認められなかった．次に各抽出DNAをサイズ排除クロマトグラフィーを用いてさらに詳細に分析した．その結果，ダイズのMini-DNAはダイズのQuicker-DNA，トウモロコシのMini-DNA，Quicker-DNAに比べてDNAに類似した紫外吸収スペクトルを有する低分子の不純物を大量に含むことが示され，これらの物質がUV法によるDNAの定量を妨害していることが明らかになった．

Keywords: DNA抽出法，ダイズ，サイズ排除クロマトグラフィー

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Koizumi D*, Shirota K*, Akita R*, Oda H*, Akiyama H: Development and validation of a lateral flow assay for the detection of crustacean protein in processed foods.

Food Chemistry. 2014;150:348-52.

We developed and validated a novel lateral flow assay for the detection of crustacean protein in processed foods. This assay had high sensitivity; the visual detection limit for shrimp protein extract was 25 μg/L, equivalent to 1 μg/g protein in a food sample, and results could be obtained within 20 min without sophisticated procedures or expensive equipment. Concordance between our assay and another validated quantitative enzyme-linked immunosorbent assay was 97% for commercially processed foods. This assay is rapid, simple, reliable, and highly correlated with validated enzyme-linked immunosorbent assays and is thus suitable for monitoring of food products, especially in food-processing facilities.

Keywords: crustacean, food allergy, lateral flow assay

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Ohtsuki T, Sato K, Sugimoto N, Akiyama H: Absolute quantification of dehydroacetic acid in processed foods using quantitative ^{1}H NMR.

Food Chemistry. 2013;141:1322-7.

An absolute quantification method for the determination of dehydroacetic acid in processed foods using quantitative ^{1}H NMR was developed and validated. The level of dehydroacetic acid was determined using the proton signals of dehydroacetic acid referenced to 1,4-bis（trimethylsilyl）benzene-d_4 after simple solvent extraction from processed foods. All the recoveries from three processed foods spiked at two different concentrations were larger than 85%. The proposed method also proved to be precise, with inter-day precision and excellent linearity. The limit of quantification was confirmed as 0.13 g/kg in processed foods, which is sufficiently low for the purposes of monitoring dehydroacetic acid. Furthermore, the method is rapid and easy to apply, and provides International System of Units traceability without the need for authentic analyte reference materials. Therefore, the proposed method is a useful and practical tool for determining the level of dehydroacetic acid in processed foods.

Keywords: absolute quantification, quantitative ^{1}H NMR, dehydroacetic acid

Wakita K*[1], Kuwabara H*[2], Furusho N, Tatebe C, Sato K, Akiyama H: A comparative study of the hydroxyl and saponification values of polysorbate 60 in international food additive specifications.

American Journal of Analytical Chemistry. 2014;5:199-204.

We investigated the hydroxyl and saponification values of 27 samples of Polysorbate 60 products that were commercially available worldwide. We observed that the values of most of the studied samples were not within the range established at the Joint FAO/WHO Expert Committee on Food Additives（JECFA），while they did agree with the specifications described in the USA, the EU and Japan. We believe that purities of the new commercial Polysorbate 60 samples are higher than those of the older products which were available when the JECFA specifications were discussed（around 1973）. The present study suggests that the hydroxyl and saponification values of the current JECFA specifications for Polysorbate 60 should be re-

evaluated.

Keywords: Polysorbate 60, polyoxyethylene sorbitan monostearate, hydroxyl value

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Tada A, Ishizuki K, Iwamura J*1, Mikami H*2, Hirao Y*2, Fujita I*3, Yamazaki T, Akiyama H, Kawamura Y: Improvement of the assay method for steviol glycosides in the JECFA specifications.
American Journal of Analytical Chemistry. 2013;4:190-6.

Steviol glycosides are natural sweetener constituents found in the leaves of *Stevia rebaudiana* Bertoni (Asteraceae). The specifications for steviol glycosides were established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, although there was a call in the following year for the modification of this assay method to enable the determination of nine steviol glycosides rather than just seven. In response, based on a proposed method by the Japan Stevia Association, we developed an improved method by changing the HPLC conditions and including the use of an octadecylsilyl column instead of an amino-bonded column to enable the rapid and reliable determination of the nine steviol glycosides by an isocratic HPLC-UV method. With the developed method, the nine steviol glycosides can be separately determined, and identified using individual reference chemicals as standards, unlike the previous identification method, which was based on the relative retention times. In addition, the single stevioside quantification standard was replaced with both stevioside and rebaudioside A quantification standards. Importantly, the validation of the developed method was successful. The limits of quantification for the nine steviol glycosides were between 0.2% and 0.6%. The developed assay method for the nine steviol glycosides was proposed to JECFA and adopted as the revised assay method for the steviol glycosides specifications at its 73rd meeting in 2010.

Keywords: steviol glycosides, stevioside, JECFA specifications

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Yoshida T*1, Terasaka K*1, Kato S*1, Bai F*1, Sugimoto N, Akiyama H, Yamazaki T*2, Mizukami H*1: Quantitative determination of carthamin in carthamus red by ^{1}H-NMR spectroscopy.
Chem Pharm Bull. 2013;61:1264-8.

Carthamus Red is a food colorant prepared from the petals of *Carthamus tinctorius* (Asteraceae) whose major pigment is carthamin. Since an authentic carthamin standard is difficult to obtain commercially for the preparation of calibration curves in HPLC assays, we applied 1H-NMR spectroscopy to the quantitative determination of carthamin in commercial preparations of Carthamus Red. Carthamus Red was repeatedly extracted in methanol and the extract was dissolved in pyridine-d_5 containing hexamethyldisilane (HMD) prior to ^{1}H-NMR spectroscopic analysis. The carthamin contents were calculated from the ratios of singlet signal intensities at approximately σ: 9.3 derived from H-16 of carthamin to those of the HMD signal at σ: 0. The integral ratios exhibited good repeatability among NMR spectroscopic analyses. Both the intra-day and inter-day assay variations had coefficients of variation of <5%. Based on the coefficient of absorption, the carthamin contents of commercial preparations determined by ^{1}H-NMR spectroscopy correlated well with those determined by colorimetry, although the latter were always approximately 1.3-fold higher than the for- mer, irrespective of the Carthamus Red preparations. In conclusion, the quantitative ^{1}H-NMR spectroscopy used in the present study is simple and rapid, requiring no carthamin standard for calibration. After HMD concentration has been corrected using certified reference materials, the carthamin contents determined by ^{1}H-NMR spectroscopy are System of Units (SI)-traceable.

Keywords: quantitative NMR, carthamin, *Carthamus tinctorius* (Asteraceae)

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Mutsuga M, Yamaguchi M, Kawamura Y: Analysis of *N*-nitrosamine migration from rubber teats and soothers.

American Journal of Analytical Chemistry. 2013;4:277-85.

A testing method for *N*-nitrosamines and *N*-nitrosatable substances in rubber teats and soothers was modified. *N*-Nitrosamines are generally analyzed using either a nitrogen chemiluminescence detector (NCD) or a thermal energy analyzer (TEA). However, because few testing laboratories are equipped with these devices, it is difficult to conduct these tests. Therefore, an analysis method for *N*-nitrosamines using the more widespread gas chromatography-mass spectrometry (GC-MS) method was improved. In addition, EN 12868 was used to prepare the test solutions because of its worldwide use and compliance with EU regulations. Using GC-MS, EN 12868 method targeting ten kinds of *N*-nitrosamines was modified. The determination limits of the method were 1.0-1.5 μg/kg for *N*-nitrosamines and 4-6 μg/kg for *N*-nitrosatable substances. Quantification was possible at 1/5 or less and 1/15 or less, respectively, of the regulation values listed in EU Directive 93/11/EEC. In terms of application, there were no problems with the selectivity of the detector. The recoveries were 58%-109% for *N*-nitrosamines and 59%-102% for *N*-nitrosatable substances. Screening and verification were possible by measuring the amount of secondary amines in the boiled solution and migration solution.

Keywords: *N*-nitrosamine, *N*-nitrosatable substances, secondary amine

Mutsuga M, Yamaguchi M, Abe Y, Akiyama H: Evaluation of the equality of non-polar capillary columns in GC/MS analysis of food contact plastics. *American Journal of Analytical Chemistry*. 2013;4:476-87.

Non-polar capillary columns for GC/MS are widely utilized in the analysis of additives for food contact materials. Though various kinds of non-polar capillary columns are commercially available, the equality of their performance has not been verified. Herein, ninety-six additives for food contact plastics were analyzed using fifteen kinds of columns, and the peak separation, retention times, and peak areas of each additive were compared. The additives, with various chemical properties, comprised forty four plasticizers, twenty lubricants, twenty antioxidants, nine ultraviolet absorbers, and three other compounds. 10 μg mL^{-1} test solutions were prepared in acetone, and injected to the GC/MS. The fifteen columns were classified into five categories based on the chromatogram pattern and peak separation. To facilitate comparison of the retention time and detection sensitivity of the columns for the additives, the relative retention time (RRT) and relative peak area (RPA) were calculated by using dibutylphthalate or 4-*tert*-butylphenylsalicylate as an internal standard. The RRTs of the additives on each column were essentially similar. However, the RRT of the additives which were detected in the later stages differed slightly. Although the RPA of the plasticizers and lubricants were roughly similar, column-to-column differences were observed for certain additives, such as antioxidants and ultraviolet absorbers. Furthermore, certain fatty acids, antioxidants, two plasticizers, and two benzophenone type ultraviolet absorbers were not detected in the chromatograms of two columns.

Keywords: non-polar capillary column, GC/MS analysis, additives for food contact plastics

Mutsuga M, Yamaguchi M, Kawamura Y: Quantification of isocyanates and amines in polyurethane foams and coated products by liquid chromatography-tandem mass spectrometry. *Food Science & Nutrition*. 2014;2:156-63.

An analytical method for the identification and quantification of 10 different isocyanates and 11 different amines in polyurethane (PUR) foam and PUR-coated products was developed and optimized. Isocyanates were extracted and derivatized with di-*n*-butylamine, while amines were extracted with methanol. Quantification was subsequently performed by liquid chromatography-tandem mass spectrometry. Using this methodology, residual levels of isocyanates and amines in commercial PUR products were quantified. Although the recoveries of certain isocyanates and amines were low, the main compounds used as monomers in the production of PUR products, and their decomposition species, were clearly identified at quantifiable levels. 2,4- and 2,6-toluenediisocyanate were detected in most PUR foam samples and a pastry bag in the range of 0.02-0.92 mg/kg, with their decomposition compounds, 2,4- and 2,6-toluenediamine, detected in all PUR foam samples in the range of 9.5-59 mg/kg. PUR-coated gloves are manufactured using 4,4'-methylenebisphenyl diisocyanate as the main raw

material, and a large amount of this compound, in addition to 4,4'-methylenedianiline and dicyclohexylmethane-4,4'-diamine were found in these samples.

Keywords: amine, isocyanate, polyurethane

Abe Y, Yamaguchi M, Mutsuga M, Akiyama H, Kawamura Y: Volatile substances in polymer toys made from butadiene and styrene.
American Journal of Analytical Chemistry. 2013;4:229-37.

The residual levels and migration behavior of volatile substances were detected using HS-GC/MS for acrylonitrile-butadiene-styrene copolymer (ABS) toys, thermoplastic elastomer toys, and rubber toys made from 1,3-butadiene and styrene found on the Japanese market. The maximum residual level of these volatile substances was 2600 μg/g of styrene in ABS toys. In particular, the levels of known carcinogens 1,3-butadiene, benzene, and acrylonitrile are 5.3, 2.5 and 55 μg/g, which are much lower than the EU limit of 0.1%. Furthermore, some volatile substances migrated from ABS toys into water in amounts of 3-40 ng/mL. Thermoplastic elastomer toys and rubber toys contained these volatile substances at significantly lower levels than ABS toys.

Keywords: volatile substance, toy

Abe Y, Yamaguchi M, Mutsuga M, Kawamura Y, Akiyama H: Survey of volatile substances in kitchen utensils made from acrylonitrile-butadiene-styrene and acrylonitrile-styrene resin in Japan.
Food Science & Nutrition. 2014;2:236-43.

Residual levels of 14 volatile substances, including 1,3-butadiene, acrylonitrile, benzene, ethylbenzene, and styrene, in 30 kitchen utensils made from acrylonitrile-butadiene-styrene resin (ABS) and acrylonitrile-styrene resin (AS) such as slicers, picks, cups, and lunch boxes in Japan were simultaneously determined using headspace gas chromatography/mass spectroscopy (HS-GC/MS). The maximum residual levels in the ABS and AS samples were found to be 2000 and 2800 μg/g of styrene, respectively. The residual levels of 1,3-butadiene ranged from 0.06 to 1.7 μg/g in ABS, and three of 15 ABS samples exceeded the regulatory limit for this compound as established by the European Union (EU). The residual levels of acrylonitrile ranged from 0.15 to 20 μg/g in ABS and from 19 to 180 μg/g in AS. The levels of this substance in seven ABS and six AS samples exceeded the limit set by the U.S. Food and Drug Administration (FDA). Furthermore, the levels of acrylonitrile in three AS samples exceeded the voluntary standard established by Japanese industries. These results clearly indicate that the residual levels of some volatile compounds are still high in ABS and AS kitchen utensils and further observations are needed.

Keywords: acrylonitrile-styrene resin (AS), acrylonitrile-styrene-butadiene resin (ABS), volatile substance

Kawamura Y, Etoh M*, Hirakawa Y*, Abe Y, Mutsuga M: Bisphenol A in domestic and imported canned foods in Japan.
Food Addit Contam A. 2014;31:330-340.

The bisphenol A (BPA) concentrations were surveyed in 100 domestic and 60 imported canned foods purchased on the Japanese market from 2011 to 2012. BPA was extracted from the canned foods, derivatized by ethylation and analysed using GC/MS. In the domestic canned foods, the maximum and average BPA concentrations were 30 and 3.4 ng/g, respectively. While, in the imported canned foods, they were 390 and 57 ng/g, respectively. The BPA level in the domestic canned foods was significantly lower than that in the imported canned foods. Based on these results, the intakes of BPA from the domestic and imported canned foods in Japan were estimated 644 ng /person/day. The Japanese BPA intake was the second-lowest following New Zealand, though the imported canned foods increased. It was sufficiently lower than the tolerable daily intake of EFSA and USEPA. The drastic reduction of BPA in the domestic canned foods should be due to the "BPA reduced cans" which Japanese can manufacturers had developed in the late of 1990s and became widely used in Japan.

Keywords: bisphenol A, canned foods, BPA reduced can

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R[*2], Arizono K[*2]: Development of novel GC/MS database for the determination of additives for food packaging into the processed foods.

Jpn J Food Chem Safety. 2013;20:42-51.

Various types of compounds are used as additives in packaging materials, and their number is increasing each year. In this study, we developed a GC/MS database containing information, such as retention times and calibration curves, for 125 additives. The extracts of several processed foods obtained by the stir-bar sportive extraction (SBSE) method were analyzed for additives, such as plasticizers and lubricants, using the database. It was found that the database was useful for the rapid and easy screening analysis of these additives.

Keywords: processed food, additives for packaging materials, simultaneous determination methods

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岸映里[*]，尾崎麻子[*]，大嶋智子[*]，清水充[*]，河村葉子：マイクロウェーブ分解およびICP-MSを用いた合成樹脂製器具・容器包装中の有害元素の迅速分析法.

日本食品化学学会誌 2013;20:105-113.

Rapid method combining microwave digestion and ICP-MS analysis was developed for the simultaneous determination of seven harmful elements (Cd, Pb, Ba, As, Hg, Cr and Ag) in food contact plastics, the former four elements are regulated by the Japanese Food Sanitation Law. After microwave digestion of 100 mg of milled sample with HNO_3, digested solution was diluted to the definite concentration and then applied to ICP-MS. The recoveries were mainly more than 80% using the standard and test solutions prepared by the equalized HNO_3 concentrations with the internal standardization. This new method was also valid for analysis of Pb in polypropylene containing barium sulfate which showed very low recovery by dry ash method adopted in Japanese official method.

Keywords: ICP-MS, microwave digestion, food package

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羽石奈穂子[*]，金子令子[*]，植松洋子[*]，河村葉子：ポリカーボネート製品中のトリエチルアミンおよびトリブチルアミン分析法.

日本食品化学学会誌 2013;20:114-118.

A method for the determination of triethylamine and tributylamine in polycarbonate products was developed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The sample was dissolved with dichloromethane, and the polymer was precipitated by addition of methanol. In order to avoid volatilization of triethylamine and tributylamine during evaporation, 2% formic acid was added to the methanol mixture. After evaporation, triethylamine and tributylamine were determined by LC-MS/MS. Recoveries of triethylamine and tributylamine in polycarbonate products at the level of 1μg were 96 and 101%, respectively. The limits of quantification were 0.05 μg/g in samples.

Keywords: triethylamine, tributylamine, LC-MS/MS

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Matsuoka H[*], Shigetomi T[*], Funabashi H[*], Saito M[*], Igimi S: Tryptic soy medium is feasible for the in situ preparation of standards containing small defined numbers of microbial cells.

J Microbiol Methods. 2013;93:49-51.

A standardmaterial comprising a fewviable cells of amicroorganismis essential for rational validation of microbiological methods. Wepropose a method of a flow cytometric sorting of cells stained with 6-carboxyfluorescein diacetate. The feasibility of tryptic soy medium in this method is demonstrated with 5 strains.

Keywords: viable cells, microorganism, flow cytometric sorting

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日本食品科学工学会誌 2013;60:347-56.

非加熱食肉製品である生ハムにおける水分活性（aW）

および乳酸ナトリウムが*Listeria monocytogenes*の挙動に及ぼす影響を明らかにするために，試験用生ハムに*L. monocytogenes* ATCC 49594（血清型4b，Scott A）を接種して5試験機関で検討した．その結果，試験用生ハムでは，いずれの機関でもaW0.93（0.930≦aw＜0.940）では，*L. monocytogenes*が10℃保管で56日間増殖しなかった．このことから，生ハムではaW0.93であれば，原料肉のpHや食塩，亜硝酸塩および低温保管（10℃）が相加，相乗的に作用し，*L. monocytogenes*の増殖を抑制することが明らかとなった．また，aW0.94（0.940≦aw＜0.950）であっても，乳酸ナトリウムを2％添加することで*L. monocytogenes*の増殖が抑制されることが示唆された．このことから，我が国の食品衛生法に従って製造される生ハムに乳酸ナトリウムを利用することは，リステリア症のリスク低減につながると考えられる．

Keywords: *Listeria monocytogenes*, Raw ham, Sodium Lactate

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生ハムにおける*Listeria monocytogenes*の増殖に及ぼす水分活性とナイシンの影響．
日本食品科学工学会誌 2013;60:619-27.

非加熱食肉製品である生ハムにおける水分活性（aW）およびナイシンの*Listeria monocytogenes*の挙動に及ぼす影響を明らかにするために，試験用生ハムに*L. monocytogenes* ATCC 49594（血清型4b，Scott A）を接種して5試験機関で検討した．試験用生ハムでは，いずれの機関でもaW 0.93（0.930≦aW＜0.940）では，*L. monocytogenes*が10℃保管28日間増殖しなかった．生ハムにナイシンを添加することによって*L. monocytogenes*の初発菌数は減少し，*L. monocytogenes*に対するナイシンの効果は，殺菌的な効果であることが示された．aW0.94（0.940≦aW＜0.950）では2試験機関で増殖が認められたが，ナイシンを12.5mg/kg添加することで*L. monocytogenes*の増殖が10℃保管28日間抑制されることが示された．これらのことから，我が国の食品衛生法に従って製造される生ハムにおいてナイシンの利用はリステリアの増殖を効果的に抑制するものと考えられる．

Keywords: *Listeria monocytogenes*, Raw ham, Nisin

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Toh H*1, Oshima K*2, Nakano A*3, Takahata M*3, Murakami M*3, Takaki T*4, Nishiyama H*4, Igimi S, Hattori M*2, Morita H*3:
Genomic adaptation of the *Lactobacillus casei* group.
PLOS ONE. 2013;0075073.

Lactobacillus casei, *L. paracasei*, and *L. rhamnosus* form a closely related taxonomic group (*Lactobacillus casei* group) within the facultatively heterofermentative lactobacilli. Here, we report the complete genome sequences of *L. paracasei* JCM 8130 and *L. casei* ATCC 393, and the draft genome sequence of *L. paracasei* COM0101, all of which were isolated from daily products. Furthermore, we re-annotated the genome of *L. rhamnosus* ATCC 53103 (also known as *L. rhamnosus* GG), which we have previously reported. We confirmed that ATCC 393 is distinct from other strains previously described as *L. paracasei*. The core genome of 10 completely sequenced strains of the *L. casei* group comprised 1,682 protein-coding genes. Although extensive genome-wide synteny was found among the *L. casei* group, the genomes of ATCC 53103, JCM 8130, and ATCC 393 contained genomic islands compared with *L. paracasei* ATCC 334. Several genomic islands, including carbohydrate utilization gene clusters, were found at the same loci in the chromosomes of the *L. casei* group. The spaCBA pilus gene cluster, which was first identified in GG, was also found in other strains of the *L. casei* group, but several *L. paracasei* strains including COM0101 contained truncated spaC gene. ATCC 53103 encoded a higher number of proteins involved in carbohydrate utilization compared with intestinal lactobacilli, and extracellular adhesion proteins, several of which are absent in other strains of the *L. casei* group. In addition to previously fully sequenced *L. rhamnosus* and *L. paracasei* strains, the complete genome sequences of *L. casei* will provide valuable insights into the evolution of the *L. casei* group.

Keywords: *Lactobacillus casei*, genome sequence, gene cluster

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Yoshida W*, Kezuka A*, Murakami Y*, Lee J*, Abe K*, Motoki H*, Matsuo T*, Shimura N*, Noda M, Igimi S, Ikebukuro K*: Automatic polymerase chain reaction product detection system for food safety monitoring using zinc finger protein fused to luciferase.
Analytica Chimica Acta. 2013;801:78-83.

An automatic polymerase chain reaction (PCR) product detection system for food safety monitoring using zinc finger (ZF) protein fused to luciferase was developed. ZF protein fused to luciferase specifically binds to target double stranded DNA sequence and has luciferase enzymatic activity. Therefore, PCR products that comprise ZF protein recognition sequence can be detected by measuring the luciferase activity of the fusion protein. We previously reported that PCR products from *Legionella pneumophila* and *Escherichia coli* (*E. coli*) O157 genomic DNA were detected by Zif268, a natural ZF protein, fused to luciferase. In this study, Zif268-luciferase was applied to detect the presence of *Salmonella* and coliforms. Moreover, an artificial zinc finger protein (B2) fused to luciferase was constructed for a Norovirus detection system. In the luciferase activity detection assay, several bound/free separation process is required. Therefore, an analyzer that automatically performed the bound/free separation process was developed to detect PCR products using the ZF-luciferase fusion protein. By means of the automatic analyzer with ZF-luciferase fusion protein, target pathogenic genomes were specifically detected in the presence of other pathogenic genomes. Moreover, we succeeded in the detection of 10 copies of *E. coli* BL21 without extraction of genomic DNA by the automatic analyzer and E. coli was detected with a logarithmic dependency in the range of 1.0 × 10 to 1.0 × 10(6) copies.

Keywords: zinc finger protein, luciferase, detection method

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日本食品科学工学会誌 2014;61:9-18.

生ハムにおいて *Listeria monocytogenes* が増殖する可能性を評価するために，生ハムで *L. monocytogenes* の増殖に対する水分活性（aw）及びくん煙成分の影響を5つの試験機関で調べた．*L. monocytogenes* をくん煙もしくはくん液処理したawの異なる生ハム（0.94, 0.92）に接種し，その挙動を嫌気条件下の10℃で56日間に渡り調べた．その結果，awが0.94の生ハムでは，無処理の生ハムでは増殖が見られたものの，くん煙処理やくん液処理をした場合では，全ての施設で増殖が見られなかった．増殖抑制が見られた aw 0.94 の生ハムのフェノール類濃度は 0.6 から12.2ppm だった．aw が0.92の生ハムでは無処理のものでさえ増殖は見られなかった．以上の結果より，aw 0.94以下で一般的なくん煙処理を施した生ハム，もしくは aw 0.92以下にした生ハムでは，*L. monocytogenes* が増殖するリスクはかなり低いことが示唆された．

Keywords: *Listeria monocytogenes*, Raw ham, Smoke compounds

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Matsuoka H*, Nakano K*, Takatani N*, Yoshida T*, Igimi S, Saito M*: Flow cytometric method for in situ preparation of standard materials of a small defined number of microbial cells with colony-forming potentiality.
J AOAC Int. 2014;97:479-83.

Standard materials of a small defined number of cells with colony-forming potentiality are essential for the rational validation of food microbiological methods. An in situ flow cytometric method using viable staining with 6-carboxyfluorescein diacetate (CFDA) and

tryptic soy agar（TSA）was previously proposed and its feasibility was demonstrated with five strains. In this study, this method was applied to 16 strains to support its broad applicability. The cell sorting gate was previously determined based on the CFDA stainability alone. Now the structural properties of cells designated by forward and side-scattering intensities have been introduced as the second gating criteria. Under the optimum gate condition, 100 cells have been selected and sorted on TSA. Consequently, a 95% or higher colony-forming rate has been attained for every strain. A successful application to microaerophilic *Campylobacter* spp. is especially of great importance because it suggests further broader applicability.

Keywords: Flow cytometric method, detection method, viable cells

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Asakura H, Masuda K, Yamamoto S＊, Igimi S: Molecular approach for tracing dissemination routes of Shiga toxin-producing *Escherichia coli* O157 in bovine offal at slaughter.

Biomed Res Int. 2014;739139.

Bovine offal is currently recognized as one of the sources of human Shiga toxin-producing Escherichia coli（STEC）infection in Japan. Here, the prevalence and genetic characterization of STEC O157 in bovine feces, offal, and carcasses at slaughtering were examined between July and October in 2006. STEC O157 was detected in 31 of 301 cattle feces（10.3%）delivered from 120 farms. Simultaneously, 60 bovine-originated offal（tongue, liver, and omasum）and carcasses were randomly selected and the detection of O157 STEC was examined as well. STEC O157 was isolated from 4 tongues（6.7%）, 1 liver（1.7%）, 3 omasam（5.0%）, and 2 carcasses（3.3%）, respectively. All the O157 isolates were positive for eae and hlyA genes, and 37 of 41 isolates（90.2%）exhibited stx2c genotype. PFGE analysis revealed the identical macrogenotypes of 4-tongue- and 1-liver-originated isolates and among 2 fecal isolates from animals slaughtered consecutively. Considering their continuous detection according to the slaughtering order, we concluded that these distributions of O157 in bovine offal and feces might be due to cross-contamination at（pre）slaughter. Our data thus reposes implication of better sanitary control in diapedesis from both upper and lower sites to prevent spread of this pathogen to bovine offal at slaughtering.

Keywords: STEC O157, Slaughter, PFGE

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Belogolova E[*1], Bauer B[*1], Pompaiah M[*1], Asakura H, Brinkmann V[*1], Ertl C[*2], Bartfeld S[*1], Nechitaylo T[*3], Haas R[*2], Machuy N, Salama N[*4], Churin Y[*1], Meyer TF[*1]: *Helicobacter pylori* HopQ identified as a novel T4SS-associated virulence factor.

Cell Microbiol. 2013;15:1896-1912.

Helicobacter pylori is a bacterial pathogen that colonizes the gastric niche of ~50% of the human population worldwide and is known to cause peptic ulceration and gastric cancer. Pathology of infection strongly depends on a *cag* pathogenicity island（cagPAI）-encoded type IV secretion system（T4SS）. Here, we aimed to identify as yet unknown bacterial factors involved in *cag*PAI effector function and performed a large-scale screen of an *H. pylori* transposon mutant library using activation of the pro-inflammatory transcription factor NF-κB in human gastric epithelial cells as a measure of T4SS function. Analysis of ~3000 *H. pylori* mutants revealed three non-*cag*PAI genes that affected NF-κB nuclear translocation. Of these, the outer membrane protein HopQ from *H. pylori* strain P12 was essential for CagA translocation and for CagA-mediated host cell responses such as formation of the hummingbird phenotype and cell scattering. Besides that, deletion of *hopQ* reduced T4SS-dependent activation of NF-κB, induction of MAPK signaling and secretion of interleukin 8（IL-8）in the host cells, but did not affect motility or the quantity of bacteria attached to host cells. Hence, we identified HopQ as a non-*cag*PAI-encoded cofactor of T4SS function.

Keywords: *Helicobacter pylori*, genome-wide screen, HopQ protein

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Asakura H, Hashii N, Uema M, Kawasaki N,Sugita-

Konishi Y*[1], Igimi S, Yamamoto S*[2]: *Campylobacter jejuni pdxA* affects flagellum-mediated motility to alter host colonization.

PLOS ONE. 2013;8:e70418.

Vitamin B6 (pyridoxal-5'-phosphate, PLP) is linked to a variety of biological functions in prokaryotes. Here, we report that the pdxA (putative 4-hydroxy-L-threonine phosphate dehydrogenase) gene plays a pivotal role in the PLP-dependent regulation of flagellar motility, thereby altering host colonization in a leading foodborne pathogen, *Campylobacter jejuni*. A *C. jejuni pdxA* mutant failed to produce PLP and exhibited a coincident loss of flagellar motility. Mass spectrometric analyses showed a 3-fold reduction in the main flagellar glycan pseudaminic acid (Pse) associated with the disruption of *pdxA*. The *pdxA* mutant also exhibited reduced growth rates compared with the WT strain. Comparative metabolomic analyses revealed differences in respiratory/energy metabolism between WT *C. jejuni* and the *pdxA* mutant, providing a possible explanation for the differential growth fitness between the two strains. Consistent with the lack of flagellar motility, the *pdxA* mutant showed impaired motility-mediated responses (bacterial adhesion, ERK1/2 activation, and IL-8 production) in INT407 cells and reduced colonization of chickens compared with the WT strain. Overall, this study demonstrated that the *pdxA* gene affects the PLP-mediated flagellar motility function, mainly through alteration of Pse modification, and the disruption of this gene also alters the respiratory/energy metabolisms to potentially affect host colonization. Our data therefore present novel implications regarding the utility of PLP and its dependent enzymes as potent target(s) for the control of this pathogen in the poultry host.

Keywords: *Campylobacter jejuni*, Vitamin B6, flagellar motility

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Asakura H, Momose Y, Ryu CH, Kasuga F, Yamamoto S, Kumagai S*, Igimi S: *Providencia alcalifaciens* causes barrier dysfunction and apoptosis in tissue cell culture: potent role of lipopolysaccharides on diarrheagenicity.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2013;30:1459-66.

Providencia alcalifaciens is a member of the Enterobacteriaceae family that occasionally causes diarrheagenic illness in humans via the intake of contaminated foods. Despite the epidemiological importance of *P. alcalifaciens*, little is known about its pathobiology. Here we report that *P. alcalifaciens* causes barrier dysfunction in Caco-2 cell monolayers and induces apoptosis in calf pulmonary artery endothelial cells. *P. alcalifaciens* infection caused a 30% reduction in transepithelial resistance in Caco-2 cell monolayers, which was greater than that for cells infected with *Shigella flexneri* or non-pathogenic *Escherichia coli*. As with viable bacteria, bacterial lysates treated with heat, benzonase or proteinase, but not with polymixin B, were also involved in the cellular response. TLR4 antibody neutralisation significantly restored the *P. alcalifaciens*-induced transepithelial resistance reduction in Caco-2 cells, suggesting that lipopolysaccharides (LPSs) might play a central role in this cellular response. Western blotting further indicated that *P. alcalifaciens* LPSs reduced occludin levels, whereas LPSs from *Shigella* or *E. coli* did not. Although the viability of Caco-2 cells was not altered significantly, the calf pulmonary artery endothelial cell line was highly sensitive to *P. alcalifaciens* infection. This sensitivity was indeed dependent on LPS, which induced rapid apoptosis. Together, these data show that *P. alcalifaciens* LPSs participate in epithelial barrier dysfunction and endothelial apoptosis. The findings give insight into the LPS-dependent cell signal events affecting diarrheagenicity during infection with *P. alcalifaciens*.

Keywords: *Providencia alcalifaciens*, lipopolysaccharides, epithelial barrier dysfunction

* Japan Food Safety Commission

Asakura H, Taguchi M*, Ekawa T, Yamamoto S, Igimi S: Continued widespread dissemination and increased poultry host fitness of *Campylobacter jejuni* ST-4526 and ST-4253 in Japan.

J Appl Microbiol. 2013;114:1529-38.

Campylobacter jejuni is a major cause of foodborne gastroenteritis. We previously reported the widespread Camp. jejuni sequence type (ST)-4526 in Japan from 2005 to 2006. This study assesses the potential for this

genotype to thrive thereafter. Fifty human *Camp. jejuni* isolates collected in 2010-2011 in Osaka, Japan, were genotyped by multilocus sequence typing (MLST). This approach identified 22 STs and 11 clonal complexes (CCs), including four novel STs. A comparative analysis to the previous data set showed the predominance of CC-21, in which ST-4526 and ST-4253 represented 39 and 63% in each of the two time frames, indicating their continued widespread presence. These two STs belong to close evolutionary lineages and are also isolated from chicken meat. The superior abilities of ST-4526/ST-4253 representatives to colonize chicken gut were demonstrated by co-infections with ST-21, ST-50 and ST-8 representatives. Data provide evidence for the continued widespread of ST-4526/ST-4253 among human clinical isolates in Japan. These STs showed adaptive fitness to chicken. This is the first evidence of the continued thriving of ST-4526/ST-4253 in Japan with their increased *in vivo* fitness. Our findings suggest that poultry mediates the microevolution of this pathogen, thereby enabling these STs to become widespread.

Keywords: *Campylobacter jejuni*, Chicken colonization, Multilocus sequence typing

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與儀健太郎*1, 大城直雅, 松田聖子*1, 佐久川さつき, 松尾敏明*2, 安元健*3：奄美大島・加計呂麻島におけるシガテラ原因魚の毒組成解析.

食品衛生学雑誌 2013;54:385-91.

2008年に鹿児島県奄美群島で発生した魚類摂食に起因する食中毒シガテラの3事例について，原因魚3試料および同時に漁獲された近海魚5種7試料のLC-MS/MSによるシガトキシン類（CTXs）一斉分析の検討を行った．食中毒の原因となったイッテンフエダイ2試料およびバラハタ1試料のすべてからCTX1B，54-deoxyCTX1B，52-epi-54-deoxyCTX1Bが検出されたが，CTX3C類縁体は認められなかった．これら2魚種のCTXs組成比は，それぞれ沖縄海域の両種における組成と共通していた．一方，宮崎県での食中毒原因試料はCTX3C類縁体が主要毒であり，毒組成の違いが示された．また，LC-MS/MSで測定した毒量はマウス毒性試験（MBA）結果（0.1～0.8 MU/g）と同程度であったが，比毒性が明確でない54-deoxyCTX1Bによる総毒力への影響も推察された．一方，MBAで陰性（<0.025 MU/g）を示した近海魚1試料からも微量のCTXsが検出された.

Keywords: シガテラ，シガトキシン，LC-MS/MS

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Suzuki H, Machii K: Effects of Injection Speed of Test Samples on the Mouse Bioassay for Paralytic Shellfish Poisoning Toxins.

Ital J Food Saf. 2013;2:70-3.

The mouse bioassay has been used as the official method for paralytic shellfish poisoning toxins detection in Japan since 1980. However, differences in the results of this assay, when performed by different investigators, have been noted despite the use of the same sample. This study was performed to examine the effect of the injection speed, a hypothetical cause of such differences, on the death time of mice. Speed-controlled injection of the toxin (at 12, 6, 3, and 1.5 mL/min) into mice was performed using a syringe pump, and the death times of mice were measured. No statistically significant differences were found among the groups, even between fast injection (5 s) and very slow injection (40 s), indicating that the injection speed may not be the crucial factor for this assay.

Keywords: mouse bioassay, paralytic shellfish poisoning toxin, injection speed

Suzuki H, Okada Y: Bacterial Translocation in Alymphoplasia (*aly/aly*) Mice.

Folia Biol. 2014;62:9-12.

Bacterial translocation (BTL) is defined as the passage of viable bacteria from the gastrointestinal tract to the organs. This study was to elucidate the roles of Peyer's patches (PPs) and/or mesenteric lymph nodes (MLNs) in BTL. Alymphoplastic mutant mice and phenotypically normal heterozygous mice were dominantly colonized with streptomycin-resistant *Escherichia coli* and BTL was examined. In PP- and MLN-competent mice, BTL to MLNs was detected in 100% of mice, but BTL to organs was rare (25%). On the other hand, in PP- and MLN-deficientmice, BTL to organs was detected in 91% of mice. The results clearly indicate that PPs are not the only site for bacterial entry.

Keywords: bacterial translocation, alymphoplasia mouse, mesenteric lymph node (MLN)

Momose Y, Okada Y, Asakura H, Ekawa T, Masuda K, Matsuoka H[*1], Yokoyama K[*2], Kai A[*2], Saito S[*3], Hiramatsu R[*4], Taguchi M[*5], Ishimura K[*6], Tominaga K[*7], Yahiro S[*8], Fujita M[*9], Igimi S: Evaluation of the culture method NIHSJ-02 alternative to ISO 10272-1:2006 for the detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken: collaborative study.

J AOAC Int. 2013;96:991-7.

For the surveillance of the prevalence of *Campylobacter jejuni* and *Campylobacter coli* in raw chicken products in Japan, a qualitative method, NIHSJ-02, has been developed alternative to ISO 10272-1:2006. A collaborative study revealed that NIHSJ-02 would work well with regard to the selective detection of *C. jejuni* and *C. coli* in chicken, which is usually contaminated with a high background level of non-campylobacters.

Keywords: Standard method, *Campylobacter*, Chicken

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Ohira T[*], Ando R[*], Okada Y, Suzuki H, Saito T[*], Nakazawa T[*], Nishihara K[*], Yamamoto S[*], Nakamura N[*], Tamura K[*]: Sequence of busulfan-induced neural progenitor cell damage in the fetal rat brain.

Exp Toxicol Pathol. 2013;65:523-30.

The sequence of neural progenitor cell (NPC) damage induced in fetal rat brain by transplacental exposure to busulfan, an antineoplastic bifunctional-alkylating agent, on gestational day 13 was examined by immunohistochemical and real-time RT-PCR analyses. Following busulfan treatment, pyknotic NPCs first appeared in the medial layer and then extended to the dorsal layer of the ventricular zone (VZ) of the telencephalon. Pyknotic NPCs that were immunohistochemically positive for cleaved caspase-3, i.e. apoptotic NPCs, began to increase at 24 hours after treatment (HAT), peaked at 48 HAT, and returned to the control levels at 96 HAT. On the other hand, the numbers of phospho-histone H3-positive NPCs, i.e. mitotic NPCs, and of BrdU-positive NPCs, i.e. S-phase cells, decreased in accordance with the increase in the number of apoptotic NPCs. Prior to the peak time of apoptotic NPCs, the numbers of p53- and p21-positive NPCs peaked at 36 HAT. In addition, the expression levels of *p21* and *Puma* (p53-target genes) mRNAs were elevated in real-time RT-PCR analysis. These findings indicated that busulfan not only induced apoptosis through the p53-mediated intrinsic pathway but also inhibited cell proliferation in NPCs, resulting in a reduction of the width of the telencephalon. On the other hand, in spite of up-regulation of *p21* expression, the expression of *cyclin D1*, part of the cell cycle machinery of the G1/S transition, and the expression levels of *Cdc20* and *cyclin B1* which are involved in G2/M transition, showed no changes, giving no possible information of busulfan-induced cell cycle arrest in NPCs.

Keywords: Busulfan, Fetal rat brain, Neural progenitor cell damage

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Okada Y, Ohnuki I[*], Suzuki H, Igimi S: Growth of *Listeria monocytogenes* in refrigerated ready-to-eat foods in Japan.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2013;30:1446-9.

We tested the ability *L. monocytogenes* to grow in a series of Japanese ready-to-eat (RTE) foods, including boiled baby sardine and Japanese pickle, at two different refrigeration temperatures. In RTE foods in which *L. monocytogenes* can grow, growth was significantly higher at 10°C than that at 4°C during their shelf lives and growth patterns varied extensively among the different types of foods. However, growth did not occur at 4°C within the shelf life of certain RTE foods, such as broiled squid. The patterns of growth were varied extensively with different sample types. These results suggest that some types of traditional Japanese RTE foods stored at

10°C may be potential sources of listeriosis. To reduce the risk of food-borne listeriosis, studies to determine the contamination levels in RTE foods and the effects of storage temperature on their shelf lives are needed.
Keywords: *Listeria monocytogenes*, refrigerating, growth

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原田誠也*1，大迫英夫*1，吉岡健太*1，西村浩一*1，清田正憲*1，李天成*2，石井孝司*2，田中智之*3，野田衛：イノシシ，シカおよびブタのE型肝炎ウイルス感染状況調査-熊本県.
病原微生検出情報 2014;35:9-10.

イノシシ，シカおよびブタのE型肝炎ウイルス（HEV）保有状況を把握することを目的として，2006年～2013年に熊本県でと畜されたそれらの獣畜から採取された肝臓，血液・血清，筋肉を対象としたHEVの遺伝子検出をRT-PCR法で，抗HEV抗体保有状況をELISA法で調べた．その結果，イノシシは253頭中17頭（6.7%），シカは63頭中0頭，豚は1,634頭中15頭（0.9%）からHEV遺伝子が検出された．イノシシから検出されたHEVの遺伝子型はG3及びG4であったが，ブタからはG3のみが検出された．系統樹解析の結果，イノシシから検出されたHEVは地域毎に，ブタから検出されたHEVは豚舎毎にクラスターを形成した．ブタの抗HEV IgG抗体の平均保有率は79.0%であり，豚舎間で抗体保有率は0～100%と大きな差がみられた．
Keyword: hepatits E virus, pig, boar

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Nidaira M*, Taira K*, Kato T*, Arakaki E*, Kyan H*, Takara T*, Okano S*, Kuba Y*, Kudaka J*, Noda M: Phylogenetic Analysis of Sapovirus Detected from an Outbreak of Acute Gastroenteritis on Ishigaki Island (Okinawa Prefecture, Japan) in 2012.
Jpn J Infect Dis. 2014;67:141-3.

From December 21 to 25, 2012, an outbreak of acute gastroenteritis occurred among adult residents of a social welfare facility on Ishigaki Island. Ten of the 50 residents of the facility and 3 of the 50 staff members exhibited symptoms of gastroenteritis. To investigate the viral agent, we collected stool samples from 3 of the symptomatic adult residents (age range, 56－75 years). Three SaV strains were detected in the 3 samples by real-time PCR. Based on phylogenetic analysis of partial nucleotide sequences of the capsid and polymerase regions, the strains were classified into GI/2. No Recombination event was detected between the capsid and polymerase regions. The partial nucleotide sequences of the capsid and polymerase regions of the present strains showed nucleotide and amino-acid identity levels and *p*-distance values were >98%, >99%, and <0.02, respectively, compared to those of GI/2 reference strains isolated in Japan, Brazil, and Hungary since 2008. This is the first report of detection of SaV in Okinawa Prefecture in Japan.
Keywords: sapovirus, outbreak, social welfare facility

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Ohnishi T, Furusawa H, Yoshinari T, Yamazaki A, Horikawa K*, Kamata Y, Sugita-Konishi Y: Electron microscopic study of *Kudoa septempunctata* infecting *Paralichthys olivaceus* (Olive Flounder).
Jpn J Infect Dis. 2013;66:348-50.

Kudoa septempunctata is a myxosporean parasite of *Paralichthys olivaceus* (olive flounder) that causes more than 50 cases of foodborne illness in Japan each year. For quantitatively assessing the presence of *K. septempunctata* spores in the causative fish at food poisoning outbreaks, both a direct observation method using microscopy and a quantitative real-time PCR (qRT-PCR) method are officially accepted in Japan. However, lower correlations have been often noticed between the number of spores counted using the direct observation method and the DNA amount determined using the qRT-PCR method. To elucidate the cause of this discrepancy, we observed muscle tissues of infected olive flounders with *K. septempunctata* by transmission electron microscopy. The images demonstrated unsynchronized development of *K. septempunctata* spores in plasmodia found within myofibers; in other words, the plasmodium contained not only developed spores with completed shell valves but also developing spores (sporoblasts) composed of spore-forming cells without shell valves. Furthermore, the ratio between developed spores and sporoblasts varied at different parts of muscles. The direct microscopic observation method could count developed spores, whereas the qRT-PCR method could quantify

the amount of not only spores but also sporoblastic cells regardless of the cellular development and differentiation. Considering that the food toxicity caused by *K. septempunctata* is induced by viable spores passing through the gastric environment, the direct observation method counting only developed spores is better than the qRT-PCR method for assessing the cause of foodborne illness at the outbreak as well as the risk of human illness in monitoring surveys of aquacultured or natural-water fish.

Keywords: Parasite, Food-borne disease, *Kudoa*

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Ohnishi T, Oyama R, Furusawa H, Ohba N, Kamata Y, Sugita-Konishi Y: *Kudoa septempunctata* was recognized by Toll-like receptor 2 on a RAW 264 macrophage-like cell line.

Food Additives & Contaminants. 2013;30:1365-69.

Kudoa septempunctata is a myxosporean parasite that infects *Paralichthys olivaceus* (olive flounder). Previously, we reported that the consumption of raw *P. olivaceus* meat containing a high concentration of *K. septempunctata* spores induces transient but severe diarrhoea and emesis. In this study, we investigated the cytokine production of mouse macrophage-like RAW 264 cells stimulated with *K. septempunctata*. When the RAW 264 cells were incubated with the spores of *K. septempunctata* for 24 h, they secreted tumour necrosis factor α (TNF-α) and several chemokines, such as IP-10, MIP-1β, and MIP-2. The secretion of TNF-α was induced in a dose-dependent manner in a bioassay using L929 cells and mouse TNF-α-specific enzyme-linked immunosorbent assay (ELISA). To identify the macrophage receptor of *K. septempunctata*, activation of HEK 293 cells expressing one of the Toll-like receptors (TLR) was measured using an NF-κB-dependent reporter assay. TLR2-expressing HEK 293 cells were strongly activated following stimulation with the spores. These results suggested that *K. septempunctata* was recognised by TLR2 on the macrophages, which were then activated and produced TNF-α.

Keywords: *Kudoa*, Toll-like receptor, Food-borne disease

Ying-Chun Li*, Sato H*, Tanaka S*, Ohnishi T,

Kamata Y, Sugita-Konishi Y: Characterization of the ribosomal RNA gene of *Kudoa neothunni* (Myxosporea: Multivalvulida) in tunas (*Thunnus* spp.) and *Kudoa scomberi* n. sp. in a chub mackerel (*Scomber japonicus*).

Parasitol Res. 2013;112:1991-2003.

Kudoa neothunni is the first described *Kudoa* species having six shell valves and polar capsules, previously assigned to the genus *Hexacapsula* Arai and Matsumoto, 1953. Since its genetic analyses remain to be conducted, the present study characterizes the ribosomal RNA gene (rDNA) using two isolates from a yellowfin tuna (*Thunnus albacares*) with post-harvest myoliquefaction and a northern bluefin tuna (*Thunnus thynnus*) without tissue degradation. Spores of the two isolates localized in the myofiber of trunk muscles, forming pseudocysts, and showed typical morphology of *K. neothunni* with six equal-sized shell valves radially arranged in apical view: spores (n = 15) measuring 9.5-11.4 μm in width, 7.3-8.6 μm in suture width, 8.9-10.9 μm in thickness, and 7.3-7.7 μm in length; and polar capsules measuring 3.6-4.1 μm by 1.8-2.3 μm. In lateral view, the spores were pyramidal in shape without apical protrusions. Their 18S and 5.8S rDNA sequences were essentially identical, but variations in the ITS1 (62.4 % similarity across 757-bp length), ITS2 (66.9 % similarity across 599-bp length), and 28S (99.0 % similarity across 2,245-bp length) rDNA regions existed between the two isolates. On phylogenetic trees based on the 18S or 28S rDNA sequence, *K. neothunni* formed a clade with *Kudoa* spp. with more than four shell valves and polar capsules, particularly *K. grammatorcyni* and *K. scomberomori*. Semiquadrate spores of a kudoid species with four shell valves and polar capsules were detected from minute cysts (0.30-0.75 mm by 0.20-0.40 mm) embedded in the trunk muscle of a chub mackerel (*Scomber japonicus*) fished in the Sea of Japan. Morphologically, it resembled *K. caudata* described from a chub mackerel fished in the southeastern Pacific Ocean off Peru; however, it lacked filamentous projections on the shell valves of spores. Additionally, it morphologically resembled *K. thunni* described from a yellowfin tuna also fished in the Pacific Ocean; spores (n = 30) measuring 8.2-10.5 μm in width, 7.0-8.8 μm in thickness, and 6.1-6.8 μm in length; and polar capsule measuring 2.5-3.4 μm by 1.3-2.0 μm. The similarities of

the 18S and 28S rDNA sequences between these two species were 98.5 % and 96.3 %, respectively. Simultaneously, the dimensions of cysts in the trunk muscle formed by *K. thunni* are clearly larger than those of the present species from a chub mackerel: 1.3-2.0 mm by 1.1-1.4 mm（n＝14）vs. 0.30-0.75 mm by 0.20-0.40 mm（n＝7）, respectively. Thus, *Kudoa scomberi* n. sp. is proposed for this multivalvulid species found in the chub mackerel.

Keywords: *Kudoa*, Parasite, Food-borne disease

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大西貴弘，古沢博子，佐古浩*1，乙竹充*1，福田譲*2，吉成知也，山﨑朗子，鎌田洋一，小西良子：クドア食中毒および*Kudoa septempunctata*の季節による特徴．
日本食品微生物学会雑誌 2013;30:125-31.

Our surveillance indicated the food-borne disease associated with *Kudoa septempunctata* has occurred on summer season. To elucidate the reasons of that, we investigated the temperature effect of food-borne disease associated with *K. septempunctata*. We continually purchased olive flounders in the same lot from the fish farm that was infected with *K. septempunctata* partly and determined the number of spores in olive flounder muscle. Both the positive ratio of *K. septempunctata* in olive flounder and the number of spores did not show the seasonal change from January to August. We discovered that the temperature of seawater in summer season was over 20℃.However, the positive ratio of *K. septempunctata*, the number of spores and the toxicity of *K. septempunctata* were not affected by high temperature of seawater. These results demonstrated that the temperature rise of sea water was not a reason why the frequency of the food-borne disease increases in summer season.

Keywords: クドア，寄生虫，食中毒

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Yoshinari T, Sakuda S*1, Watanabe M, Kamata Y*2, Ohnishi T, Sugita-Konishi Y*3: New Metabolic Pathway for Converting Blasticidin S in *Aspergillus flavus* and Inhibitory Activity of Aflatoxin Production by Blasticidin S Metabolites.
J Agric Food Chem. 2013;61:7925-31.

Blasticidin S（BcS）, a protein synthesis inhibitor, inhibits aflatoxin production of *Aspergillus flavus* without affecting fungal growth. Analysis of metabolites in BcS-treated *A. flavus* using quadrupole time-of-flight liquid chromatography-mass spectrometry showed that BcS was metabolized into a novel metabolite, *N*-acetyldeaminohydroxyblasticidin S（AcDahBcS）.

Conversion of BcS to AcDahBcS via deaminohydroxyblasticidin S（DahBcS）or *N*-acetylblasticidin S（AcBcS）was observed in in vivo and in vitro *A. flavus* systems. BcS and AcBcS inhibited the growth of *Aspergillus niger* strongly and weakly, respectively, but DahBcS and AcDahBcS did not inhibit its growth. On the other hand, DahBcS sustained the inhibition of aflatoxin production whereas AcBcS and AcDahBcS did not. These results suggest that the free amino group at C-13 of blasticidin S and DahBcS may be important for the inhibitory activity of aflatoxin production.

Keywords: Aflatoxin, Blasticidin S, Metabolome

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Yoshinari T, Tanaka T*1, Ishikuro E*2, Horie M*3, Nagayama T*4, Nakajima M*5, Naito S*6, Ohnishi T, Sugita-Konishi Y: Inter-laboratory study of an LC-MS/MS method for simultaneous determination of fumonisin B_1, B_2 and B_3 in corn.
Shokuhin Eiseigaku Zasshi 2013;54:266-76.

To validate an LC-MS/MS method using a strong anion exchange cartridge for simultaneous determination of fumonisin B_1, B_2 and B_3 in corn, an inter-laboratory study was performed in 9 laboratories using one fumonisin-negative corn sample, three spiked corn samples and two naturally contaminated corn samples. The recoveries were in the ranges of 79.7-87.2% for FB_1, 78.6-103.2% for FB_2 and 80.1-92.8% for FB_3. Surveillance for fumonisins in corn grits was performed using the validated method. These results indicated that the method for simultaneous determination of FB_1, FB_2 and FB_3 in corn was successfully developed and validated.

Keywords: Fumonisin, Inter-laboratory study, Corn

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Yoshinari T, Sakuda S*1, Furihata K*1, Furusawa H, Ohnishi T, Sugita-Konishi Y*2, Ishizaki N*2, Terajima J: Structural determination of a nivalenol glucoside and development of an analytical method for the simultaneous determination of nivalenol and deoxynivalenol, and their glucosides, in wheat.
J Agric Food Chem. 2014;62:1174-80.

Trichothecene mycotoxins such as nivalenol (NIV) and deoxynivalenol (DON) frequently contaminate foodstuffs. Recently, several trichothecene glucosides have been found in trichothecene-contaminated foods, and information about their chemistry, toxicity, and occurrence is required. In this study, a glucoside of NIV was isolated from NIV-contaminated wheat and was identified as nivalenol-3-*O*-*β*-D-glucopyranoside (N3G). Analytical methods using an immunoaffinity column have been developed for the simultaneous determination of NIV, N3G, DON, and deoxynivalenol-3-*O*-*β*-D-glucopyranoside in wheat. The methods were validated in a single laboratory, and recovery from wheat samples spiked at four levels ranged between 86.4 and 103.5%. These mycotoxins in contaminated wheat samples were quantitated by the validated method. N3G was detected in the NIV-contaminated wheat, and the percentage of N3G to NIV ranged from 12 to 27%. This result indicates that the analytical method developed in this study is useful for obtaining data concerning the state and level of food contamination by NIV, DON, and their glucosides.
Keywords: Nivalenol, Glucoside, Wheat

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Sakuma H, Watanabe Y, Furusawa H, Yoshinari T, Akashi H*1, Kawakami H*2, Saito S*3, Sugita-Konishi Y: Estimated dietary exposure to mycotoxins after taking into account the cooking of staple foods in Japan.
Toxins (Basel). 2013;21:1032-42.

This study examined the retention of aflatoxin (AFL) and ochratoxin A (OTA) during the cooking of rice and pasta. AFL was retained at 83%-89% the initial level after the cooking of steamed rice. In pasta noodles, more than 60% of the OTA was retained. These results show that AFL and OTA are relatively stable during the cooking process, suggesting that a major reduction in the exposure to these mycotoxins cannot be expected to occur by cooking rice and pasta. The estimated exposure assessment at the high consumer level (95th percentile) and the mycotoxin contamination level determined by taking into account these reductions in the present study should be useful for the establishment of practical regulations for mycotoxins in staple foods.
Keywords: Ochratoxin A, Exposure

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Sakamoto N*, Tsuyuki R*, Yoshinari T, Usuma J*, Furukawa T*, Nagasawa H*, Sakuda S*: Correlation of ATP citrate lyase and acetyl CoA levels with trichothecene production in *Fusarium graminearum*.
Toxins (Basel). 2013;5:2258-69.

The correlation of ATP citrate lyase (ACL) and acetyl CoA levels with trichothecene production in *Fusarium graminearum* was investigated using an inhibitor (precocene II) and an enhancer (cobalt chloride) of trichothecene production by changing carbon sources in liquid medium. The results suggest that ACL expression is activated in the presence of sucrose and that acetyl CoA produced by the increased ALC level may be used for trichothecene production in the fungus. These findings also suggest that sucrose is important for the action of cobalt chloride in activating trichothecene production and that precocene II may affect a step down-stream of the target of cobalt chloride.
Keywords: Deoxynivalenol, ATP citrate lyase

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Matsutani S: Evolution of the B-block binding subunit of TFIIIC that binds to the internal promoter for RNA polymerase III.

Int J Evol Biol. 2014;2014:609865.

Eukaryotic RNA polymerase III transcribes tRNA genes, and this requires the transcription factor TFIIIC. Promoters are within genes, with which the B-block binding subunit of TFIIIC associates to initiate transcription. The binding subunits are more than 1000 amino acids in length in various eukaryotic species. There are four regions with conserved sequence similarities in the subunits. The helix-turn-helix motif is included in one of these regions and has been characterized as the B-block_TFIIIC family in the Pfam database. In the NCBI and EMBL translated protein databases, there are archaeal proteins (approximately 100 amino acids in length) referred to as B-block binding subunits. Most of them contain a B-block_TFIIIC motif. DELTA-BLAST searches using these archaeal proteins as queries showed significant multiple blast hits for many eukaryotic B-block binding subunits on the same proteins. This result suggests that eukaryotic B-block binding subunits were constituted by repeating a small unit of B-block_TFIIIC over a long evolutionary period. Bacterial proteins have also been annotated as B-block binding subunits in the databases. Here, some of them were confirmed to have significant similarities to B-block_TFIIIC. These results may imply that part of the RNAP III transcription machinery existed in the common ancestry of prokaryotes and eukaryotes.

Keywords: RNA polymerase III transcription machinery, Evolution, Common ancestry of prokaryotes and eukaryotes

Hara-Kudo Y, Kumagai S[*1], Konuma H[*2], Miwa N[*3], Masuda T[*4], Ozawa K[*5], Nishina T[*3]:

Decontamination of *Vibrio parahaemolyticus* in fish by washing with hygienic seawater and impacts of the high level contamination in the gills and viscera.

J Vet Med Sci. 2013;75:589-96.

The effect of washing in *Vibrio parahaemolyticus* contaminated and hygienic seawater on fish, and the frequency and level of natural *V. parahaemolyticus* contamination in fish were investigated. In the first experiment, live horse mackerel was experimentally kept in seawater artificially contaminated with *V. parahaemolyticus*. After washing in contaminated and hygienic seawater, the contamination in fish was quantitatively analyzed. Washing fish in the seawater contaminated with *V. parahaemolyticus* increases the contamination level on the surface and in the gills of the fish. Washing in hygienic seawater was effective in reducing the contamination in fish and cooking board surfaces, but not in the gills or viscera. In the second experiment, natural *V. parahaemolyticus* contamination in various fish caught by us was analyzed. *V. parahaemolyticus* was detected in 6 of 28 gill samples and 10 of 28 viscera samples of naturally contaminated fish. The means of *V. parahaemolyticus* level on gills were 3.3 and 3.9 log cfu/g, and those in viscera were 2.6 and 4.4 log cfu/g by culture method and a real-time PCR assay, respectively. These results indicate that the gills and viscera are able to spread the pathogens to fish meat as well as fish surface contamination by washing in the contaminated seawater. Washing with hygienic seawater and control of contamination from gills and viscera are critically important to prevent *V. parahaemolyticus* infections.

Keywords: fish, *Vibrio parahaemolyticus*, washing

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Hara-Kudo Y, Konuma H[*1], Kamata K, Miyahara M, Takatori K[*2], Onoue Y[*3], Sugita-Konishi Y, Ohnishi T:

Prevalence of main foodborne pathogens in retail food under the National Food Surveillance System in Japan.

Food Addit Contam Part A, Chem Anal Control Expo Risk Assess. 2013;30(8):1450-58.

The National Food Surveillance System in Japan was formed in 1998 to monitor contamination of retail foods with bacterial pathogens. Approximately 2,000-3,000 samples were tested annually, and the data from food categories which more than 400 samples were collected during 1998-2008 were analyzed. With regard to meat, the frequency of positive samples for *Salmonella* in chicken for raw consumption and ground chicken were 12.7% and 33.5%, respectively. Moreover, Shiga toxin-producing *Escherichia coli* (STEC O157) was found in ground meat, organ meat and processed meat, although at low frequency (0.1%). The prevalence of *Campylobacter jejuni/coli* was 13.3% and 20.9% in

chicken for raw consumption and ground chicken, respectively. In vegetables and fruits, *Salmonella* was detected in cucumber, lettuce, sprout and tomato samples at a frequency of around 0.1%-0.2%. With regard to seafood, *Salmonella* was found in 0.5% of oysters for raw consumption. Seafood was not contaminated with STEC O157 or *Shigella*. Serotype Infantis was the most frequently detected serotype of *Salmonella* in seafood, followed by the serotypes Typhimurium, Schwarzengrund and Manhattan. In ground chicken, 72.2% of the strains were identified as the serotype Infantis. *E. coli*, as an indicator of food hygiene, was detected in all food categories. The results show the prevalence of the above-mentioned pathogens in the retail food supplied in Japan; further, they indicate that consumption of raw food carries the risk of contracting foodborne infections.

Keywords: *Salmonella*, Shiga toxin-producing *Escherichia coli O157, Campylobacter*

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Wang L*1, Wakushima M*1, Aota T*1, Yoshida Y*1, Kita T*2, Maehara T*3, Ogasawara J*4, Choi C*5, Kamata Y, Hara-Kudo Y, Nishikawa Y*1: Specific properties of enteropathogenic *Escherichia coli* strains isolated from diarrheal patients: comparison between strains from foods, and fecal specimens from cattle, swine and healthy carriers in Osaka City, Japan.

Appl Environ Microbiol. 2013;79:1232-40.

For exhaustive detection of diarrheagenic *Escherichia coli*, we previously developed a colony-hybridization method using hydrophobic grid-membrane filters in combination with multiplex real-time PCR. To assess the role of domestic animals as the source of atypical enteropathogenic *E. coli* (aEPEC), a total of 679 samples (333 from foods, fecal samples from 227 domestic animals, and 119 from healthy people) were examined. Combining 48 strains previously isolated from patients and carriers, 159 aEPEC strains were classified by phylogroup, virulence profile, and intimin typing. Phylogroup B1 was significantly more prevalent among aEPEC from patients (50%) and bovine samples (79%) than from healthy carriers (16%) and swine strains (23%), respectively. Intimin type β1 was predominant in phylogroup B1; B1-β1 strains comprised 26% of bovine strains and 25% of patient strains. The virulence profile groups Ia and Ib were also observed more frequently among bovine strains than among porcine strains. Similarly, virulence group Ia was detected more frequently among patient strains than strains of healthy carriers. A total of 85 strains belonged to virulence group I, and 63 of these strains (74%) belonged to phylogroup B1. The present study suggests that the etiologically important aEPEC in diarrheal patients could be distinguished from aEPEC strains indigenous to humans based on type, such as B1, Ia, and β1/γ1, which are shared with bovine strains, while the aEPEC strains in healthy humans are different, and some of these were also present in porcine samples.

Keywords: Enteropathogenic *Escherichia coli*, diarrheal patients, Osaka City

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Hasegawa A*1, Hara-Kudo Y, Ogata K*2, Saito S*3, Sugita-Konishi Y, Kumagai S*4: Differences in the stress tolerances of *Vibrio parahaemolyticus* strains due to their source and harboring of virulence genes.

J Food Prot. 2013;76:1456-62.

To investigate the diversity of stress tolerance levels in *Vibrio parahaemolyticus*, 200 *V. parahaemolyticus* strains isolated from various coastal environments, seafood and patients were exposed to acid, low osmolality, freezing/thawing, and heat stresses. Tolerance against acid stress was higher in the virulent (*tdh*- and/or *trh*-positive) strains than in the avirulent (*tdh*- and *trh*-negative) strains. Tolerance against low osmolality, freezing/thawing, and heat stresses was higher in the clinical strains of *tdh*- and/or *trh*-positive *V. parahaemolyticus* than in the coastal environment/seafood-originated strains of *tdh*- and/or

trh-positive *V. parahaemolyticus*. Tolerance against acid stress was higher in the strains isolated from coastal seawater at ≤15°C than in the strains isolated at ≥20°C. Tolerance against heat stress was higher in the avirulent strains than the virulent strains and in the strains isolated from coastal seawater at ≥20°C than the strains isolated from coastal seawater at ≤15°C. Therefore, this study demonstrated that the diversity of stress tolerance levels in *V. parahaemolyticus* strains depended on their source and whether they harbored virulence genes. In particular, there was significantly greater tolerance against acid in the virulence gene-harboring strains and strains isolated from low temperature seawater. Because the stress tolerances of *V. parahaemolyticus* have direct influences for the survival in environment and food, it is important for the prevention of foodborne infection to control the stress tolerant strains.

Keywords: acid, osmolality, *Vibrio parahaemolyticus*

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Kobayashi N, Lee K*1, Yamazaki A, Saito S*2, Furukawa I*3, Kono T*4, Maeda E*5, Isobe J*6, Sugita-Konishi Y, Hara-Kudo Y: Virulence gene profiles and population genetic analysis for exploration of pathogenic serogroups of Shiga toxin-producing *Escherichia coli*.

J Clin Microbiol. 2013;51:4022-28.

Infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is a serious public health concern, causing severe diarrhea and hemolytic-uremic syndrome. Patient symptoms are varied among STEC strains, potentially implying the presence of additional markers for STEC virulence other than Stx. To reveal the genotypic traits responsible for STEC virulence, we investigated 282 strains of various serogroups for the presence of 17 major virulence genes: *stx1*, *stx2a*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, *eae*, *tir*, *espB*, *espD*, *iha*, *saa*, *subA*, *ehxA*, *espP*, *katP*, and *stcE*. Next, we examined the prevalence of virulence genes according to the seropathotypes in which serotypes were classified into 5 groups (A through E) based on the reported frequencies in human illness, as well as known associations with outbreaks and with severe disease. Our result demonstrated that the harboring of both *katP* and *stcE* in STEC, in addition to the genes located in locus of enterocyte effacement (LEE), including *eae*, *tir*, *espB*, and *espD*, may represent the most pathogenic genotype of STECs. A population structure analysis of the profile of virulence genes statistically supported the pathogenic genotype and, furthermore, revealed that there are potentially higher pathogenic serogroups than previously thought. A segment of serogroups O26, O145, and O165 strains may have high a virulence equivalent to serogroup O157. Several serogroups, including the serogroups O14, O16, O45, O63, O74, 119, O128, and O untypable, also may be potentially pathogenic, although rarely in humans.

Keywords: Virulence gene, genetic analysis, Shiga toxin-producing *Escherichia coli*

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Jones JL*, Benner RA*, DePaola A*, Hara-Kudo Y: *Vibrio* densities in the intestinal contents of finfish from coastal Alabama.

Agric Food Anal Bacteriol. 2013;3:186-94.

Vibrio vulnificus, *Vibrio parahaemolyticus* and *Vibrio cholerae*, are human pathogens ubiquitous in the marine and estuarine environments. Correlation between abundance of these pathogens and increased water temperature is well established, but little is known about their environmental persistence. Previous studies have identified finfish intestines as a potential reservoir of *V. vulnificus*; however, the data for other pathogenic *Vibrios* is sparse. The objective of this study was to simultaneously enumerate the three *Vibrio* spp. of greatest human health concern in finfish intestines collected from the Gulf of Mexico and estuarine sites in Mobile Bay, Alabama. *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* levels in fish

intestines were enumerated using a microtiter plate most probable number (MPN)-real-time polymerase chain reaction (Rti-PCR) method. Of the 21 finfish samples examined, 62%, 76%, and 19% had detectable levels (≥3 MPN/g) of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*, respectively. The highest levels of *V. vulnificus* (7.63 log MPN/g), *V. parahaemolyticus* (7.97 log MPN/g), and *V. cholerae* (4.58 log MPN/g) were found in sheepshead (*Archosargus probatocephalus*) collected from estuarine sites. There was a greater detection frequency of all three organisms in the estuarine samples, compared to the Gulf of Mexico samples; *V. cholerae* was only detected in the estuarine samples. Additionally, the levels of *V. vulnificus* and *V. parahaemolyticus* were significantly higher in the estuarine samples. This is the first report for simultaneous enumeration of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in their environmental reservoir of finfish intestines.

Keywords: *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae*

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早川亮太[*1]，小林直樹，加藤登[*1]，工藤由起子，荒木惠美子[*2]：日本産海産魚におけるヒスタミン生成魚種および凍結保存によるヒスタミン生成の低減の検討.

食品衛生学雑誌 2013;54(6):402-9.

日本産海産魚におけるヒスタミンが生成される魚種を明らかにするために，ヒスタミン生成モデルを構築し検討したところ，73魚種中35種においてヒスタミン生成が認められた．また，凍結がヒスタミン生成に及ぼす影響を検討したところ，-45℃で1ヵ月の凍結保存にてヒスタミン生成が低下することが判明した．さらに，凍結しても生残し，ヒスタミンを生成する菌として*P. damselae*および*P. iliopiscarium*が分離された．本研究の結果から，日本で流通する多種の日本産海産魚はヒスタミン食中毒の原因となる可能性があることが明らかになった．また，ヒスタミン生成菌の生残はあるものの水産食品製造に凍結原料を用いることによってヒスタミン生成を低減させることが考えられた．今後，ヒスタミン食中毒の予防のために，さらに凍結温度および凍結時間を詳細に解析することが必要であると思われる．

Keywords: Japanese marine fish, histamine forming bacterium, frozen storage

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Kikuchi Y, Ohnishi T, Furusawa H, Kawai T[*1], Fukuda Y[*2], Yokoyama H[*3], Sugita-Konishi Y: ELISA Detection of *Kudoa septempunctata* in Raw *Paralichthys olivaceus* (Olive Flounder) using a Chicken Anti-*Kudoa* Antiserum.

Biocontrol Sci. 2013;18:193-7.

Kudoa septempunctata is the causative agent of a foodborne disease associated with the consumption of raw *Paralichthys olivaceus* (olive flounder). Chickens were used to establish specific antibodies against *K. septempunctata* spores. A specific antiserum, CS#3, raised against sonicated spores, also recognized intact spores. The CS#3 antiserum showed high titers for sonicated and intact *K. septempunctata* spores and was suitable for both ELISA and immunohistochemical staining. Using homogenated raw olive flounder meat, the ELISA system detected more than 5.0×105 spores in 1 g of tissue, which was consistent with the number determined by microscopic examination. The preparation of rapid detection kits for *K. septempunctata* spores in *P. olivaceus* muscle tissue using immunochromatography with CS#3 antiserum should be useful for preventing the foodborne disease in the field.

Keywords: *Kudoa septempunctata*, *Paralichthys olivaceus*, Chicken serum

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Tamura C, Nakamura M[*1], Furusawa H, Kadota T[*2], Kamata Y, Nishijima M[*3], Itoh S[*4], Sugita-Konishi Y: Formulation of a pectin gel that efficiently traps mycotoxin deoxynivalenol and reduces its bioavailability.

Carbohydr Polym. 2013;93:747-52.

We aimed to develop a new food-processing approach using pectin to reduce gastrointestinal absorption of mycotoxins. When Ca2+ is added to low-methoxyl pectin, a gel resembling an egg box-like structure forms that is able to trap certain molecules. We examined whether or not low-methoxyl amidated pectin (LMA) and low-methoxyl non-amidated pectin

（LMNA）trapped the mycotoxin deoxynivalenol（DON）after being ingested. We first determined the trapping effects of LMA and LMNA on DON in vitro under conditions similar to those in the human stomach, with results showing that LMA gel trapped DON to a greater extent than the LMNA gel. We then performed in vivo experiments and demonstrated that the LMA gel containing DON reduced DON's absorption from the gastrointestinal tract. This new food-processing technique holds great promise for reducing the bioavailability of DON in contaminated food and may be useful in mitigating the effects of other mycotoxins.
Keywords: Low-methoxyl amidated pectin, Low-methoxyl non-amidated pectin, Deoxynivalenol

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Wu W*1, Bates MA*2, Bursian SJ*3,4, Flannery BM*2,3, Sugita-Konishi Y, Watanabe M, Zhang H*1, Pestka HJ*2,3,5: Comparison of Emetic Potencies of the 8-Ketotrichothecenes Deoxynivalenol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Fusarenon X and Nivalenol.
Toxicol Sci. 2013;131:279-91.

We compared potencies of DON, 15-acetyldeoxynivalenol（15-ADON）, 3-acetyldeoxynivalenol（3-ADON）, fusarenon X（FX）, and nivalenol（NIV）in the mink emesis model following intraperitoneal（ip）and oral administration. All five congeners dose-dependently induced emesis by both administration methods. The effective doses resulting in emetic events in 50% of the animals for ip exposure to DON, 15-ADON, 3-ADON, FX, and NIV were 80, 170, 180, 70, and 60 μg/kg bw, respectively, and for oral exposure, they were 30, 40, 290, 30, and 250 μg/kg bw, respectively. The emetic potency of DON determined here was comparable to that reported in analogous studies conducted in pigs and dogs, suggesting that the mink is a suitable small animal model for investigating acute trichothecene toxicity. The use of a mouse pica model, based on the consumption of kaolin, was also evaluated as a possible surrogate for studying emesis but was found unsuitable. From a public health perspective, comparative emetic potency data derived from small animal models such as the mink should be useful for establishing toxic equivalency factors for DON and other trichothecenes.
Keywords: emesis, trichothecene, vomitoxin

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青木佳代*1，石川和彦*1，林賢一*1，斉藤守弘*2，小西良子，渡辺麻衣子，鎌田洋一：シカ肉中の*Sarcosystis*が原因として疑われた有症苦情.
日本食品微生物学会雑誌 2013;30:28-32.

平成23年12月２日に滋賀県内の飲食店で食事をしたグループの中に，食中毒様症状を呈している者が複数名いるとの連絡があった．調査を行ったところ，１グループの18 名中４名が食後５時間から16時間後に下痢や嘔吐などの食中毒様症状を呈していることが判明した．すべての検体で，既知の食中毒原因菌およびノロウイルスは検出されなかった．患者便を対象に，*Sarcocystis*属の遺伝子の増幅を試みたが，確認できなかった．シカ肉より抽出したDNAから定性PCRを行ったところ，３ブロックすべてから，約1,100 bpの位置に*Sarcocystis*属の遺伝子の増幅を確認し，PCR 陽性となった．光学顕微鏡下でシストおよびブラディゾイトを探索したところ，シストおよびブラディゾイトを検出することができた．光学顕微鏡による生鮮シストおよび組織標本の形態的特徴から，*S. sybillensis*，*S. wapiti*および新種と推察される*Sarcocystis*属のシストと同定された．*S. fayeri*の15 kDa蛋白質に対するウサギ抗血清による免疫組織化学染色を行ったところ，シカ肉中の*S. sybillensis*および*S. wapiti*のブラディゾイトが染色され，*S. fayeri*と同様の病原性を担っている15 kDa蛋白質の存在が確認された．肝炎ウイルスや*Sarcocystis*属を含めた寄生虫の感染防止および食肉としての安全性を保つためには，肉の生食を避け，十分に加熱調理することが重要であると考える．
Keywords: *Sarcocystis*, deer meat, food poisoning

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Oshikata C[*1], Tsurikisawa N[*1], Saito A[*1], Watanabe M, Kamata Y, Tanaka M[*2], Tsuburai T[*1], Mitomi H[*1], Takatori K[*2], Yasueda H, Akiyama K: Fatal pneumonia caused by Penicillium digitatum: a case report.

BMC Pul Med. 2013;13:16.

Penicillium digitatum is a plant pathogen that commonly causes a postharvest fungal disease of citrus called green mould; it very rarely causes systemic mycosis in humans. A cavity was found in the left upper lung on routine chest X-ray in a 78-year-old undernourished male who had been diagnosed at age 66 with bronchial asthma and pulmonary emphysema. The patient was treated over a period of months with itraconazole, micafungin, voriconazole, amphotericin B, and antibacterials. However, the cavity enlarged, the pleural effusion increased, and the patient began producing purulent sputum. He died from progressive renal failure. From sputum culture only one fungus was isolated repeatedly on potato-dextrose agar in large quantities. This fungus was confirmed to be P. digitatum by molecular identification. To our knowledge, this is the first report of pulmonary infection with P. digitatum. In his case, antimycotics were ineffective in treating the lung involvement. Although human infection with P. digitatum is considered rare, it appears that this organism can be very virulent and resistant to antimycotics.

Keywords: *Penicillium digitatum*, Immunocompromised host, Pulmonary emphysema

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原田誠也[*1]，古川真斗[*1]，徳岡英亮[*1]，松本一俊[*2]，八尋俊輔[*3]，宮坂次郎[*4]，斉藤守弘[*5]，鎌田洋一，渡辺麻衣子，入倉大祐，松本博[*1]，小西良子：馬肉中に含まれる住肉胞子虫の危害性消失条件の検討による生食用馬肉を共通食とする食中毒事例の発生防止対策に関する研究.

食品衛生学雑誌 2013;54:198-203.

熊本県では，馬刺しを共通食とする原因不明の一過性嘔吐下痢症事例が最近３年間で毎年27件以上発生していた．同事例の原因は*Sarcocystis fayeri*住肉胞子虫で，本研究では一定時間の冷凍処理で住肉胞子虫のシストがペプシンにより消化されその毒性を失うことを見いだした．同胞子虫シストを含んだ馬肉を-20℃で48時間以上冷凍したところ，シスト由来の毒性タンパク質の消失も確認された．本研究で確立した冷凍条件を用いての冷凍処理の普及により，平成23年10月以降，馬刺しが原因と考えられる食中毒の発生報告はなく，この冷凍処理基準が，馬刺しによる食中毒防止対策として有効であることが示唆された．

Keywords: *Sarcocystis fayeri*，冷凍処理，馬肉

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Watanabe M, Yonezawa T[*], Sugita-Konishi Y, Kamata Y: Utility of phylotoxigenic relationships among trichothecene-producing *Fusarium* species to the prediction about the potential mycotoxin-productivity.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2013;30:1370-81.

There is a need to understand the mechanisms of mycotoxin production by *Fusarium* species and to predict which produce mycotoxins. In this study, the *Fusarium* phylogenetic tree was first inferred among trichothecene producers and related species. We reconstructed the maximum likelihood (ML) tree based on the combined data from nucleotide sequences of several genetic regions. Second, based on this tree topology, the ancestral states of the producing potential of TriA and TriB, ZEN, MON, BEA and ENN were reconstructed using the maximum parsimony method based on the observed production by extant species as reported in the literature. Finally, the species having the potential to produce each of these six mycotoxins was predicted on the basis of the parsimonious analysis. The parsimony reconstruction suggested that the potential for producing MON and ZEN was gained or lost only once, and that the producing potential for TriA and TriB, BEA and ENN was repeatedly gained and lost during the evolutionary history of the *Fusarium* species analysed in this study. The results showed the possibility that several species, about which reports were scarce with regard to mycotoxin production, have the potential to produce one or more

of the six evaluated in this study. The phylogenetic information therefore helps one to predict the mycotoxin-producing potential by Fusarium species.
Keywords: *Fusarium*, phylotoxigenic relationship, mycotoxin production

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Kamata Y, Saito M[*1], Irikura D, Yahata Y[*2], Ohnishi T, Bessho T[*3], Inui T[*3], Watanabe M, Sugita-Konishi Y: A Toxin Isolated from *Sarcocystis fayeri* Following the Consumption of Raw Horsemeat Causes Food Poisoning.
J Food Prot. 2014;77:814-9.

Food poisoning has been reported after the consumption of raw horsemeat in Japan. Diarrhea with a short incubation period is a common symptom in such cases of food poisoning. Cysts found in horsemeat ingested by patients have been identified as *Sarcocystis fayeri* based on morphological and genetic evaluation and findings from experimental feeding of cysts to dogs, which resulted in the excretion of sporocysts. The extracts of the horsemeat containing the cysts produced a positive enterotoxic response in the rabbit ileal loop test. Intravenous injection of a 15-kDa protein isolated from the cysts induced diarrhea and lethal toxicity in rabbits, and the protein produced enterotoxicity in the ileal loop test as did the extracts of the horsemeat containing the cysts. The partial amino acid sequence of the 15-kDa protein was homologous to the actin-depolymerizing factor of *Toxoplasma gondii* and *Eimeria tenella*. These findings indicate that the 15-kDa protein of *S. fayeri* is a toxin that causes food poisoning after consumption of parasitized horsemeat.
Keywords: horse meet, *Sarcocystis fayeri*, 15-kDa protein

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Watanabe M, Ohnishi T, Araki E[*1], Kanda T[*2], Tomita A[*3], Ozawa K[*4], Goto K[*5], Sugiyama K[*2], Konuma H[*1], Hara-Kudo Y: Characteristics of bacterial and fungal growth in plastic bottled beverages under a consuming condition model.
J Environ Sci Health A. 2014;49:819-26.

In this study, we elucidated the characteristics of microorganism growth in bottled beverages under consuming condition models. Furthermore, we provide insight into the safety of partially consumed bottled beverages with respect to food hygiene. We inoculated microorganisms, including foodborne pathogens, into various plastic bottled beverages and analysed the dynamic growth of microorganisms as well as bacterial toxin production in the beverages. Eight bottled beverage types were tested in this study, namely green tea, apple juice drink, tomato juice, carbonated drink, sport drink, coffee with milk, isotonic water and mineral water, and in these beverages several microorganism types were used: nine bacteria including three toxin producers, three yeasts, and five moulds. Following inoculation, the bottles were incubated. During the incubation period, the number of bacteria and yeasts and visible changes in mould-growth were determined over time. Our results indicated that combinations of the beverage types and microorganism species correlated with the degree of growth. Our results suggest that various types of unfinished beverages have microorganism growth and can include food borne pathogens and bacterial toxins. Therefore, our results indicate that in terms of food hygiene it is necessary to consume beverages immediately after opening the bottle.
Keywords: Beverage, microorganism, plastic bottle

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Wu W[*1,2], Zhou H R[*2], He K[*3,4], Pan X[*3], Sugita-Konishi Y, Watanabe M, Zhang H[*1], Pestka JJ[*2-5]: Role of Cholecystokinin in Anorexia Induction Following Oral Exposure to the 8-Ketotrichothecenes Deoxynivalenol, 15Acetyldeoxynivalenol, 3Acetyldeoxynivalenol, Fusarenon X and Nivalenol.
Toxicol Sci. 2014;138:278-89.

The head blight fungus *Fusarium graminearum* elaborates five closely related 8-ketotrichothecene congeners: (1) deoxynivalenol (DON), (2)

3-acetyldeoxynivalenol (3-ADON), (3) 15-acetyldeoxynivalenol (15-ADON), (4) fusarenon X (FX), and (5) nivalenol (NIV). The purpose of this study was to (1) compare the anorectic responses to the aforementioned 8-ketotrichothecenes following oral gavage at a common dose (2.5 mg/kg bw) and (2) relate these effects to changes plasma CCK and PYY_{3-36} concentrations. Elevation of plasma CCK markedly corresponded to anorexia induction by DON and all other 8-ketotrichothecenes tested. Furthermore, the CCK1 receptor antagonist SR 27897 and the CCK2 receptor antagonist L-365,260 dose-dependently attenuated both CCK- and DON-induced anorexia, which was consistent with this gut satiety hormone being an important mediator of 8-ketotrichothecene-induced food refusal. In contrast to CCK, PYY_{3-36} was moderately elevated by oral gavage with DON and NIV but not by 3-ADON, 15-ADON, or FX. Taken together, the results suggest that CCK plays a major role in anorexia induction following oral exposure to 8-ketotrichothecenes, whereas PYY_{3-36} might play a lesser, congener-dependent role in this response.

Keywords: cholecystokinin, anorexia, trichothecene

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Sakakibara N*1, Hamasaki T*2, Baba M*2, Demizu Y, Kurihara M, Irie K*1, Iwai M*1, Asada R*1, Kato Y*1, Maruyama T*1: Synthesis and evaluation of novel 3-(3,5-dimethylbenzyl)uracil analogs as potential anti-HIV-1 agents.

Bioorg Med Chem. 2013;21:5900-6.

A novel series of uracil derivatives with a 3,5-dimethylbenzyl group at the N^3-position were synthesized and evaluated as non-nucleoside HIV-1 reverse transcriptase inhibitors. Some of these compounds showed good-to-moderate activity with EC50 values in the submicromolar range. Among them, com- pound 10c showed significant potency against HIV-1 activity with an EC50 value of 0.03 lM and a high selectivity index of 2863. Preliminary structure-activity relationships and molecular modeling analyses were used to explore the major interactions between HIV-1 reverse transcriptase and the potent inhibitor 10c, which may serve as an important lead for further optimization.

Keywords: anti-HIV-1 agents, uracil analogs, HIV-1RT

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Demizu Y, Nagoya S, Shirakawa M, Kawamura M, Yamagata N, Sato Y, Doi M*, Kurihara M: Development of stapled short helical peptides capable of inhibiting vitamin D receptor (VDR)-coactivator interactions.

Bioorg Med Chem Lett. 2013;23:4292-6.

We synthesized stapled helical leucine-based peptides (DPI-01-07) containing 2-aminoisobutyric acid and a covalent cross-linked unit as inhibitors of vitamin D receptor (VDR)-coactivator interactions. The effects of these peptides on the human VDR were examined in an inhibition assay based on the recep- tor cofactor assay system, and one of them, DPI-07, exhibited potent inhibitory activity (IC50: 3.2 μM).

Keywords: vitamin D receptor, VDR-coactivator interaction inhibitor, stapled helical peptide

* 大阪薬科大学

Yamazaki N, Demizu Y, Sato Y, Doi M*, Kurihara M: Helical foldamer containing a combination of cyclopentane-1,2-diamine and 2,2-dimethylmalonic acid.

J Org Chem. 2013;78:9991-4.

We have developed new helical oligomers using a combination of (1*S*,2*S*)-cyclopentane-1,2-diamine [(*S*,*S*)-CPDA] and 2,2-dimethylmalonic acid (DMM) residues as building blocks. In solution, the preferred secondary structure of the (*S*,*S*) tetramer 6 was a right-handed (*P*) helix, and that of the (*R*,*R*) tetramer ent-6 was a left-handed (*M*) helix. In the crystalline state, both 6 and the (*S*,*S*) pentamer 7 folded into (*P*) 11-helices, and ent-6 folded into an (*M*) 11-helix with hydrogen bonds that were oriented in alternating directions.

Keywords: helical foldamer, diamine, dicarboxylic acid

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Oba M＊1, Ishikawa N＊2, Demizu Y, Kurihara M, Suemune H＊2, Tanaka M＊1: Helical oligomer with changeable chiral acetal moiety.
Eur J Org Chem. 2013;7679-82.

(*R*,*R*)-Ac_6c^{4BD}homopeptides form helical structures with slight control of the helical screw sense to the right-handed form. The chiral acetal moieties in (*R*,*R*)-Ac_6c^{4BD} are changeable in the peptide state.
Keywords: amino acid, chirality, helical structure

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Demizu Y, Yamashita H, Yamazaki N, Sato Y, Doi M＊1, Tanaka M＊2, Kurihara M: Oligopeptides with equal amounts of L- and D-amino acids may prefer a helix screw sense.
J Org Chem. 2013;78:12106-13.

We investigated the preferred conformations of two nonapeptides, Boc-(L-Leu-D-Leu-Aib)$_3$-OMe (2) and its enantiomer Boc-(D-Leu-L-Leu-Aib)$_3$-OMe (ent-2), four dodec- apeptides, Boc-(L-Leu-D-Leu-Aib)$_4$-OMe (3), Boc-(L-Leu-Aib- D-Leu)$_4$-OMe (4), Boc-(Aib-L-Leu-D-Leu)$_4$-OMe (5), and Boc-(L-Leu-Aib-D-Leu-Aib)$_3$-OMe (6), and a decapeptide, Boc-L-Leu-(D-Leu-L-Leu-Aib)$_3$-OMe (7), in solution and in the crystalline state. The nonapeptide 2 formed a right-handed (*P*) α-helix, and its enantiomer ent-2 formed a left-handed (*M*) α-helix. The dodecapeptides 3 and 5 were folded into (*P*) helices, and 4 formed an (*M*) helical structure. As for 6, roughly equivalent amounts of (*P*) and (*M*) helices were observed in solution, and two (*M*) α-helices were detected in the crystalline state. Furthermore, the decapeptide 7, which possesses four L-Leu residues and three D-Leu residues, was folded into an (*M*) α-helix.
Keywords: amino acid, peptide, conformation

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Fukuhara K, Ohno A, Ota Y, Senoo Y, Maekawa K, Okuda H, Kurihara M, Okuno A＊, Niida S＊, Saito Y, Takikawa O＊: NMR-based metabolomics of urine in a mouse model of Alzheimer's disease: identification of oxidative stress biomarkers.
J Clin Biochem Nutr. 2013;52:133-8.

Alzheimer's disease (AD) is the most common cause of neurodegenerative dementia among elderly patients. A biomarker for the disease could make diagnosis easier and more accurate, and accelerate drug discovery. In this study, NMR-based metabolomics analysis in conjunction with multivariate statistics was applied to examine changes in urinary metabolites in transgenic AD mice expressing mutant tau and β-amyloid precursor protein. These mice showed significant changes in urinary metabolites throughout the progress of the disease. Levels of 3-hydroxykynurenine, homogentisate and allantoin were significantly higher compared to control mice in 4 months (prior to onset of AD symptoms) and reverted to control values by 10 months of age (early/middle stage of AD), which highlights the relevance of oxidative stress to this neurodegenerative disorder even prior the onset of dementia. The level of these changed metabolites at very early period may provide an indication of disease risk at asymptomatic stage.
Keywords: Alzheimer's disease, metabolomics, NMR

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Zaima K, Wakana D, Demizu Y, Kumeta Y, Kamakura H, Maruyama T, Kurihara M, Goda Y: Isoheleproline: a new amino acid-sesquiterpene adduct from Inula helenium.
J Nat Med. 2014;68:432-5.

A new amino acid-sesquiterpene adduct, isoheleproline (1), was isolated from the roots of Inula helenium (elecampane), together with four known sesqui- terpene lactones (2-5). The planar configuration of 1 was elucidated on the basis of spectroscopic data analysis, and the relative configuration of 1 was determined by per- forming a detailed analysis of NOESY correlations and comparing its physicochemical data with the D- and L-proline adducts of 2 obtained by Michael addition. This is the first report of a new amino acid-sesquiterpene adduct from Inula plants.
Keywords: Inula helenium, asteraceae, amino acid-sequiterpene adduct

Shoda T, Okuhira K, Kato M, Demizu Y, Inoue H*, Naito M, Kurihara M: Design and synthesis of a tamoxifen derivative of selective estrogen receptor down-regulator.

Bioorg Med Chem Lett. 2014;24:87-9.

We designed and synthesized an estrogen receptor (ER) down-regulator (5), which is a derivative of tamoxifen with a long alkyl side chain. Compound 5 effectively reduced ER protein levels in MCF-7 cells and had an antagonistic effect.

Keywords: Breast cancer, Estrogen receptor, Tamoxifen.

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Yamashita H, Demizu Y, Shoda T, Sato Y, Oba M*, Tanaka M*, Kurihara M: Amphipathic short helix-stabilized peptides with cell-membrane penetration.

Bioorg Med Chem. 2014;22:2403-8.

We synthesized four types of arginine-based amphipathic nonapeptides, including two homochiral pep- tides, R-(L-Arg-L-Arg-Aib)$_3$-NH$_2$ (R = 6-FAM-β-Ala: FAM-1; R = Ac: Ac-1) and R-(D-Arg-D-Arg-Aib)$_3$-NH$_2$ (R = 6-FAM-β-Ala: ent-FAM-1; R = Ac: ent-Ac-1); a heterochiral peptide, R-(L-Arg-D-Arg-Aib)$_3$-NH$_2$ (R = 6-FAM-β-Ala: FAM-2; R = Ac: Ac-2); and a racemic mixture of diastereomeric peptides, R-(rac-Arg- rac-Arg-Aib)$_3$-NH$_2$ (R = 6-FAM-β-Ala: FAM-3; R = Ac: Ac-3), and then investigated the relationship between their secondary structures and their ability to pass through cell membranes. Peptides 1 and ent-1 formed stable one-handed α-helical structures and were more effective at penetrating HeLa cells than the non-helical peptides 2 and 3.

Keywords: cell-penetrating peptide, helical structure, DDS carrier.

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栗原正明：コンピュータシミュレーションによる違法ドラッグの活性予測.

YAKUGAKU ZASSHI 2013;133:13-6.

Prediction method of biological activities of chemicals has been developed as drug discovery technology. In recent years, a wide distribution of non-controlled psychotropic substances has become a serious problem in Japan. It takes a long time to evaluate their bioactivity in vitro and in vivo. Computer simulation could regulate new designer drugs in a short time. Prediction of biological activities of these drugs was performed by QSAR (Quantitative Structure-Activity Relationship) and pharmacophore-fingerprint method. Demonstration to predict the bioactivity of 4-methcathinone, one of cathinone derivatives which have been widely distributed by two methods was described.

Keywords: designer drugs, pharmacophore-fingerprint, QSAR(Quantitative Structure-Activity Relationship)

Okuhira K, Demizu Y, Hattori T, Ohoka N, Shibata N, Nishimaki-Mogami T, Okuda H, Kurihara M, Naito M: Development of hybrid small molecules that induce degradation of estrogen receptor-alpha and necrotic cell death in breast cancer cells.

Cancer Sci. 2013;104:1492-8.

Manipulation of protein stability with small molecules has a great potential for both basic research and clinical therapy. Recently, we have developed a series of hybrid small molecules named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser) that induces degradation of target proteins via ubiquitin-proteasome system. Here we report the activities of SNIPER(ER) that targets estrogen receptor alpha (ERα) for degradation. SNIPER(ER) induced degradation of ERα and inhibited estrogen-dependent expression of pS2 gene in an estrogen-dependent breast cancer cell line MCF-7. A proteasome inhibitor MG132 and siRNA-mediated downregulation of cIAP1 abrogated the SNIPER(ER)-induced ERα degradation, suggesting that the ERα is degraded by proteasome subsequent to cIAP1-mediated ubiquitylation. Intriguingly, after the ERα degradation, the SNIPER (ER)-treated MCF-7 cells undergo rapid cell death. Detailed analysis indicated that SNIPER(ER) caused necrotic cell death accompanied by a release of HMGB1, a marker of necrosis, from the cells. Following the ERα degradation, reactive oxygen species (ROS) was produced in the SNIPER(ER)-treated MCF-7 cells, and an anti-oxidant N-acetylcysteine inhibited the necrotic cell death. These results indicate that SNIPER (ER) induces ERα degradation, ROS production and necrotic cell death, implying a therapeutic potential of SNIPER(ER) as a lead for the treatment of ERα-positive breast cancers.

Keywords: estrogen receptor alpha, IAP, breast cancer

Shibata N, Carlin AF[*1], Spann NJ[*1], Saijo K[*1], Morello CS[*1], McDonald JG[*2], Romanoski CE[*1], Maurya MR[*1], Kaikkonen MU[*1], Lam MT[*1], Crotti A[*1], Reichart D[*1], Fox JN[*1], Quehenberger O[*1], Raetz CR[*3], Sullards MC[*4], Murphy RC[*5], Merrill AH Jr[*4], Brown HA[*6], Dennis EA[*1], Fahy E[*1], Subramaniam S[*1], Cavener DR[*7], Spector DH[*1], Russell DW[*2], Glass CK[*1]: 25-Hydroxycholesterol activates the integrated stress response to reprogram transcription and translation in macrophages.

J Biol Chem. 2013;288:35812-23.

25-Hydroxycholesterol（25OHC）is an enzymatically derived oxidation product of cholesterol that modulates lipid metabolism and immunity. 25OHC is synthesized in response to interferons and exerts broad antiviral activity by as yet poorly characterized mechanisms. To gain further insights into the basis for antiviral activity, we evaluated time-dependent responses of the macrophage lipidome and transcriptome to 25OHC treatment. In addition to altering specific aspects of cholesterol and sphingolipid metabolism, we found that 25OHC activates integrated stress response（ISR）genes and reprograms protein translation. Effects of 25OHC on ISR gene expression were independent of liver X receptors and sterol-response element-binding proteins and instead primarily resulted from activation of the GCN2/eIF2α/ATF4 branch of the ISR pathway. These studies reveal that 25OHC activates the integrated stress response, which may contribute to its antiviral activity.

Keywords: macrophages, stress response, 25-Hydroxycholesterol

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Hattori T, Uchida C[*1], Takahashi H[*2], Yamamoto N[*3], Naito M, Taya Y[*3]: Distinct and Site-Specific Phosphorylation of the Retinoblastoma Protein at Serine 612 in Differentiated Cells.

PLOS ONE. 2014;9:e80769.

The retinoblastoma susceptibility protein（pRB）is a phosphoprotein that regulates cell cycle progression at the G1/S transition. In quiescent and early G1 cells, pRB predominantly exists in the active hypophosphorylated form. The cyclin/cyclin-dependent protein kinase complexes phosphorylate pRB at the late G1 phase to inactivate pRB. This event leads to the dissociation and activation of E2F family transcriptional factors. At least 12 serine/threonine residues in pRB are phosphorylated*in vivo*. Although there have been many reports describing bulk phosphorylation of pRB, detail research describing the function of each phosphorylation site remains unknown. Besides its G1/S inhibitory function, pRB is involved in differentiation, prevention of cell death and control of tissue fate. To uncover the function of phosphorylation of pRB in various cellular conditions, we have been investigating phosphorylation of each serine/threonine residue in pRB with site-specific phospho-serine/threonine antibodies. Here we demonstrate that pRB is specifically phosphorylated at Ser612 in differentiated cells in a known kinase-independent manner. We also found that pRB phosphorylated at Ser612 still associates with E2F-1 and tightly binds to nuclear structures including chromatin. Moreover, expression of the Ser612Ala mutant pRB failed to induce differentiation. The findings suggest that phosphorylation of Ser612 provides a distinct function that differs from the function of phosphorylation of other serine/threonine residues in pRB.

Keywords: pRB, phosphorylation, differentiation

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Uchida C[*1], Hattori T, Takahashi H[*2], Yamamoto N[*3], Kitagawa M[*1], Taya Y[*3]: Distinct and Site-Specific Interaction between RB protein and NuMA is required for proper alignment of spindle microtubules.

Genes to Cells. 2014;19:89-96.

Retinoblastoma protein（pRB）controls cell cycle progression and cell cycle exit through interactions with cellular proteins. Many pRB-binding proteins,

which function in gene transcription or modulation of chromatin structure, harbor LXCXE motifs in their binding domain for pRB. In this study, we found that nuclear mitotic apparatus protein (NuMA), a mitotic spindle organizer, interacts with pRB through LSCEE sequences located in its C-terminal region. siRNA-mediated down-regulation of pRB caused aberrant distribution of NuMA and alignment of spindle microtubules in mitotic cells. Abnormal organization of spindle microtubules was also accompanied by misalignment of an over-expressed NuMA mutant (mut-NuMA) with a defect in pRB binding caused by an LSGEK mutation. The mut-NuMA-over-expressing cells showed lower potency for survival than wild-type NuMA (wt-NuMA)-over-expressing cells during 2 weeks of culture. Interestingly, knockdown of pRB reduced the population of wt-NuMA-over-expressing cells to the same level as mut-NuMA cells after 2 weeks. Taken together, pRB may have a novel function in regulating the mitotic function of NuMA and spindle organization, which are required for proper cell cycle progression.

Keywords: pRB, NuMA, mitosis

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Kikuchi R*1, Ohata H*2, Ohoka N, Kawabata A*1, Naito M: APOLLON protein promotes early mitotic CYCLIN A degradation independent of the spindle assembly checkpoint.
J Biol Chem. 2014;289:3457-67.

In the mammalian cell cycle, both CYCLIN A and CYCLIN B are required for entry into mitosis, and their elimination is also essential to complete the process. During mitosis, CYCLIN A and CYCLIN B are ubiquitylated by the anaphase-promoting complex/cyclosome (APC/C) and then subjected to proteasomal degradation. However, CYCLIN A, but not CYCLIN B, begins to be degraded in the prometaphase when APC/C is inactivated by the spindle assembly checkpoint (SAC). Here, we show that APOLLON (also known as BRUCE or BIRC6) plays a role in SAC-independent degradation of CYCLIN A in early mitosis. APPOLON interacts with CYCLIN A that is not associated with cyclin-dependent kinases. APPOLON also interacts with APC/C, and it facilitates CYCLIN A ubiquitylation. In APPOLON-deficient cells, mitotic degradation of CYCLIN A is delayed, and the total, but not the cyclin-dependent kinase-bound, CYCLIN A level was increased. We propose APPOLON to be a novel regulator of mitotic CYCLIN A degradation independent of SAC.

Keywords: Apollon, cyclin A, ubiquitin

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Nakajima O, Nakamura K, Kondo K, Akiyama H, Teshima R: Method of detecting genetically modified chicken containing human erythropoietin gene.
Biol Pharm Bull. 2013;36:1454-9.

Genetically modified (GM) chickens carrying the human erythropoietin (hEpo) gene have been developed to produce recombinant hEpo protein in eggs. However, such animals have not been approved as food sources in Japan. We developed a method for detecting the hEpo gene in chicken meat using a real-time polymerase chain reaction (real-time PCR). The hEpo gene was clearly detected in genomic DNA extracted from magnum and heart of a chimeric chicken containing the hEpo gene. A plasmid containing the hEpo gene was used as a standard reference molecule as well. The results clearly showed that our method was capable of detecting the hEpo gene contained in the plasmid in the presence of genomic DNA extracted from a raw chicken meat sample. We successfully used this method to test six samples of raw chicken meat and six samples of chicken meat in processed foods. This method will be useful for monitoring chicken meat that might have originated from GM chickens carrying the hEpo gene to assure food safety.

Keywords: genetically modified chicken, erythropoietin, real-time polymerase chain reaction

Kurokawa S*1,4, Kuroda M*2, Mejima M*1, Nakamura R, Takahashi Y*1, Sagara H*3, Takeyama N*1, Satoh S*4, Kiyono H*1,5, Teshima R, Masumura T*4, Yuki Y*1,5: RNAi-mediated suppression of endogenous storage proteins leads to a change in localization of overexpressed cholera toxin B-subunit and the allergen protein RAG2 in rice seeds.

Plant Cell Rep. 2014;33:75-87.

A purification-free rice-based oral cholera vaccine (MucoRice-CTB) was previously developed by our laboratories using a cholera toxin B-subunit (CTB) overexpression system. Recently, an advanced version of MucoRice-CTB was developed (MucoRice-CTB-RNAi) through the use of RNAi to suppress the production of the endogenous storage proteins 13-kDa prolamin and glutelin, so as to increase CTB expression. The level of the α-amylase/trypsin inhibitor-like protein RAG2 (a major rice allergen) was reduced in MucoRice-CTB-RNAi seeds in comparison with wild-type (WT) rice. To investigate whether RNAi-mediated suppression of storage proteins affects the localization of overexpressed CTB and major rice allergens, we generated an RNAi line without CTB (MucoRice-RNAi) and investigated gene expression, and protein production and localization of two storage proteins, CTB, and five major allergens in MucoRice-CTB, MucoRice-CTB-RNAi, MucoRice-RNAi, and WT rice. In all lines, glyoxalase I was detected in the cytoplasm, and 52- and 63-kDa globulin-like proteins were found in the aleurone particles. In WT, RAG2 and 19-kDa globulin were localized mainly in protein bodies II (PB-II) of the endosperm cells. Knockdown of glutelin A led to a partial destruction of PB-II and was accompanied by RAG2 relocation to the plasma membrane/cell wall and cytoplasm. In MucoRice-CTB, CTB was localized in the cytoplasm and PB-II. In MucoRice-CTB-RNAi, CTB was produced at a level six times that in MucoRice-CTB and was localized, similar to RAG2, in the plasma membrane/cell wall and cytoplasm. Our findings indicate that the relocation of CTB in MucoRice-CTB-RNAi may contribute to down-regulation of RAG2.

Keywords: allergen, localization, MucoRice

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Kurokawa S*1,2, Nakamura R, Mejima M*1, Kozuka-Hata H*3, Kuroda M*4, Takeyama N*1, Oyama M*3, Satoh S*2, *5, Kiyono H*1,6, Masumura T*2,5, Teshima R, Yuki Y*1,6: MucoRice-cholera toxin B-subunit, a rice-based oral cholera vaccine, down-regulates the expression of α-amylase/trypsin inhibitor-like protein family as major rice allergens.

J Proteome Res. 2013;12:3372-82.

To develop a cold chain- and needle/syringe-free rice-based cholera vaccine (MucoRice-CTB) for human use, we previously advanced the MucoRice system by introducing antisense genes specific for endogenous rice storage proteins and produced a molecularly uniform, human-applicable, high-yield MucoRice-CTB devoid of plant-associated sugar. To maintain the cold chain-free property of this vaccine for clinical application, we wanted to use a polished rice powder preparation of MucoRice-CTB without further purification but wondered whether this might cause an unexpected increase in rice allergen protein expression levels in MucoRice-CTB and prompt safety concerns. Therefore, we used two-dimensional fluorescence difference gel electrophoresis and shotgun MS/MS proteomics to compare rice allergen protein expression levels in MucoRice-CTB and wild-type (WT) rice. Both proteomics analyses showed that the only notable change in the expression levels of rice allergen protein in MucoRice-CTB, compared with those in WT rice, was a decrease in the expression levels of α-amylase/trypsin inhibitor-like protein family such as the seed allergen protein RAG2. Real-time PCR analysis showed mRNA of RAG2 reduced in MucoRice-CTB seed. These results demonstrate that no known rice allergens appear to be up-reregulated by genetic modification of MucoRice-CTB, suggesting that MucoRice-CTB has potential as a safe oral cholera vaccine for clinical application.

Keywords: proteomics, 2D-DIGE, MucoRice

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Nakamura R, Nakamura R, Adachi R, Hachisuka A, Yamada A*, Ozeki Y*, Teshima R: Differential

analysis of protein expression in RNA-binding-protein transgenic and parental rice seeds cultivated under salt stress.

J Proteome Res. 2014;13:489-95.

Transgenic plants tolerant to various environmental stresses are being developed to ensure a consistent food supply. We used a transgenic rice cultivar with high saline tolerance by introducing an RNA-binding protein (RBP) from the ice plant (Mesembryanthemum crystallinum); differences in salt-soluble protein expression between nontransgenic (NT) and RBP rice seeds were analyzed by 2D difference gel electrophoresis (2D-DIGE), a gel-based proteomic method. To identify RBP-related changes in protein expression under salt stress, NT and RBP rice were cultured with or without 200 mM sodium chloride. Only two protein spots differed between NT and RBP rice seeds cultured under normal conditions, one of which was identified as a putative abscisic acid-induced protein. In NT rice seeds, 91 spots significantly differed between normal and salt-stress conditions. Two allergenic proteins of NT rice seeds, RAG1 and RAG2, were induced by high salt. In contrast, RBP rice seeds yielded seven spots and no allergen spots with significant differences in protein expression between normal and salt-stress conditions. Therefore, expression of fewer proteins was altered in RBP rice seeds by high salt than those in NT rice seeds.

Keywords: proteomics, RNA binding protein, transgenic rice

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Nakamura K, Maeda Y*, Morimoto K*, Katayama S*, Kondo K, Nakamura S*: Functional expression of amyloidogenic human stefins A and B in *Pichia pastoris* using codon optimization.

Biotech Appl Biochem. 2013;60:283-8.

Complementary DNAs encoding human stefins A (HSA) and B (HSB) were synthesized using *Pichia*-preferred codons by overlap extension PCR. The full-length genes were ligated downstream of the *glyceraldehyde-3-phosphate dehydrogenase* promoter in the *Pichia* expression vector pGAPZ$_{\alpha}$C and successfully expressed in *Pichia pastoris* strain X-33. Functional recombinant HSA and HSB proteins were purified from culture medium at yields of 121.3 ± 13.5 (n = 3) and 95.4 ± 4.1 (n = 3) mg/L, respectively. Using this expression strategy, we demonstrated that high levels of bioactive recombinant HSA and HSB can be produced by fermentation in *P. pastoris*.

Keywords: human stefins, molecular stability, papain-inhibitory activity

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Nakamura K, Akiyama H, Kawano N*1, Kobayashi T, Yoshimatsu K*1, Mano J*2, Kitta K*2, Ohmori K*3, Noguchi A, Kondo K, Teshima R: Evaluation of real-time PCR detection methods for detecting rice products contaminated by rice genetically modified with a CpTI-KDEL-T-nos transgenic construct.

Food Chem. 2013;141:2618-24.

Genetically modified (GM) rice (*Oryza sativa*) lines, such as insecticidal Kefeng and Kemingdao, have been developed and found unauthorised in processed rice products in many countries. Therefore, qualitative detection methods for the GM rice are required for the GM food regulation. A transgenic construct for expressing cowpea (*Vigna unguiculata*) trypsin inhibitor (CpTI) was detected in some imported processed rice products contaminated with Kemingdao. The 3' terminal sequence of the identified transgenic construct for expression of CpTI included an endoplasmic reticulum retention signal coding sequence (KDEL) and nopaline synthase terminator (T-nos). The sequence was identical to that in a report on Kefeng. A novel construct-specific real-time polymerase chain reaction (PCR) detection method for detecting the junction region sequence between the CpTI-KDEL and T-nos was developed. The imported processed rice products were evaluated for the contamination of the GM rice using the developed construct-specific real-time PCR methods, and detection frequency was compared with five event-specific detection methods. The construct-specific detection methods detected the GM rice at higher frequency than the event-specific detection methods. Therefore, we propose that the construct-specific detection method is a beneficial tool for screening the contamination of GM rice lines, such as Kefeng, in processed rice products for the GM food regulation.

Keywords: genetically modified rice, detection method, trypsin inhibitor

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Mano J*1, Hatano S*2, Futo S*2, Minegishi Y*3, Ninomiya K*4, Nakamura K, Kondo K, Teshima R, Takabatake R*1, Kitta K*1: Development of direct real-time PCR system applicable to a wide range of foods and agricultural products.

Shokuhin Eiseigaku Zasshi. 2014;55:25-33.

To improve the efficiency of DNA analysis of foods and agricultural products, we investigated a direct real-time PCR based on the real-time monitoring of DNA amplification directly from crude cell lysates of analytical samples. We established a direct real-time PCR system comprising sample pretreatment with a specified lysis buffer and real-time PCR using the developed master mix reagent. No PCR inhibition was observed in the analysis of crude cell lysates from 50 types of samples, indicating that the direct real-time PCR system is applicable to a wide range of materials. The specificity of the direct real-time PCR was evaluated by means of a model assay system for single nucleotide discrimination. Even when crude cell lysates coexisted in the reaction mixtures, the primer selectivity was not affected, suggesting that the sequence specificity of the direct real-time PCR was equivalent to that of PCR from purified DNA templates. We evaluated the sensitivity and quantitative performance of the direct real-time PCR using soybean flour samples including various amounts of genetically modified organisms. The results clearly showed that the direct real-time PCR system provides sensitive detection and precise quantitation.

Keywords: PCR, direct real-time PCR, crude cell lysate

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Nakamura K, Kondo K, Kobayashi T, Noguchi A, Ohmori K*1, Takabatake R*2, Kitta K*2, Akiyama H, Teshima R, Nishimaki-Mogami T: Identification and detection of genetically modified papaya resistant to papaya ringspot virus strains in Thailand.

Biol Pharm Bull. 2014;37:1-5.

Many lines of genetically modified (GM) papaya (*Carica papaya Linnaeus*) have been developed worldwide to resist infection from various strains of papaya ringspot virus (PRSV). We found an unidentified and unauthorized GM papaya in imported processed papaya food. Transgenic vector construct that provides resistance to the PRSV strains isolated in Thailand was detected. An original and specific real-time polymerase chain reaction method was generated to qualitatively detect the PRSV-Thailand-resistant GM papaya.

Keywords: genetically modified organism, papaya, polymerase chain reaction

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Noguchi A, Nakamura K, Sakata K, Kobayashi T, Ohmori K*1, Kasahara M*2, Takabatake R*3, Kitta K*3, Akiyama H, Kondo K, Teshima R: Interlaboratory validation study of an event-specific real-time polymerase chain reaction detection method for genetically modified 55-1 papaya.

J AOAC Int. 2013;96:1054-8.

Genetically modified (GM) papaya line 55-1 (55-1) is resistant to papaya ringspot virus infection, and is commercially available in several countries. A specific detection method for 55-1 is required for mandatory labeling regulations. An event-specific real-time PCR method was developed by our laboratory. To validate the method, interlaboratory validation of event-specific qualitative real-time PCR analysis for 55-1 was performed in collaboration with 12 laboratories. DNA extraction and real-time PCR reaction methods were evaluated using 12 blind samples: six non-GM papayas and six GM papayas in each laboratory. Genomic DNA was highly purified from all papayas using an ion-exchange column, and the resulting DNA sample was analyzed using real-time PCR. Papaya endogenous reference gene chymopapain (CHY) and the event-specific 55-1 targeted sequence were detected in GM papayas whereas CHYalone was detected in non-GM papayas in all laboratories. The cycle threshold values of CHYand the 55-1 targeted sequence showed high repeatability (RSD, 0.6-0.8%) and reproducibility (RSDR 2.2-3.6%). This study demonstrates that the

55-1 real-time PCR detection method is a useful and reliable method to monitor 55-1 papaya in foods.
Keywords: food composition, polymerase chain reaction

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Takabatake R*1, Noritake H*2, Noguchi A, Nakamura K, Kondo K, Akiyama H, Teshima R, Mano J*1, Kitta K*1: Comparison of DNA extraction methods for sweet corn and processed sweet corns.
Shokuhin Eiseigaku Zasshi. 2013;54:309-15.

DNA was extracted from sweet corn and its processed products using four DNA extraction methods: the CTAB method, the DNeasy Plant Maxi kit, GM Quicker 3, and Genomic-tip 20/G. DNA was successfully extracted from raw sweet corn and baby corn samples using all four methods. Meanwhile, from frozen, canned, and dry pack products, DNA was well extracted using the DNeasy Plant Maxi kit, GM Quicker 3, and Genomic-tip 20/G, but not enough with the CTAB method. The highest yield of DNA was obtained with Genomic-tip 20/G. The degree of degradation of extracted DNA was observed to increase in the order of raw, frozen, canned, dry pack, and baby corn samples. To evaluate the quality of extracted DNA, real-time PCR analyses were conducted using three maize endogenous genes. The DNAs extracted using GM Quicker 3 had high purity, suggesting that GM Quicker 3 would be the most suitable method for DNA extraction from processed sweet corn products.
Keywords: sweet corn, DNA extraction, real-time PCR

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Obitsu S, Sakata K, Teshima R, Kondo K: Eleostearic acid induces RIP1-mediated atypical apoptosis in a kinase-independent manner via ERK phosphorylation, ROS generation and mitochondrial dysfunction.
Cell Death Dis. 2013;4:e674.

RIP1 is a serine/threonine kinase, which is involved in apoptosis and necroptosis. In apoptosis, caspase-8 and FADD have an important role. On the other hand, RIP3 is a key molecule in necroptosis. Recently, we reported that eleostearic acid (ESA) elicits caspase-3- and PARP-1-independent cell death, although ESA-treated cells mediate typical apoptotic morphology such as chromatin condensation, plasma membrane blebbing and apoptotic body formation. The activation of caspases, Bax and PARP-1, the cleavage of AIF and the phosphorylation of histone H2AX, all of which are characteristics of typical apoptosis, do not occur in ESA-treated cells. However, the underlying mechanism remains unclear. To clarify the signaling pathways in ESA-mediated apoptosis, we investigated the functions of RIP1, MEK, ERK, as well as AIF. Using an extensive study based on molecular biology, we identified the alternative role of RIP1 in ESA-mediated apoptosis. ESA mediates RIP1-dependent apoptosis in a kinase independent manner. ESA activates serine/threonine phosphatases such as calcineurin, which induces RIP1 dephosphorylation, thereby ERK pathway is activated. Consequently, localization of AIF and ERK in the nucleus, ROS generation and ATP reduction in mitochondria are induced to disrupt mitochondrial cristae, which leads to cell death. Necrostatin (Nec)-1 blocked MEK/ERK phosphorylation and ESA-mediated apoptosis. Nec-1 inactive form (Nec1i) also impaired ESA-mediated apoptosis. Nec1 blocked the interaction of MEK with ERK upon ESA stimulation. Together, these findings provide a new finding that ERK and kinase-independent RIP1 proteins are implicated in atypical ESA-mediated apoptosis.
Keywords: apoptosis, AIF, RIP1

Ohmori K*1, Nakamura K, Kasahara M*2, Takabatake R*3, Kitta K*3, Fujimaki T*1, Kondo K, Teshima R, Akiyama H: A novel DNA extraction and purification method using an ion-exchange resin type kit for the detection of genetically modified papaya in processed papaya products.
Food Control. 2013;32:728-35.

A method for the extraction and purification of genomic DNA from processed papaya products is essential for the detection of approved genetically modified (GM) papaya, according to GM labeling regulations, and unapproved GM papaya, to restrict the import or sale of products containing it. Here, we investigated methods for the extraction of DNA from processed papaya products, including dried papaya,

canned papaya and papaya jam. The extraction of DNA from dried papaya and canned papaya required a pre-digestion step, using RNase, cellulase and proteinase K. In the case of papaya jam, α-amylase was found to be indispensable to obtain DNA with high yield and purity. The DNA yield was considerably higher when an ion-exchange resin type kit (IER-100G) was used, compared with other five methods (IER-20G, QIAamp DNA Stool Mini Kit, DNeasy Plant Maxi Kit, GM Quicker 3 Kit and Wizard Cleanup Resin System). We developed a suitable method for the extraction and purification of DNA from processed papaya products, which could be used to detect GM papaya.

Keywords: genetically modified (GM) papaya, real-time PCR, DNA extraction

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Nakamura K, Minamitake Y*1, Nakamura K, Kobayashi T, Noguchi A, Takabatake R*2, Kitta K*2, Hashimoto H*3, Kawakami H*1, Kondo K, Teshima R, Akiyama H: Development of PCR primers designed for sensitive detection of genetically modified potato DNA in processed foods.

Jpn J Food Chem Safety. 2013;20:161-9.

The degree of DNA fragmentation in commercially processed potato products was investigated using qualitative polymerase chain reaction (PCR) with primers designed to amplify amplicons of different lengths. The PCR amplified the amplicons up to 301 bp using 25 ng of the DNA purified from snack foods, frozen potatoes, dried potatoes and pre-cooked potatoes. In contrast, the DNA from potato starch and processed potato products, such as vermicelli, were amplifiable up to 51-101 bp. The amplicons with 63 bp using the real-time PCR from the DNA extracted from all processed potato products were detected. The study suggests that the primers that are designed to produce amplicons less than 51-101 bp are required for detecting genetically modified potatoes in processed potato products.

Keywords: processed potato products, genetically modified potato, amplicon length

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Nakamura K, Sakagami H*1, Asanuma-Date K*1, Nagasawa N*1, Nakahara Y*2, Akiyama H, Ogawa H*1: Immobilized glycosylated Fmoc-amino acid for SPR: comparative studies of lectin-binding to linear or biantennary diLacNAc structures.

Carbohydr Res. 2013;382:77-85.

A method to immobilize glycan-linked amino acids with protected α-amino groups to a Biacore sensor chip and their utility for interaction analyses were demonstrated. Two types of diN-acetyllactosamine (diLacNAc)-containing glycans, a core 2 hexasaccharide involving linear diLacNAc that is O-linked to N-(9-fluorenyl)methoxycarbonyl (Fmoc) -Thr and a biantennary diLacNAc that is N-linked to Fmoc-Asn, were used as ligands. For immobilization, the free carboxyl groups of the amino acid residues were activated with EDC/NHS, then reacted with the ethylenediamine-derivatized carboxymethyldextran sensor chip to obtain the desired ligand concentrations. Interactions of the ligands with five plant lectins were analyzed by surface plasmon resonance and bindings were compared. The resonance unit of each lectin was corrected by subtracting that of the reference cell at which the core 1-Thr-Fmoc or Asn-Fmoc was immobilized as a ligand. The carbohydrate-specific interactions were verified by preincubating lectins with their respective inhibitory sugar before injection. By steady state analysis, *Lycopersicon esculentum* lectin showed 27-fold higher affinities to linear diLacNAc than to biantennary diLacNAc, while *Datura stramonium* lectin and *Solanum tuberosum* lectin showed similarly low $K_{a,app}$s of $10^6 M^{-1}$ for the two ligands. In contrast, *Ricinus communis* agglutinin-120 showed 3.2-fold higher $K_{a,app}$ to the biantennary LacNAc than to the linear diLacNAc. A lectin purified from *Pleurocybella porrigens* mushroom interacted at equally high affinity of $10^8 M^{-1}$ with linear and biantennary diLacNAcs, which revealed it as a unique probe. This method provides a useful and sensitive system to analyze interactions by simulating the glycans on the cell surface.

Keywords: N-acetyllactosamine-binding lectin, polylactosaminoglycan, glycosyl Fmoc-amino acid

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Igarashi N*, Takeguchi A*, Sakai S, Akiyama H, Higashi K*, Toida T*: Effect of molecular sizes of chondroitin sulfate on interaction with L-selectin.
Int J Carbohydr Chem. 2013;2013:1-9. article ID 856142.

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) side chain of proteoglycans (PGs) which are widely distributed in the extracellular matrix and at cell surface. CS shows a highly structural diversity in not only molecular weight (MW) but sulfonation pattern. CS has been reported to exert anti-inflammatory activity by having effects on cytokine production by helper T cells. In this study, we focused on the structures of CS chains, especially MW of CS, and investigated effect of the different MW of CS on binding affinity with L-selectin and cytokine production by murine splenocytes. Firstly, we fractionated CS by employing gel filtration chromatography and obtained several CS fractions with different MW. Then the interaction between fractionated CS and L-selectin was analyzed by surface plasmon resonance (SPR). Finally, the influence of MW of CS on cytokine production by murine splenocytes was investigated in vitro. The results showed that interferon-gamma production was significantly increased by mouse splenocytes cocultivated with CS. On the contrary, CS inhibited interleukin 5 production by murine splenocytes depending on MW of the cocultivated CS. These results strongly indicate the existence of the optimal molecular size for an anti-inflammatory effect of CS through cytokine production by murine splenocytes.
Keywords: Chondroitin sulfate, L-Selectin, splenpcytes

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Sakai S, Adachi R, Akiyama H, Teshima R: Validation of Quantitative and Qualitative Methods for Detecting Allergenic Ingredients in Processed Foods in Japan.
J Agric Food Chem. 2013;61:5675-80.

A labeling system for food allergenic ingredients was established in Japan in April 2002. To monitor the labeling, the Japanese government announced official methods for detecting allergens in processed foods in November 2002. The official methods consist of quantitative screening tests using enzyme-linked immunosorbent assays (ELISAs) and qualitative confirmation tests using Western blotting or polymerase chain reactions (PCR). In addition, the Japanese government designated 10 μg protein/g food (the corresponding allergenic ingredient soluble protein weight/food weight), determined by ELISA, as the labeling threshold. To standardize the official methods, the criteria for the validation protocol were described in the official guidelines. This paper, which was presented at the Advances in Food Allergen Detection Symposium, ACS National Meeting and Expo, San Diego, CA, Spring 2012, describes the validation protocol outlined in the official Japanese guidelines, the results of interlaboratory studies for the quantitative detection method (ELISA for crustacean proteins) and the qualitative detection method (PCR for shrimp and crab DNAs), and the reliability of the detection methods.
Keywords: food allergy, detection method, validation

Shimizu Y*1, Kishimura H*1, Kanno G*1, Nakamura A, Adachi R, Akiyama H, Watanabe K*2, Hara A*1, Ebisawa M*3, Saeki H*1: Molecular and immunological characterization of β'-component (Onc k 5), a major IgE-binding protein in chum salmon roe.
Int Immunol. 2014;26:139-47.

Salmon roe has a high allergic potency and often causes anaphylaxis in Japan. The major allergic protein of salmon roe is β'-component, which is a 35kDa vitellogenin fragment consisting of two subunits. To elucidate structural information and immunological characteristics, β'-component and the subunit components were purified from chum salmon (Onchorhincus keta) roe and vitellogenin-encoding mRNA was used to prepare β'-component subunit-encoding cDNA. This was PCR-amplified, cloned and sequenced and the deduced amino acid sequence compared with partial sequences of β'-component obtained by peptide mapping. The recombinant β'-component subunit was produced by bacterial expression in Escherichia coli and its IgE-binding ability was measured by ELISA using the sera of a patient allergic to salmon roe. This was then compared

with that of the native β'-component with and without carboxymethylation. Following successful cloning of the cDNA encoding the β'-component subunit, 170 amino acid residues were deduced and matched with the amino acid sequences of 121 and 88 residues in the 16kDa and 18kDa subunits, respectively. The sequences of both β'-component subunits were almost identical, and the predicted secondary structure of the β'-component showed a high content of β-pleated sheets and no α-helices. There was no difference in IgE-binding ability between the native and recombinant β'-component subunits at the same protein concentration, regardless of carboxymethylation. In conclusion, β'-component is a homodimer protein composed of two isoform subunits having the same level of IgE-binding ability and, therefore, allergenic identity.

Keywords: β'-component, IgE-binding ability, vitellogenin

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登田美桜，畝山智香子，春日文子：過去50年間のわが国の高等植物による食中毒事例の傾向.
食品衛生学雑誌 2014;55:55-63.

厚生労働省（旧厚労省）監修の「全国食中毒事件録」をもとに，昭和36年～平成22年に日本国内で発生した高等植物による食中毒事例の傾向を分析した．発生件数では，チョウセンアサガオ類，バイケイソウ類及びトリカブト類が多かった．主な原因施設は「家庭」であり，事例の多くは患者が自ら採取した原因植物を摂取していた．さらに，発生件数の推移で最近10年間に顕著な増加が見られた原因植物，近年の主な特徴，リスク管理上の今後の課題などについて考察した.

Keywords: plant toxin, food poisoning, descriptive epidemiology

Kaniwa N, Sugiyama E, Saito Y, Kurose K, Maekawa K, Hasegawa R, Furuya H*1, Ikeda H*2, Takahashi Y*2, Muramatsu M*3, Tohkin M*4, Ozeki T*5, Mushiroda T*5, Kubo M, Kamatani N*5, Abe M*6, Yagami A*6, Ueta M*7, Sotozono C*7, Kinoshita S*7, Ikezawa Z*8, Matsunaga K*6, Aihara M*9, Japan Pharmacogenomics Data Science Consortium: Specific HLA types are associated with antiepileptic drug-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese subjects.
Pharmacogenomics. 2013;14:1821-31.

AIM: This preliminary study investigated genomic biomarkers for Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), related to three antiepileptic drugs, zonisamide, phenobarbital and phenytoin. PATIENTS & METHODS: HLA class I and HLA-DRB1 loci were genotyped for Japanese patients with zonisamide-, phenobarbital- or phenytoin-induced SJS/TEN (n = 12, 8 and 9, respectively) and for healthy Japanese volunteers (n = 2878). RESULTS: Carrier frequencies of HLA-A*02:07 in patients with zonisamide-induced SJS/TEN and in the general Japanese population were 41.7 and 6.81%, respectively. Carrier frequencies of HLA-B*51:01 in patients with phenobarbital- and phenytoin-induced SJS/TEN and in controls were 75.0, 55.6 and 15.2%, respectively. HLA-A*02:07 and HLA-B*51:01, in a dominant model, were significantly associated with zonisamide- and phenobarbital-induced SJS/TEN, respectively (Pc = 0.0176 and 0.0042, respectively). CONCLUSION: Our data suggest that HLA-A*02:07 and HLA-B*51:01 are potential biomarkers for zonisamide- and phenobarbital-induced SJS/TEN, respectively, in Japanese individuals.

Keywords: phenobarbital, zonisamide, SJS/TEN

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Sai K, Hanatani T, Azuma Y, Segawa K, Tohkin M*1, Omatsu H*2, Makimoto H*2, Hirai M*2, Saito Y: Development of a detection algorithm for statin-induced myopathy using electronic medical records.
J Clin Pharm Ther. 2013;38:230-5.

Statin-induced myopathy (SIM) is a clinically important ADE, but pharmacoepidemiological methodology for detecting this ADE with high predictability has not yet been established. The

current study aimed to develop a detection algorithm, highly selective for SIM using electronic medical records (EMRs). We collected EMRs on prescriptions, laboratory tests, diagnoses and medical practices from the hospital information system of Kobe University Hospital (Japan) for a total of 5,109 patients who received prescription of statins from April 2006 to March 2009. Our developed algorithm for extracting SIM-suspected patients consisted of three steps: 1) event detection: increase of creatine kinase (CK) and subsequent statin discontinuation, 2) filtration by exclusion factors (disease diagnosis/medical practices), and 3) judgment on the time course of CK values (baseline, event and recovery). A causal relationship between the event and statin prescription (probable/possible/unlikely) was judged by review of patient medical charts. Among 5,109 statin-treated patients, five SIM-suspected subjects were identified by the current algorithm at a frequency of 0.1%. Review of the medical charts revealed that the causality of statin use for SIM for all five suspected patients was judged as "Likely (probable/possible)"; thus, positive predictive value was estimated as 100% (95% confidential interval: 56.6-100%). We successfully developed a detection algorithm for SIM with high PPV. Further study is needed to confirm the utility of the current algorithm and its applicability to PV in a larger population.

Keywords: electronic medical records, statin, myopathy

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Hanatani T, Sai K, Tohkin M*1, Segawa K, Kimura M*2, Hori K*2, Kawakami J*2, Saito Y: An algorithm for the identification of heparin-induced thrombocytopenia using a medical information database.

J Clin Pharm Ther. 2013;38:423-8.

The current study was aimed to develop and validate a novel algorithm for detecting heparin-induced thrombocytopenia (HIT) using a medical information database (MID) and to identify possible risk-factors for HIT. We developed a new HIT-detecting algorithm based on platelet-count at distinct time-points and diagnostic information from patients treated with unfractionated heparin (UFH) from April 2008 through March 2012 at Hospital of Hamamatsu University School of Medicine, Japan. Definitive diagnoses of HIT were made through reviews of the medical records by a skilled hematologist. This algorithm detected 47 patients with suspected HIT in the source population (n = 2 875). Of these, 41 were identified as definitive HIT patients after review of the medical records. The positive predictive value for the algorithm was 87.2%, and the frequency of definitive HIT was 1.4%. Multivariate logistic regression analysis revealed that longer-term treatment (>4 days) with UFH was a risk factor for HIT, with an odds ratio of 5.38 (95% CI: 2.35 to 12.32) for definitive HIT. We developed a novel, high-PPV detection algorithm for HIT and identified longer-term treatment with UFH as a risk-factor for HIT. Our results support the utility of MIDs for improving pharmacovigilance.

Keywords: Pharmacovigilance, medical information database, heparin-induced thrombocytopenia

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Sai K, Kurose K, Koizumi T, Katori N, Sawada J*1, Matsumura Y*2, Saijo N*2, Yamamoto N*3, Tamura T*3, Okuda H, Saito Y: Distal promoter regions are responsible for differential regulation of human orosomucoid-1 and -2 gene expression and acute phase responses.

Biol Pharm Bull. 2014;37(1):164-8.

Human orosomucoid (ORM), a major acute-phase plasma protein, is encoded by 2 highly homologous genes, *ORM1* and *ORM2*. Human *ORM* induction is regulated by each proximal promoter region, where putative glucocorticoid responsive elements and C/EBPβ binding sites are located. However, the details of the differential regulation of these genes remain unknown. In this study, we assessed the role of the distal promoter region of each *ORM* in HeLa cells. Luciferase-reporter activities of full constructs, containing approximately 1.1 kbp (FULL), and those of deletion constructs, containing up to 188 bp region (DEL) upstream of the transcription start sites of *ORM1* and *ORM2* were compared under both basal and inducer-treated conditions. For *ORM1* and *ORM2* DEL constructs, significantly increased activities after dexamethasone (DEX) treatments (alone and

combined with IL-1β were observed. Significantly higher FULL construct activities than DEL construct activities were observed for *ORM1* after IL-1β treatment, while those for *ORM2* were significantly lower at basal level and after DEX treatments. Upon C/EBPβ overexpression, FULL construct activities were significantly higher than those of DEL constructs at basal level and after IL-1β treatment for *ORM1*, and at basal level and after inducer-treatments for *ORM2*. Higher transcription-induction activity in the distal promoter region was evident for *ORM1* in the absence of C/EBPβ overexpression, and for *ORM2* under C/EBPβ overexpression conditions. These findings suggest that the *ORM* distal promoter region differentially regulates expression of *ORM* genes at basal level and in acute phase responses.

Keywords: orosomucoid, alpha 1 acid glycoprotein, distal promoter region

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Nakamura R, Nakamura R, Sakai S, Adachi R, Hachisuka A, Urisu A*1, Fukutomi Y*2, Teshima R: Tissue transglutaminase generates deamidated epitopes on gluten, increasing reactivity with hydrolyzed wheat protein-sensitized IgE.
J Allergy Clin Immunol. 2013;132:1436-8.

Patients sensitized with hydrolyzed wheat protein (HWP)-containing facial soap present with an exercise-induced systemic allergic reaction after ingestion of HWP-free wheat. Tissue transglutaminase can generate deamidated epitopes on gluten, which are cross-reactive with HWP.

Keywords: Hydrolyzed wheat protein, Wheat gluten, Tissue transglutaminase

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Kamemura N*1, Kawamoto N*2, Nakamura R, Teshima R, Fukao T*2, Kido H*1: Low-affinity allergen-specific IgE in cord blood and affinity maturation after birth.
J Allergy Clin Immunol. 2014;133:904-5.

The low-affinity allergen-specific IgEs in the cord blood and affinity maturation of them in the peripheral blood of 6- and 14-month-old infants were identified. We also describe here the methods for detection of low- and high-affinity allergen-specific IgE, using a diamond-like-carbon-coated chip and a luciferase reporter system.

Keywords: IgE, affinity maturation, diamond-like-carbon-coated chip

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Hino M*1, Shimojo N*1, Ochiai H*1, Inoue Y*1, Ando K*1, Chikaraishi K*1, Ota S*2, Okimoto Y*3, Sunami S*4, Nakamura R, Teshima R, Sato Y*5, Kohno Y*1: Expression of CD203c on basophils as a marker of immunoglobulin E-mediated l-asparaginase allergy.
Leuk Lymphoma. 2014;55:92-6.

Immediate allergy to L -asparaginase (ASP) is a major obstacle in treating lymphoid malignancies. ASP-specific immunoglobulin G (ASP-IgG) has been used as a surrogate marker. Recently, the CD203c-basophil activation test (BAT) was found to be useful in diagnosing IgE-mediated allergies. We compared the diagnostic utility of the CD203c-BAT to that of ASP-IgG levels in determining ASP allergies in children. Eight ASP allergic reactions occurred over 75 ASP treatment courses. The sensitivity, specifi city and area under the receiver operating characteristic curve of CD203c-BAT were similar to the ASP-IgG levels (0.75 vs. 0.85, 0.82 vs. 0.78 and 0.81 vs. 0.85, respectively). Positive skin prick test results in patients with ASP allergy suggested that ASP-IgE was one of the key players in ASP allergy. A combination of the BAT with the ASP-IgG level had the highest specifi city (0.95) and positive predictive value (0.62), which permitted us to identify ASP allergy more eff ectively.

Keywords: L -asparaginase, IgE-mediated allergy, basophil activation test

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Ishikawa M, Tajima Y, Murayama M, Senoo Y,

Maekawa K, Saito Y: Plasma and serum from nonfasting men and women differ in their lipidomic profiles.

Biol Pharm Bull. 2013;36:682-5.

Biomarkers will play important roles in disease diagnosis, drug development, and the proper use of drugs. Blood is considered the best biofluid for biomarker research because it is easy to access and a wealth of data are available. However, previous studies revealed that several ionic metabolites showed different levels (including presence or absence) in plasma and serum. Thus, attention should be paid to selecting the best biofluid for biomarker exploration. Many lipid molecules have biological significance and thus would be candidate biomarkers. However, no comprehensive study revealing differences in lipid metabolite levels between plasma and serum has been undertaken. Furthermore, gender differences have not been reported. To clarify the difference in the levels of lipid metabolites between human plasma and serum from both genders, we performed lipid metabolomic analysis using liquid chromatography-mass spectrometry-based systems for phospholipids (PLs), lysoPLs, sphingomyelins, ceramides and oxidative fatty acids. Our results revealed that most of the lipid metabolites were present at similar levels in plasma and serum and in males and females. However, several oxidative fatty acid metabolites showed differences. Of the metabolites related to clotting processes, three showed higher levels in serum than in plasma, and three were detected only in serum. Furthermore, four metabolites were present at different levels between males and females, and two were detected only in males. Thus, attention should be paid to the selection of plasma or serum when utilizing these lipid metabolites as biomarkers.

Keywords: Lipid metabolites, Biomarker, Plasma and Serum

Tajima Y, Ishikawa M, Maekawa K, Murayama M, Senoo Y, Nishimaki-Mogami T, Nakanishi H[*1], Ikeda, K[*2], Arita M[*3], Taguchi R[*4], Okuno A[*5], Mikawa R[*5], Niida S[*5], Takikawa O[*5], Saito Y: Lipidomic analysis of brain tissues and plasma in a mouse model expressing mutated human amyloid precursor protein/tau for Alzheimer's disease.

Lipids in Health and Disease. 2013;12:68.

Alzheimer's disease (AD), the most common cause of dementia among neurodegenerative diseases, afflicts millions of elderly people worldwide. In addition to amyloid-beta (Aβ) peptide and phosphorylated tau, lipid dysregulation is suggested to participate in AD pathogenesis. However, alterations in individual lipid species and their role in AD disease progression remain unclear. We performed a lipidomic analysis using brain tissues and plasma obtained from mice expressing mutated human amyloid precursor protein (APP) and tau protein (Tg2576 × JNPL3) (APP/tau mice) at 4 (pre-symptomatic phase), 10 (early symptomatic) and 15 months (late symptomatic). Levels of docosahexaenoyl (22:6) cholesterol ester (ChE) were markedly increased in APP/tau mice compared to controls at all stages examined. Several species of ethanolamine plasmalogens (pPEs) and sphingomyelins (SMs) showed different levels between brains from APP/tau and control mice at various stages of AD. Increased levels of 12-hydroxyeicosatetraenoic acid (12-HETE) during the early symptomatic phase were consistent with previous reports using human AD brain tissue. In addition, 19,20-dihydroxy-docosapentaenoic acid (19,20-diHDoPE) and 17,18-dihydroxy-eicosatetraenoic acid (17,18-diHETE), which are produced from docosahexaenoic acid and eicosapentaenoic acid via 19,20-epoxy-docosapentaenoic acid (19,20-EpDPE) and 17,18-epoxy-eicosatetraenoic acid (17,18-EpETE), respectively, were significantly increased in APP/tau brains during the pre-symptomatic phase, and concomitant increases occurred in plasma. Several arachidonic acid metabolites such as prostaglandin D2 (PGD2) and 15-hydroxyeicosatetraenoic acid (15-HETE), which have potential deteriorating and protective actions, respectively, were decreased in the early symptomatic phase of APP/tau mice. Significant decreases in phosphatidylcholines and PEs with polyunsaturated fatty acids were also detected in the late symptomatic phase, indicating a perturbation of membrane properties. Our results provide fundamental information on lipid dysregulation during various stages of human AD.

Keywords: Lipidomic analysis, Alzheimer's disease, Mice model

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Ishikawa M, Maekawa K, Saito K, Senoo Y, Urata M, Murayama M, Tajima Y, Kumagai Y*, Saito Y: Plasma and Serum Lipidomics of Healthy White Adults Shows Characteristic Profiles by Subjects' Gender and Age.
PLOS One. 2014;9:e91806.

Blood is a commonly used biofluid for biomarker discovery. Although blood lipid metabolites are considered to be potential biomarker candidates, their fundamental properties are not well characterized. We aimed to (1) investigate the matrix type (serum vs. plasma) that may be preferable for lipid biomarker exploration, (2) elucidate age- and gender-associated differences in lipid metabolite levels, and (3) examine the stability of lipid metabolites in matrix samples subjected to repeated freeze-thaw cycles. Using liquid chromatography-mass spectrometry, we performed lipidomic analyses for fasting plasma and serum samples for four groups (15 subjects/group) of young and elderly (25-34 and 55-64 years old, respectively) males and females and for an additional aliquot of samples from young males, which were subjected to repeated freeze-thaw cycles. Lysophosphatidylcholine and diacylglycerol levels were higher in serum than in plasma samples, suggesting that the clotting process influences serum lipid metabolite levels. Gender-associated differences highlighted that the levels of many sphingomyelin species were significantly higher in females than in males, irrespective of age and matrix (plasma and serum). Age-associated differences were more prominent in females than in males, and in both matrices, levels of many triacylglycerols were significantly higher in elderly females than in young females. Plasma and serum levels of most lipid metabolites were reduced by freeze-thawing. Our results indicate that plasma is an optimal matrix for exploring lipid biomarkers because it represents the original properties of an individual's blood sample. In addition, the levels of some blood lipid species of healthy adults showed gender- and age-associated differences; thus, this should be considered during biomarker exploration and its application in diagnostics. Our fundamental findings on sample selection and handling procedures for measuring blood lipid metabolites is important for ensuring the quality of biomarkers identified and its qualification process.
Keywords: Lipid metabolites, Biomarker, Plasma

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Maekawa K, Futagami T*1, Kusunoki Y*1, Matsuzaki Y*2, Takikawa H*3: Identification of a novel HLA-B allele *HLA-B*07:185* in a Japanese individual.
Tissue Antigens. 2013;82:434-6.

*HLA-B*07:185* differs from *B*07:02:01* by one nucleotide substitution in exon 2 at position 300G>C.
Keywords: Human leukocyte antigen-B, Novel allele, Sequence-based typing

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Watanabe C*1, Fukuzawa K*2, Okiyama Y*1, Tsukamoto T*2, Kato A*2, Tanaka S*3, Mochizuki Y*4, Nakano T: Three- and four-body corrected fragment molecular orbital calculations with a novel subdividing fragmentation method applicable to structure-based drug design.
J Mol Gr Mod. 2013;41:31-42.

3体および4体効果を考慮した，フラグメント分子軌道法に基づいたstructure-based drug designについて検討した.
Keywords: Three- and four-body corrected, fragment molecular orbital calculation, structure-based drug design

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Fukuzawa K*1, Watanabe C*2, Kurisaki I*3, Taguchi N*4, Mochizuki Y*3, Nakano T, Tanaka S*5, Komeiji Y*6: Accuracy of the fragment molecular orbital (FMO) calculations for DNA: Total energy, molecular orbital, and inter-fragment interaction energy.
Comp Theor Chem. 2014;1034:7-16.

フラグメント分子軌道法を用いたDNAのベンチマーク計算を行った.

Keywords: Fragment molecular orbital method, DNA, benchmark

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Takahashi H*1, Kaniwa N, Saito Y, Sai K, Hamaguchi T*1, Shirao K*1, Shimada Y*1, Matsumura Y*1, Ohtsu A*1, Yoshino T*1, Takahashi A*2, Odaka Y*1, Okuyama M*1, Sawada J, Sakamoto H*1, Yoshida T*1: Identification of a candidate single-nucleotide polymorphism related to chemotherapeutic response through a combination of knowledge-based algorithm and hypothesis-free genomic data.

J Biosci Bioengineering. 2013;116:768-73.

Inter-individual variations in drug responses among patients are known to cause serious problems in medicine. Genome-wide association study (GWAS) is powerful for examining single-nucleotide polymorphisms (SNPs) and their relationships with drug response variations. However, no significant SNP has been identified using GWAS due to multiple testing problems. Therefore, we propose a combination method consisting of knowledge-based algorithm, two stages of screening, and permutation test for identifying SNPs in the present study. We applied this method to a genome-wide pharmacogenomics study for which 109,365 SNPs had been genotyped using Illumina Human-1 BeadChip for 119 gastric cancer patients treated with fluoropyrimidine. We identified rs2293347 in epidermal growth factor receptor (EGFR) is as a candidate SNP related to chemotherapeutic response. The p value for the rs2293347 was $2.19 \times 10^{(-5)}$ for Fisher's exact test, and the p value was 0.00360 for the permutation test (multiple testing problems are corrected). Additionally, rs2293347 was clearly superior to clinical parameters and showed a sensitivity value of 55.0% and specificity value of 94.4% in the evaluation by using multiple regression models. Recent studies have shown that combination chemotherapy of fluoropyrimidine and EGFR-targeting agents is effective for gastric cancer patients highly expressing EGFR. These results suggest that rs2293347 is a potential predictive factor for selecting chemotherapies, such as fluoropyrimidine alone or combination chemotherapies.

Keywords: Genome-wide association study, Bioinformatics, Gastric cancer

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Knights J*1, Sato Y*2, Kaniwa N, Saito Y, Ueno H*3, Ramanathan M*1: Genetic factors associated with gemcitabine pharmacokinetics, disposition, and toxicity.

Pharmacogenet Genomics. 2014;24:15-25.

The goal of this work was to investigate the associations of genetic and environmental factors with gemcitabine disposition and toxicity from genome-wide data using a novel information theoretic approach. We utilized the information theoretic K-way interaction information (KWII) metric to detect gene-gene and gene-environment interactions associated with gemcitabine disposition and gemcitabine-induced neutropenia in genomic and clinical data from Japanese cancer patients. The information theoretic KWII analyses identified age and four genes - DMD, HEXDC, CNTN4, and ALOX5AP - to be associated with gemcitabine pharmacokinetics (PK). The rs4769060 single-nucleotide polymorphism in the ALOX5AP gene was associated with all PK parameters studied. For gemcitabine-induced neutropenia, multiple associations with long intergenic noncoding RNA regions were detected. Pathway analysis identified leukotriene and eoxin synthesis, platelet homeostasis, and L1CAM interactions as potential pathways associated with gemcitabine disposition. The KWII analyses detected novel associations with gemcitabine PK and toxicity. These results could be used to inform future investigations involving gemcitabine efficacy in clinical settings.

Keywords: Bioinformatics, Gemcitabine, Genome-wide association study

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Ohba T[*1], Xu J[*2], Alexander DB[*2], Yamada A[*1], Kanno J, Hirose A, Tsuda H[*2], Imaizumi Y[*1]: MWCNT causes extensive damage to the ciliated epithelium of the trachea of rodents.

J Toxicol Sci. 2014;39(3):499-505.

The ciliated tracheobronchial epithelium plays an important role in the excretion of inhaled dust. While many reports indicate that inhaled multi-walled carbon nanotubes (MWCNT) induce inflammation and proliferative changes in the lung and pleura, their effects on the upper airway have not been reported. Two different types of MWCNTs, MWCNT-L (8 μm in length and 150 nm in diameter) and MWCNT-S (3 μm in length and 15 nm in diameter), were examined for their effect on the trachea as well as the bronchus and lung. In vitro, the movement of the cilia of primary tracheal epithelial cells was impaired by treatment with the 2 MWCNTs. Rats were treated with 0.3 ml of a 250 μg/ml suspension of MWCNTs on days 1, 4, and 7, and sacrificed on day 8. Extensive loss of ciliated cells and replacement by flat cells without cilia was observed in the trachea. Deposition of MWCNTs and occasional squamous cell metaplasia were found in the regenerative granulation tissue. The proportion of the lesion to the transverse section of the trachea was vehicle, 0; MWCNT-L, 27.2 ± 10.5; MWCNT-S, 32.1 ± 15.8 (both MWCNTs, $p < 0.001$ vs vehicle). The amount of cilia showed significant decrease in the MWCNT-L treated rats ($p < 0.05$). In contrast to the trachea lesions, the number of inflammatory foci in the lung was greater in the MWCNT-S than in the MWCNT-L treated rats. Our results indicate that both MWCNTs caused extensive damage to the ciliated epithelium of the trachea. This damage may prolong the deposition of inhaled MWNCT in the lung.

Keywords: MWCNT, Tracheal damage, Rat

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Janesick A[*1], Nguyen TT[*1], Aisaki K, Igarashi K, Kitajima S, Chandraratna RA[*2], Kanno J, Blumberg B[*3]: Active repression by RARγ signaling is required for vertebrate axial elongation.

Development. 2014;141(11):2260-70.

Retinoic acid receptor gamma 2 (RARγ2) is the major RAR isoform expressed throughout the caudal axial progenitor domain in vertebrates. During a microarray screen to identify RAR targets, we identified a subset of genes that pattern caudal structures or promote axial elongation and are upregulated by increased RAR-mediated repression. Previous studies have suggested that RAR is present in the caudal domain, but is quiescent until its activation in late stage embryos terminates axial elongation. By contrast, we show here that RARγ2 is engaged in all stages of axial elongation, not solely as a terminator of axial growth. In the absence of RA, RARγ2 represses transcriptional activity in vivo and maintains the pool of caudal progenitor cells and presomitic mesoderm. In the presence of RA, RARγ2 serves as an activator, facilitating somite differentiation. Treatment with an RARγ-selective inverse agonist (NRX205099) or overexpression of dominant-negative RARγ increases the expression of posterior Hox genes and that of marker genes for presomitic mesoderm and the chordoneural hinge. Conversely, when RAR-mediated repression is reduced by overexpressing a dominant-negative co-repressor (c-SMRT), a constitutively active RAR (VP16-RARγ2), or by treatment with an RARγ-selective agonist (NRX204647), expression of caudal genes is diminished and extension of the body axis is prematurely terminated. Hence, gene repression mediated by the unliganded RARγ2-co-repressor complex constitutes a novel mechanism to regulate and facilitate the correct expression levels and spatial restriction of key genes that maintain the caudal progenitor pool during axial elongation in Xenopus embryos.

Keywords: Active repression, Posterior Hox, Retinoic acid receptor

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Numano T[*1,3], Xu J[*2], Futakuchi M[*1], Fukamachi K[*1], Alexander DB[*2], Furukawa F[*3], Kanno J,

Hirose A, Tsuda H[*2], Suzu, M[*1]: Comparative Study of Toxic Effects of Anatase and Rutile Type Nanosized Titanium Dioxide Particles *in vivo* and *in vitro*.

Asian Pac J Cancer Prev. 2014;15(2):929-35.

Two types of nanosized titanium dioxide, anatase ($anTiO_2$) and rutile ($rnTiO_2$), are widely used in industry, commercial products and biosystems. TiO_2 has been evaluated as a Group 2B carcinogen. Previous reports indicated that $anTiO_2$ is less toxic than $rnTiO_2$, however, under ultraviolet irradiation $anTiO_2$ is more toxic than $rnTiO_2$ *in vitro* because of differences in their crystal structures. In the present study, we compared the *in vivo* and *in vitro* toxic effects induced by $anTiO_2$ and $rnTiO_2$. Female SD rats were treated with 500 μg/ml of $anTiO_2$ or $rnTiO_2$ suspensions by intra-pulmonary spraying 8 times over a two week period. In the lung, treatment with $anTiO_2$ or $rnTiO_2$ increased alveolar macrophage numbers and levels of 8-hydroxydeoxyguanosine (8-OHdG); these increases tended to be lower in the $anTiO_2$ treated group compared to the $rnTiO_2$ treated group. Expression of MIP1αmRNA and protein in lung tissues treated with $anTiO_2$ and $rnTiO_2$ was also significantly up-regulated, with MIP1αmRNA and protein expression significantly lower in the $anTiO_2$ group than in the $rnTiO_2$ group. In cell culture of primary alveolar macrophages (PAM) treated with $anTiO_2$ and $rnTiO_2$, expression of MIP1α mRNA in the PAM and protein in the culture media was significantly higher than in control cultures. Similarly to the *in vivo* results, MIP1αmRNA and protein expression was significantly lower in the $anTiO_2$ treated cultures compared to the $rnTiO_2$ treated cultures. Furthermore, conditioned cell culture media from PAM cultures treated with $anTiO_2$ had less effect on A549 cell proliferation compared to conditioned media from cultures treated with $rnTiO_2$. However, no significant difference was found in the toxicological effects on cell viability of ultra violet irradiated $anTiO_2$ and $rnTiO_2$. In conclusion, our results indicate that $anTiO_2$ is less potent in induction of alveolar macrophage infiltration, 8-OHdG and MIP1α expression in the lung, and growth stimulation of A549 cells *in vitro* than $rnTiO_2$.

Keywords: Nanosized titanium dioxide, lung toxicity, MIP1α

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Kondoh S[*1], Inoue K[*1-3], Igarashi K, Sugizaki H[*4], Shirode-Fukuda Y[*1], Inoue E[*1], Yu T[*1,2], Takeuchi JK[*4,5], Kanno J, Bonewald LF[*6], Imai Y[*1,2]: Estrogen receptor α in osteocytes regulates trabecular bone formation in female mice.

Bone. 2014;60:68-77.

Estrogens are well known steroid hormones necessary to maintain bone health. In addition, mechanical loading, in which estrogen signaling may intersect with the Wnt/β-catenin pathway, is essential for bone maintenance. As osteocytes are known as the major mechanosensory cells embedded in mineralized bone matrix, osteocyte ERα deletion mice ($ER\alpha^{\Delta Ocy/\Delta Ocy}$) were generated by mating ERα floxed mice with Dmp1-Cre mice to determine the role of ERα in osteocytes. Trabecular bone mineral density of female, but not male $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice was significantly decreased. Bone formation parameters in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ were significantly decreased while osteoclast parameters were unchanged. This suggests that ERα in osteocytes exerts osteoprotective function by positively controlling bone formation. To identify potential targets of ERα, gene array analysis of Dmp1-GFP osteocytes sorted by FACS from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and control mice was performed. Gene expression microarray followed by gene ontology analyses revealed that osteocytes from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ highly expressed genes categorized in 'Secreted' when compared to control osteocytes. Among them, expression of Mdk and Sostdc1, both of which are Wnt inhibitors, was significantly increased without alteration of expression of the mature osteocyte markers such as Sost and β-catenin. Moreover, hindlimb suspension experiments showed that trabecular bone loss due to unloading was greater in $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice without cortical bone loss. These data suggest that ERα in osteocytes has osteoprotective functions in trabecular bone formation through regulating expression of Wnt antagonists, but conversely plays a negative role in cortical bone loss

due to unloading.

Keywords: Estrogen receptor α, Osteocyte, Wnt signaling

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Xu J*1,2, Futakuchi M*2, Alexander DB*1, Fukamachi K*2, Numano T*2, Suzui M*2, Shimizu H*3, Omori T*4, Kanno J, Hirose A, Tsuda H*1: Nanosized zinc oxide particles do not promote DHPN-induced lung carcinogenesis but cause reversible epithelial hyperplasia of terminal bronchioles.

Arch Toxicol. 2014;88(1):65-75.

Zinc oxide (ZnO) is known to induce lung toxicity, including terminal bronchiolar epithelial hyperplasia, which gives rise to concerns that nanosized ZnO (nZnO) might lead to lung carcinogenesis. We studied the tumor promoting activity of nZnO by an initiation-promotion protocol using human c-Ha-*ras* proto-oncogene transgenic rats (H*ras*128 rats). The rats were given 0.2 % N-nitrosobis(2-hydroxypropyl)amine (DHPN) in the drinking water for 2 weeks and then treated with 0.5 ml of 250 or 500 μg/ml nZnO suspension by intra-pulmonary spraying once every 2 weeks for a total of 7 times. Treatment with nZnO particles did not promote DHPN-induced lung carcinogenesis. However, nZnO dose-dependently caused epithelial hyperplasia of terminal bronchioles (EHTB) and fibrosis-associated interstitial pneumonitis (FAIP) that were independent of DHPN treatment. Tracing the fate of EHTB lesions in wild-type rats indicated that the hyperplastic lesions almost completely disappeared within 12 weeks after the last nZnO treatment. Since nZnO particles were not found in the lung and $ZnCl_2$ solution induced similar lung lesions and gene expression profiles, the observed lesions were most likely caused by dissolved Zn^{2+}. In summary, nZnO did not promote carcinogenesis in the lung and induced EHTB and FAIP lesions that regressed rapidly, probably due to clearance of surplus Zn^{2+} from the lung.

Keywords: Nanosized zinc oxide particles, Lung toxicity, Interstitial pneumonitis

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Hirabayashi Y: Radiation-induced, cell cycle-related gene expression in aging hematopoietic stem cells: enigma of their recovery.

Annals of the New York Academy of Sciences. 2014;1310:69-73.

This paper reviews quantitative and qualitative studies conducted to identify changes in the characteristics of hematopoietic stem/progenitor cells (HSCs/HPCs) with or without radiation exposure. The numerical recovery of HSCs/HPCs after radiation exposure is lower than for other types of cells, an effect that may depend on hierarchical ordering of generation age during blood cell differentiation, from primitive HSCs to various differentiated HPCs. Studies are in progress to evaluate gene expression in bone marrow cells and cells in the lineage-negative, c-kit (+), stem cell antigen (+) (LKS) fraction from 21-month-old mice, with or without radiation exposure. Preliminary data suggest that cell cycle-related genes, that is, cyclin D1 (Ccnd1), phosphatidylinositol 3 kinase regulatory subunit polypeptide 1 (PiK3r1), and Fyn, are upregulated solely in the LKS fraction from 21-month-old mice irradiated at 6 weeks of age, compared with the LKS fraction from age-matched nonirradiated control mice. Additional studies may provide evidence that the aging phenotype is exaggerated following exposure to ionizing radiation,

specifically in the LKS fraction.
Keywords: Radiation late effects, LKS fraction, PiK3r1

Taquahashi Y, Ogawa Y, Takagi A, Tsuji M, Morita K, Kanno J: An improved dispersion method of multi-wall carbon nanotube for inhalation toxicity studies of experimental animals.
J Toxicol Sci. 2013;38(4):619-28.

A multiwall carbon nanotube (MWCNT) product Mitsui MWNT-7 is a mixture of singular fibers, their agglomerates and aggregates. In the rodent lungs, it has been experienced that the administration of MWCNT as a mixture induced inflammatory lesions triggered predominantly by the aggregates and agglomerates at the level of terminal bronchiole. In case of human, because of two reasons below, pulmonary toxicity induced by singular fibers that reached the lung alveoli is most important to assess; Human respiratory tract is longer than the rodents and the aggregates/agglomerates are likely to be trapped before they reach the lung alveoli, and in the human ambient conditions, the air flow is generally milder than in the animal inhalation chamber and therefore the aggregates and agglomerates are likely to precipitate faster than the singular fibers. Therefore, for the precise assessment of human inhalation toxicity of the MWCNT, it is important to develop a method to generate aerosol predominantly consisting of singular fibers without changing the length and width. Here, we report a method to effectively remove the aggregate/agglomerates and disperse Mitsui MCWNT-7 into single fibers in dry condition without dispersant and without significant selection/changes in fiber length and width of the singular fibers. The MCWNT-7 was suspended in Tert-butyl alcohol, freeze-and-thawed, filtered by a vibrating 25 μm mesh Metallic Sieve, snap-frozen by liquid nitrogen, and vacuum-dried in order to avoid re-aggregation of the singular fibers by surface tension during drying.
Keywords: Multiwall carbon nanotube, dispersion, Sublimation drying.

Kanno J, Aisaki K, Igarashi K, Kitajima S, Matsuda N, Morita K, Tsuji M, Moriyama N, Furukawa Y, Otsuka M, Tachihara E, Nakatsu N*, Kodama Y: Oral administration of pentachlorophenol induces interferon signaling mRNAs in C57BL/6 male mouse liver.
J Toxicol Sci. 2013;38(4):643-54.

Pentachlorophenol (PCP) was monitored for transcriptome responses in adult mouse liver at 2, 4, 8 and 24 hr after a single oral administration at four dose levels, 0, 10, 30 and 100 mg/kg. The expression data obtained using Affymetrix GeneChip MOE430 2.0 were absolutized by the Percellome method and expressed as three dimensional (3D) surface graphs with axes of time, dose and copy numbers of mRNA per cell. We developed the programs RSort, for comprehensive screening of the 3D surface data and PercellomeExploror for cross-referencing and confirmed the significant responses by visual inspection. In the first 8 hr, approximately 100 probe sets (PSs) related to PXR/SXR and Cyp2a4 and other metabolic enzymes were induced whereas Fos and JunB were suppressed. At 24 hr, about 1,200 PSs were strongly induced. We cross-referenced the Percellome database consisting of 111 chemicals on the liver transcriptome and found that about half of the PSs belonged to the metabolic pathways including Nrf2-mediated oxidative stress response networks shared with some of the 111 chemicals. The other half of the induced genes were interferon signaling network genes (ISG) and their induction was unique to PCP. Toll like receptors and other pattern recognition receptors, interferon regulatory factors and interferon alpha itself were included but inflammatory cytokines were not induced. In summary, these data indicated that functional symptoms of PCP treatment, such as hyperthermia and profuse sweating might be mediated by the ISG rather than the previously documented mitochondrial uncoupling mechanism. PCP might become a hint for developing low molecular weight orally available interferon mimetic drugs following imiquimod and RO4948191 as agonists of toll-like receptor and interferon receptor.
Keywords: Pentachlorophenol, Interferon signaling genes, Percellome toxicogenomics

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Okuno Y*[1], Ohtake F*[1], Igarashi K, Kanno J, Matsumoto T*[1], Takada I*[1], Kato S*[2], Imai Y*[1]: Epigenetic Regulation of Adipogenesis by PHF2

Histone Demethylase.

Diabetes. 2013;62(5):1426-1434.

PHF2 is a JmjC family histone demethylase that removes the methyl group from H3K9me2 and works as a coactivator for several metabolism-related transcription factors. In this study, we examined the in vivo role of PHF2 in mice. We generated Phf2 floxed mice, systemic Phf2 null mice by crossing Phf2 floxed mice with CMV-Cre transgenic mice, and tamoxifen-inducible Phf2 knockout mice by crossing Phf2 floxed mice with Cre-ERT2 transgenic mice. Systemic Phf2 null mice had partial neonatal death and growth retardation and exhibited less adipose tissue and reduced adipocyte numbers compared with control littermates. Tamoxifen-induced conditional knockout of PHF2 resulted in impaired adipogenesis in stromal vascular cells from the adipose tissue of tamoxifen-inducible Phf2 knockout mice as well as of Phf2 knocked-down 3T3-L1 cells. PHF2 interacts with CEBPA and demethylates H3K9me2 in the promoters of CEBPA-regulated adipogenic genes. These findings suggest that PHF2 histone demethylase potentiates adipogenesis through interaction with CEBPA in vivo. Taken together, PHF2 may be a novel therapeutic target in the treatment of obesity and the metabolic syndrome.

Keywords: Adipogenesis, PHF2, Epigenetic

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Si Y*1, Inoue K*1,2,3, Igarashi K, Kanno J, Imai Y*1,3: Autoimmune regulator, Aire, is a novel regulator of chondrocyte differentiation.

Biochem Biophys Res Commun. 2013;437(4):579-84.

Chondrocyte differentiation is controlled by various regulators, such as Sox9 and Runx2, but the process is complex. To further understand the precise underlying molecular mechanisms of chondrocyte differentiation, we aimed to identify a novel regulatory factor of chondrocyte differentiation using gene expression profiles of micromass-cultured chondrocytes at different differentiation stages. From the results of microarray analysis, the autoimmune regulator, Aire, was identified as a novel regulator. Aire stable knockdown cells, and primary cultured chondrocytes obtained from Aire(-/-) mice, showed reduced mRNA expression levels of chondrocyte-related genes. Over-expression of Aire induced the early stages of chondrocyte differentiation by facilitating expression of Bmp2. A ChIP assay revealed that Aire was recruited on an Airebinding site (T box) in the Bmp2 promoter region in the early stages of chondrocyte differentiation and histone methylation was modified. These results suggest that Aire can facilitate early chondrocyte differentiation by expression of Bmp2 through altering the histone modification status of the promoter region of Bmp2. Taken together, Aire might play a role as an active regulator of chondrocyte differentiation, which leads to new insights into the regulatory mechanisms of chondrocyte differentiation.

Keywords: Aire, BMP2, Histone modification

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Takahashi Y, Yasuhiko Y, Takahashi J*1, Takada S*1, Johnson RL*2, Saga Y*3, Kanno J: Metameric pattern of intervertebral disc/vertebral body is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somites in the mouse embryo.

Developmental Biology. 2013;380:172-84.

The vertebrae are derived from the sclerotome of somites. Formation of the vertebral body involves a process called resegmentation, by which the caudal half of a sclerotome is combined with the rostral half of the next sclerotome. To elucidate the relationship between resegmentation and rostro-caudal patterning of somite, we used the *Uncx4.1-LacZ* transgene to characterize the resegmentation process. Our observations suggested that in the thoracic and lumbar vertebrae, the Uncx4.1-expressing caudal sclerotome gave rise to the intervertebral disc (IVD) and rostral portion of the vertebral body (VB). In the cervical vertebrae, the Uncx4.1-expressing caudal sclerotome appeared to contribute to the IVD and both caudal and rostral ends of the VB. This finding suggests that the rostro-caudal gene expression boundary does not necessarily coincide with the resegmentation boundary. This conclusion was supported by analyses of *Mesp2* KO and *Ripply1/2* double KO embryos lacking rostral and caudal properties, respectively. Resegmentation

was not observed in *Mesp2* KO embryos, but both the IVD and whole VB were formed from the caudalized sclerotome. Expression analysis of IVD marker genes including *Pax1* in the wild-type, *Mesp2* KO, and *Ripply1/2* DKO embryos also supported the idea that a metameric pattern of IVD/VB is generated independently of Mesp2/Ripply-mediated rostro-caudal patterning of somite. However, in the lumbar region, IVD differentiation appeared to be stimulated by the caudal property and suppressed by the rostral property. Therefore, we propose that rostro-caudal patterning of somites is required to stimulate IVD differentiation in the caudal half of the sclerotome.

Keywords: Uncx4.1, vertebra, resegmentation

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Mizui T, Sekino Y, Yamazaki Y, Ishizuka H, Takahashi H, Kojima N, Kojima M, Shirao T: Myosin II ATPase activity mediates the long-term potentiation-induced exodus of stable F-actin bound by drebrin A from dendritic spines.
PLOS ONE. 2014;9(1):e8536722.

The neuronal actin-binding protein drebrin A forms a stable structure with F-actin in dendritic spines. NMDA receptor activation causes an exodus of F-actin bound by drebrin A (DA-actin) from dendritic spines, suggesting a pivotal role for DA-actin exodus in synaptic plasticity. We quantitatively assessed the extent of DA-actin localization to spines using the spine-dendrite ratio of drebrin A in cultured hippocampal neurons, and found that (1) chemical long-term potentiation (LTP) stimulation induces rapid DA-actin exodus and subsequent DA-actin re-entry in dendritic spines, (2) Ca(2+) influx through NMDA receptors regulates the exodus and the basal accumulation of DA-actin, and (3) the DA-actin exodus is blocked by myosin II ATPase inhibitor, but is not blocked by myosin light chain kinase (MLCK) or Rho-associated kinase (ROCK) inhibitors. These results indicate that myosin II mediates the interaction between NMDA receptor activation and DA-actin exodus in LTP induction. Furthermore, myosin II seems to be activated by a rapid actin-linked mechanism rather than slow MLC phosphorylation. Thus the myosin-II mediated DA-actin exodus might be an initial event in LTP induction, triggering actin polymerization and spine enlargement.

Keywords: drebrin, myosin II ATPase, myosin light chain kinase

Yamazaki H, Kojima N, Kato K, Hirose H, Iwasaki T, Mizui T, Takahashi H, Hanamura K, Roppongi R.T, Koibuchi N, Sekino Y, Mori N, Shirao T: Spikar, a novel drebrin-binding protein, regulates the formation and stabilization of dendritic spines.
J Neurochem. 2014;128(4):507-22.

Dendritic spines are small, actin-rich protrusions on dendrites, the development of which is fundamental for the formation of neural circuits. The actin cytoskeleton is central to dendritic spine morphogenesis. Drebrin is an actin-binding protein that is thought to initiate spine formation through a unique drebrin-actin complex at postsynaptic sites. However drebrin overexpression in neurons does not increase the final density of dendritic spines. In this study, we have identified and characterized a novel drebrin-binding protein, spikar. Spikar is localized in cell nuclei and dendritic spines, and accumulation of spikar in dendritic spines directly correlates with spine density. A reporter gene assay demonstrated that spikar acts as a transcriptional co-activator for nuclear receptors. We found that dendritic spine, but not nuclear, localization of spikar requires drebrin. RNA-interference knockdown and overexpression experiments demonstrated that extranuclear spikar regulates dendritic spine density by modulating de novo spine formation and retraction of existing spines. Unlike drebrin, spikar does not affect either the morphology or function of dendritic spines. These findings indicate that drebrin-mediated postsynaptic accumulation of spikar regulates spine density, but is not involved in regulation of spine morphology.

Keywords: drebrin, spiker, dendritic spines

Ishikawa M, Shiota J, Ishibashi Y, Hakamata T, Shoji S, Fukuchi M, Tsuda M, Shirao T, Sekino Y, Ohtsuka T, Baraban J.M, Tabuchi A: Identification, expression and characterization of rat isoforms of the SRF coactivator MKL1.
FEBS Open Bio. 2013;3:387-93.

Megakaryoblastic leukemia 1 (MKL1) is a member

of the MKL family of serum response factor (SRF) coactivators. Here we have identified three rat MKL1 transcripts: two are homologues of mouse MKL1 transcripts, full-length MKL1 (FLMKL1) and basic, SAP, and coiled-coil domains (BSAC), the third is a novel transcript, MKL1-elongated derivative of yield (MELODY). These rat MKL1 transcripts are differentially expressed in a wide variety of tissues with highest levels in testis and brain. During brain development, these transcripts display differential patterns of expression. The FLMKL1 transcript encodes two isoforms that utilize distinct translation start sites. The longer form possesses three actin-binding RPXXXEL (RPEL) motifs and the shorter form, MKL1met only has two RPEL motifs. All four rat MKL1 isoforms, FLMKL1, BSAC, MKL1met and MELODY increased SRF-mediated transcription, but not CREB-mediated transcription. Accordingly, the differential expression of MKL1 isoforms may help fine-tune gene expression during brain development.

Keywords: Megakaryoblastic leukemia 1, serum response factor, coiled-coil domains

Shigemoto-Mogami Y, Hoshikawa K, Goldman JE*, Sekino Y, Sato K: Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone.

J Neurosci. 2014;34(5):2231-43.

Although microglia have long been considered as brain resident immune cells, increasing evidence suggests that they also have physiological roles in the development of the normal central nervous system (CNS). In this study, we found large numbers of activated microglia in the forebrain subventricular zone (SVZ) of the rat from P1 to P10. Pharmacological suppression of the activation, which produces a decrease in levels of a number of proinflammatory cytokines, i.e., IL-1β, IL-6, TNF-α, and IFN-γ, significantly inhibited neurogenesis and oligodendrogenesis in the SVZ. In vitro neurosphere assays reproduced the enhancement of neurogenesis and oligodendrogenesis by activated microglia and showed that the cytokines revealed the effects complementarily. These results suggest that activated microglia accumulate in the early postnatal SVZ and that they enhance neurogenesis and oligodendrogenesis via released cytokines.

Keywords: microglia, subventricular zone, neurogenesis

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* Columbia University

Takahashi K, Ishii-Nozawa R*, Takeuchi K*, Nakazawa K, Sekino Y, Sato K: Niflumic acid activates additional currents of the human glial L-glutamate transporter EAAT1 in a substrate-dependent manner.

Biol Pharm Bull. 2013;36(12):1996-2004.

The astrocytic L-glutamate (L-Glu) transporter EAAT1 participates in the removal of L-Glu from the synaptic cleft and maintenance of non-toxic concentrations in the extracellular fluid. We have shown that niflumic acid (NFA), a non-steroidal anti-inflammatory drug (NSAIDs), alters L-Glu-induced EAAT1 currents in a voltage-dependent manner using the two-electrode voltage clamp technique in Xenopus oocytes expressing EAAT1. In this study, we characterised the effects of NFA on each type of ion-flux through EAAT1. NFA modulated currents induced by both L-Glu and L-aspartate (L-Asp) in a voltage-dependent manner. Ion-substitution experiments revealed that the activation of additional H+ conductance was involved in the modulation of currents induced by L-Asp and L-Glu, but Cl- was involved only with the L-Asp currents. NFA activated additional currents of EAAT1 in a substrate-dependent manner.

Keywords: astrocytic L-glutamate transporter, niflumic acid

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Oguchi-Katayama A, Monma A*, Sekino Y, Moriguchi T*, Sato K: Comparative gene expression analysis of the amygdalae of juvenile rats exposed to valproic acid at prenatal and postnatal stages.

J Toxicol Sci. 2013;38(3):381-402.

Gene expression profiles in the amygdala of juvenile rats were compared between the two autistic rat models for mechanistic insights into impaired social behavior and enhanced anxiety in autism. The rats exposed to VPA by intraperitoneal administration to their dams at embryonic day (E) 12 were used as a model for autism (E2IP), and those by subcutaneous

administration at postnatal day (P) 14 (P14SC) were used as a model for regressive autism; both of the models show impaired social behavior and enhanced anxiety as symptoms. Gene expression profiles in the amygdala of the rats (E12IP and P14SC) were analyzed by microarray and compared to each other. Only two genes, Neu2 and Mt2a, showed significant changes in the same direction in both of the rat models, and there were little similarities in the overall gene expression profiles between them. It was considered that gene expression changes per se in the amygdala might be an important cause for impaired social behavior and enhanced anxiety, rather than expression changes of particular genes.

Keywords: valproic acid, amygdala, microarray

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* Azabu University

Kinoshita M*, Nasu-Tada K*, Fujishita K*, Sato K, Koizumi S*: Secretion of matrix metalloproteinase-9 from astrocytes by inhibition of Tonic P2Y14-receptor-mediated signal(s).

Cell Mol Neurobiol. 2013;33(1):47-58.

Glial cells have various important roles in regulation of brain functions. For such events, extracellular nucleotides/P2 receptors have central roles. Although there have been huge amount of literature about activation of P2 receptors and glial functions, little is known about what happens in glia or the brain if glial P2 receptor is inhibited. Here we show that the inhibition of P2 receptors in astrocytes, the most abundant glial cells and cause a constitutive release of nucleotides, resulted in secretion of metalloproteinase-9 (MMP-9), a metal-dependent endopeptidase that degrades extracellular matrix molecules and is important in regulation of brain remodeling. When cultured astrocytes were treated with apyrase (ecto-nucleotidase), reactive blue 2 (P2 receptor antagonist), and pertussis toxin, they secreted MMP-9, suggesting that Gi-coupled P2Y receptor-mediated signals constitutively suppress the production of MMP-9. Among Gi-coupled P2Y receptors, we found that an inhibition of P2Y14 receptor, a receptor for nucleotide-sugars such as UDP-glucose, is responsible for the production of MMP-9 by pharmacological and molecular biochemical analysis. As for the mechanisms, the inhibition of P2Y14 receptors resulted in the release of tumor necrosis factor (TNF)-α which then acted on astrocytes to induce MMP-9. Taken together, our results suggest that the constitutive releases of nucleotide-sugars in astrocytes should play an important role in maintaining the normal status of the cell, through Gi-coupled P2Y14 receptors, and when the signal is removed, the cells start to release TNF-α, which then acts on astrocytes in a feedback fashion to boost MMP-9 synthesis and secretion.

Keywords: Astrocytes, Nucleotide-sugar, MMP-9

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Yamada S, Kotake Y*, Sekino Y, Kanda Y: AMP-activated protein kinase-mediated glucose transport as a novel target of tributyltin in human embryonic carcinoma cells.

Metallomics. 2013;5:484-91.

Organotin compounds such as tributyltin (TBT) are known to cause various forms of cytotoxicity, including developmental toxicity and neurotoxicity. However, the molecular target of the toxicity induced by nanomolar levels of TBT has not been identified. In the present study, we found that exposure to 100 nM TBT induced growth arrest in human pluripotent embryonic carcinoma cell line NT2/D1. Since glucose provides metabolic energy, we focused on the glycolytic system. We found that exposure to TBT reduced the levels of both glucose-6-phosphate and fructose-6-phosphate. To investigate the effect of TBT exposure on glycolysis, we examined glucose transporter (GLUT) activity. TBT exposure inhibited glucose uptake via a decrease in the level of cell surface-bound GLUT1. Furthermore, we examined the effect of AMP-activated protein kinase (AMPK), which is known to regulate glucose transport by facilitating GLUT translocation. Treatment with the potent AMPK activator, AICAR, restored the TBT-induced reduction in cell surface-bound GLUT1 and glucose uptake. In conclusion, these results suggest that exposure to nanomolar levels of TBT causes growth arrest by targeting glycolytic systems in human embryonic carcinoma cells. Thus, understanding the energy metabolism may provide new insights into the mechanisms of metal-induced cytotoxicity.

Keywords: Tin compound, Metabolomics, Neurotoxicity

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Ishida K*, Kotake Y*, Miyara M*, Aoki K*, Sanoh S*, Kanda Y, Ohta S*: Involvement of GluR2 decrease in lead-induced neuronal cell death.
J Toxicol Sci. 2013;38:513-21.

Ead is known to induce neurotoxicity, particularly in young children, and GluR2, an AMPA-type glutamate receptor subunit, plays an important role in neuronal cell survival. Therefore, we hypothesized that altered GluR2 expression plays a role in lead-induced neuronal cell death. To test this idea, we investigated the effect of exposure to 5 and 20 μM lead for 1-9 days on the viability and GluR2 expression of primary-cultured rat cortical neurons. The number of trypan-blue stained cells was increased by exposure to 5 μM lead for 9 days or 20 μM lead for 7-9 days, and LDH release was increased after exposure to 20 μM lead for 9 days. GluR2 expression was reduced by exposure to 5-100 μM lead, but not 0.1-1 μM lead, for 9 days. Immunocytochemistry also confirmed that GluR2 expression was decreased in the presence of lead. Application of 50 ng/ml brain-derived neurotrophic factor (BDNF) led to a recovery of lead-induced neuronal cell death, accompanied with increased GluR2 expression. Our results suggest that long-term exposure to lead induces neuronal cell death, in association with a decrease of GluR2 expression.
Keywords: Lead, GluR2, Neurotoxicity

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Usami M, Mitsunaga K*[1], Irie T, Miyajima A, Doi O*[2]: Proteomic analysis of ethanol-induced embryotoxicity in cultured post-implantation rat embryos.
J Toxicol Sci. 2014;39(2):285-92.

Protein expression changes were examined in day 10.5 rat embryos cultured for 24 hr in the presence of ethanol by using two-dimensional electrophoresis and mass spectrometry. Exposure to ethanol resulted in quantitative changes in many embryonic protein spots (16 decreased and 28 increased) at in vitro embryotoxic concentrations (130 and 195 mM); most changes occurred in a concentration-dependent manner. For these protein spots, 17 proteins were identified, including protein disulfide isomerase A3, alpha-fetoprotein, phosphorylated cofilin-1, and serum albumin. From the gene ontology classification and pathway mapping of the identified proteins, it was found that ethanol affected several biological processes involving oxidative stress and retinoid metabolism.
Keywords: Ethanol, Embryotoxicity, Ptroteomics

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Irie T, Matsuzaki Y*, Sekino Y, Hirai H*: Kv3.3 channels harbouring a mutation of spinocerebellar ataxia type 13 alter excitability and induce cell death in cultured cerebellar Purkinje cells.
J Physiol. 2014;592:229-47.

The cerebellum plays crucial roles in controlling sensorimotor functions. The neural output from the cerebellar cortex is transmitted solely by Purkinje cells (PCs), whose impairment causes cerebellar ataxia. Spinocerebellar ataxia type 13 (SCA13) is an autosomal dominant disease, and SCA13 patients exhibit cerebellar atrophy and cerebellar symptoms. Recent studies have shown that missensemutations in the voltage-gated K+ channel Kv3.3 are responsible for SCA13. In the rodent brain, Kv3.3 mRNAs are expressed most strongly in PCs, suggesting that the mutations severely affect PCs in SCA13 patients. Nevertheless, how these mutations affect the function of Kv3.3 in PCs and, consequently, the morphology and neuronal excitability of PCs remains unclear. To address these questions, we used lentiviral vectors to express mutant mouse Kv3.3 (mKv3.3) channels harbouring an R424H missense mutation, which corresponds to the R423Hmutation in theKv3.3 channels ofSCA13patients, inmousecerebellar cultures. TheR424H mutant-expressing PCs showed decreased outward current density, broadened action potentials and elevated basal [Ca2+]i compared with PCs expressing wild-type mKv3.3 subunits or those expressing green fluorescent protein alone. Moreover, expression of R424H mutant subunits induced impaired dendrite development and cell death selectively in PCs, both of which were rescued by blocking P/Q-type Ca2+ channels in the culture conditions. We therefore concluded that expression of R424H mutant subunits in

PCs markedly affects the function of endogenous Kv3 channels, neuronal excitability and, eventually, basal [Ca2+]i, leading to cell death. Theseresults suggest that PCs in SCA13 patients also exhibit similar defects in PC excitability and induced cell death, which may explain the pathology of SCA13.

Keywords: Cerebellum, Purkinje cells, SCA13

* Gunma University

Kanto H*[1], Washizaki K*[1], Ito M*[1], Matsunaga K*[2], Akamatsu H*[2], Kawai K*[3], Katoh N*[4], Natsuaki M*[5], Yoshimura I*[6], Kojima H, Okamoto Y*[7], Okuda M*[8], Kuwahara H*[9], Sugiyama M*[10], Kinoshita S*[11], Mori F*[11]: Optimal patch application time in the evaluation of skin irritation.
J Dermatol. 2013;40(5):363-9.

We investigated the optimum application for evaluating skin irritation response by using samples of irritants commonly used as additives in cosmetics and other common household products. We studied 47 volunteers (16 men and 31 women). We selected three types of surfactant, one moisturizer, one anti-infective agent and one oil solution. Using Finn chambers on Scanpor tape, we performed the patch test. A total of 0.015 mL of each sample was applied to the Finn chamber. For liquids, circular filter paper was soaked in 0.015 mL of the sample. Samples were placed on the upper back of participants, and closed for 4, 24 or 48 h. A patch application time of 24 h is sufficient to detect primary skin irritation from irritants in cosmetics and other common household products. In addition, we found that skin irritation reactions were strongest at 24 h after patch removal and that the reaction tended to be weaker at 48 h after patch removal. Patch testing to evaluate irritants should be performed by means of a 24-h patch test with a follow-up reading at 24 h after patch removal. An application time of 24 h places less of a burden on patients than a 48-h patch test.

Keywords: Patch test, Skin irritation, Application time

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*[2] Fujita Health University School of Medicine
*[3] Keiichi Kawai Skin Clinic
*[4] Kyoto Prefectural University of Medicine
*[5] Hyogo College of Medicine
*[6] Tokyo University of Science
*[7] Kose Corporation
*[8] Kao Corporation
*[9] Kanebo Cosmetics Inc.
*[10] Shiseido Co., Ltd.
*[11] Pola Chemical Industries Inc.

Kojima H, Hayashi K*[1], Sakaguchi H*[1], Omori T*[2], Otoizumi T*[2], Sozu T*[3], Kuwahara H*[4], Hayashi T*[4], Sakaguchi M*[5], Toyoda A*[5], Goto H*[5], Watanabe S*[6], Ahiko K*[6], Nakamura T*[6], Morimoto T*[7]: Second-phase validation study of short time exposure test for assessment of eye irritation potency of chemicals.
Toxicol In Vitro. 2013;27(6):1855-69.

A Short Time Exposure (STE) test is a cytotoxicity test that uses SIRC cells (rabbit corneal cell line) to assess eye irritation potency following a 5-min chemical exposure. This second-phase validation study assessed the predictive capacity of the STE test using 40 coded test substances at three laboratories. A Validation Management Team (VMT) then evaluated the predictivity of the STE test for United Nation (UN) Globally Harmonized System (GHS) categories using 63 test substances including the results of the first-phase validation study. The STE test can assess not only the severe or corrosive ocular irritants (corresponding to the UN GHS Category 1) but also non-irritant (corresponding to UN GHS Non Category) from other toxicity classes, especially for limited types of test substances. The predictivity by STE test, however, was insufficient for identification of UN GHS categories (Category 1, Category 2, or Non Category). These results suggest that the STE test can be recommended as an initial step in a top-down approach to identification of severe irritants and test substances that require classification for eye irritation (UN GHS Category 1) as well as an initial step in a bottom-up approach to identification of test substances that do not require classification for eye irritation (UN GHS Non Category) from other toxicity classes, especially for limited types of test substances. On the other hand, the STE test is not considered adequate for the identification of mild or moderate irritants (i.e., UN GHS Categories 2A and 2B) and severe irritants (UN GHS Category 1).

Keywords: Alternative method, Eye irritation, Validation

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Yamaguchi H*1,2, Kojima H, Takezawa T*1: Vitrigel-Eye Irritation Test Method using HCE-T cells.
Toxicological Sciences. 2013;135(2):347-55.

A previous multi-center validation study demonstrated high transferability and reliability of reactive oxygen species (ROS) assay for photosafety evaluation. The present validation study was undertaken to verify further the applicability of different solar simulators and assay performance. In 7 participating laboratories, 2 standards and 42 coded chemicals, including 23 phototoxins and 19 non-phototoxic drugs/chemicals, were assessed by the ROS assay using two different solar simulators (Atlas Suntest CPS series, 3 labs; and Seric SXL-2500V2, 4 labs). Irradiation conditions could be optimized using quinine and sulisobenzone as positive and negative standards to offer consistent assay outcomes. In both solar simulators, the intra- and inter-day precisions (coefficient of variation; CV) for quinine were found to be below 10%. The inter-laboratory CV for quinine averaged 15.4% (Atlas Suntest CPS) and 13.2% (Seric SXL-2500V2) for singlet oxygen and 17.0% (Atlas Suntest CPS) and 7.1% (Seric SXL-2500V2) for superoxide, suggesting high inter-laboratory reproducibility even though different solar simulators were employed for the ROS assay. In the ROS assay on 42 coded chemicals, some chemicals (ca. 19-29%) were unevaluable because of limited solubility and spectral interference. Although several false positives appeared with positive predictivity of ca. 76-92% (Atlas Suntest CPS) and ca. 75-84% (Seric SXL-2500V2), there were no false negative predictions in both solar simulators. A multi-center validation study on the ROS assay demonstrated satisfactory transferability, accuracy, precision, and predictivity, as well as the availability of other solar simulators.

Keywords: collagen vitrigel membrane, corneal epithelium, eye irritation test

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Stokes W*1,12, Srinivas G*2, McFarland R*3, Kulpa-Eddy J*4, Casey W*1, Walker A*2, Draayer H*5, Sebring R*6, Brown K*7, Balks E*8, Stirling C*9, Klaasen E*10, Hill R*2, Rippke B*2, Ruby K*2, Alt D*11, Mukhopadhyay S*12, Kojima H, Johnson N*13, Rinckel L*13, Doelling V*13, Jones B*13: Report on the international workshop on alternative methods for Leptospira vaccine potency testing: state of the science and the way forward.
Biologicals. 2013;41(5):279-94.

Routine potency testing of Leptospira vaccines is mostly conducted using a vaccination-challenge test that involves large numbers of hamsters and unrelieved pain and distress. NICEATM, ICCVAM, and their international partners organized a workshop to review the state of the science of alternative methods that might replace, reduce, and refine the use of animals for veterinary Leptospira vaccine potency testing and to identify ways to advance improved alternative methods. Vaccine manufacturers were encouraged to initiate or continue product-specific validation using in vitro enzyme-linked immunosorbent assays as replacements for potency testing of four common Leptospira serogroups. Participants discussed the potential for eliminating the back-titration procedure in the hamster challenge assay, which could reduce animal use by 50% for each individual potency test. Further animal reduction may also be possible by using cryopreserved Leptospira stock to replace continual passaging through hamsters. Serology assays were identified as a way to further reduce and refine animal use but should be considered only after attempting in vitro assays. Workshop participants encouraged consideration of analgesics and use of earlier humane endpoints when the hamster vaccination-challenge potency assay is used. International harmonization of alternative potency methods was recommended to avoid duplicative potency testing to meet regionally different requirements.

Keywords: Alternative methods, Leptospira vaccines, Potency

*1 National Institutes of Health

*2 U.S. Department of Agriculture (USDA)
*3 U.S. Food and Drug Administration
*4 USDA Animal and Plant Health Inspection Service
*5 Gourdneck View Consulting, LLC
*6 Colorado Serum Company
*7 Pair O'Docs Consultants
*8 Paul-Ehrlich-Institut
*9 Pfizer Ltd.
*10 MSD Animal Health
*11 USDA Agricultural Research Service
*12 National Institute of Allergy and Infectious Diseases
*13 Integrated Laboratory Systems Inc.

Ogawa K, Murasaki T*, Sugiura S*, Nakanishi M*, Shirai T*: Organ differences in the impact of $p27^{kip1}$ deficiency on carcinogenesis induced by *N*-methyl-*N*-nitrosourea.

J Appl Toxicol. 2013;33:471-9.

To evaluate the impact of p27 on carcinogenesis in various organs, *N*-methyl-*N*-nitrosourea (MNU), a direct-acting alkylating agent, was given to p27 knock-out mice. Groups of 20-40 male and female mice with null, hetero- or wild-type p27 alleles were given drinking water containing 240 ppm MNU or distilled water every other week for five cycles. The incidence and multiplicity of the induced proliferative lesions were then histologically evaluated at weeks 14 and 20. MNU treatment induced various lesions including squamous hyperplasia and squamous cell carcinoma in the forestomach, atypical hyperplasia and adenocarcinomas in the fundic and pyloric glands, adenomas and adenocarcinomas in the duodenum, malignant lymphomas in the thymus, liver, kidney and spleen and alveolar hyperplasia, adenomas, adenocarcinomas and malignant lymphomas in the lung. Although the incidences of the lesions in the forestomach, fundic and pyloric glands did not differ among the p27 genotypes, those of alveolar hyperplasia of the lung and malignant lymphoma of the thymus were significantly increased in p27-null males as compared with both wild- and hetero-type animals. Moreover, in both $p27^{+/+}$ and $p27^{+/-}$ cases, the rates for p27-positive cells were obviously increased in proliferative lesions of the pyloric gland and the lung. However, an increased rate of p27-positive cells was not observed in malignant lymphoma of the thymus. These findings suggest that p27 does not control the cell cycle equally in all organs affected by MNU-induced carcinogenesis.

Keywords: p27, MNU, stomach

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Tasaki M, Kuroiwa Y, Inoue T, Hibi D, Matsushita K, Ishii Y, Maruyama S*, Nohmi T, Nishikawa A, Umemura T: Oxidative DNA damage and *in vivo* mutagenicity caused by reactive oxygen species generated in the livers of *p*53-proficient or -deficient *gpt* delta mice treated with non-genotoxic hepatocarcinogens.

J Appl Toxicol. 2013;33:1433-41.

Oxidative stress is thought to participate in chemical carcinogenesis and may trigger gene mutations. To accurately assess the carcinogenesis risk posed to humans by chemical exposure, it is important to understand the pathways by which reactive oxygen species (ROS) are generated and the effects of the resulting oxidative stress. In the present study, p53-proficient and -deficient *gpt* delta mice were given pentachlorophenol (PCP), phenobarbital (PhB) or piperonyl butoxide (PBO), which are classified as non-genotoxic hepatocarcinogens in rodents, at the respective carcinogenic doses for 13 weeks. Exposure to PCP or PBO, but not PhB, invoked significant increases in liver DNA 8-hydroxydeoxyguanosine (8-OHdG) levels. Treatment with PCP significantly increased mRNA levels of the gene encoding NAD(P):quinone oxidoreductase 1 (NQO1) in the liver, suggesting that redox cycling of the PCP metabolite tetrachlorohydroquinone gave rise to ROS. Exposure to PhB or PBO significantly elevated CYP 2B10 mRNA levels while NQO1 levels were also significantly increased in PBO-treated mice. Therefore, in addition to involvement of the CYP catalytic pathway in the ROS-generated system of PBO, catechol derivatives produced from the opening of the PBO functional group methylenedioxy ring probably resulted in ROS generation. However, PCP, PBO and PhB failed to increase *gpt* and red/gam gene mutations in the liver independently of p53. Overall, the action of oxidative stress by ROS derived from the metabolism of these carcinogens might be limited to cancer-promoting activity, which supports the previous classification of these carcinogens as non-genotoxic.

Keywords: non-genotoxic hepatocarcinogen, reactive oxygen species, oxidative DNA damage

* Nihon University

Niwa T*[1], Toyoda T, Tsukamoto T*[2], Mori A*[1], Tatematsu M*[3], Ushijima1 T*[1]: Prevention of *Helicobacter pylori*-induced gastric cancers in gerbils by a DNA demethylating agent.
Cancer Prev Res. 2013;6:263-70.

Suppression of aberrant DNA methylation is a novel approach to cancer prevention, but, so far, the efficacy of the strategy has not been evaluated in cancers associated with chronic inflammation. Gastric cancers induced by *Helicobacter pylori* infection are known to involve aberrant DNA methylation and associated with severe chronic inflammation in their early stages. Here, we aimed to clarify whether suppression of aberrant DNA methylation can prevent *H. pylori*-induced gastric cancers using a Mongolian gerbil model. Administration of a DNA demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), to gerbils (0.125 mg/kg for 50-55 weeks) decreased the incidence of gastric cancers induced by *H. pylori* infection and *N*-methyl-*N*-nitrosourea (MNU) treatment from 55.2% to 23.3% ($P < 0.05$). In gastric epithelial cells, DNA methylation levels of six CpG islands (HE6, HG2, SB1, SB5, SF12, and SH6) decreased to 46% to 68% ($P < 0.05$) of gerbils without 5-aza-dC treatment. Also, the global DNA methylation level decreased from 83.0% ± 4.5% to 80.3% ± 4.4% (mean ± SD) by 5-aza-dC treatment ($P < 0.05$). By 5-aza-dC treatment, *Il1b* and *Nos2* were downregulated (42% and 58% of gerbils without, respectively) but *Tnf* was upregulated (187%), suggesting that 5-aza-dC treatment induced dysregulation of inflammatory responses. No obvious adverse effect of 5-aza-dC treatment was observed, besides testicular atrophy. These results showed that 5-aza-dC treatment can prevent *H. pylori*-induced gastric cancers and suggested that removal of induced DNA methylation and/or suppression of DNA methylation induction can become a target for prevention of chronic inflammation-associated cancers.
Keywords: *Helicobacter pylori*, DNA methylation, epigenetics

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Matsushita K, Kijima A, Ishii Y, Takasu S, Jin M, Kuroda K, Kawaguchi H*, Miyoshi N*, Nohmi T, Ogawa K, Umemura T: Development of a medium-term animal model using *gpt* delta rats to evaluate chemical carcinogenicity and genotoxicity.
J Toxicol Pathol. 2013;26:19-27.

In this study, the potential for development of an animal model (GPG46) capable of rapidly detecting chemical carcinogenicity and the underlying mechanisms of action were examined in *gpt* delta rats using a reporter gene assay to detect mutations and a medium-term rat liver bioassay to detect tumor promotion. The tentative protocol for the GPG46 model was developed based on the results of dose-response exposure to diethylnitrosamine (DEN) and treatment with phenobarbital over time following DEN administration. Briefly, *gpt* delta rats were exposed to various chemicals for 4 weeks, followed by a partial hepatectomy (PH) to collect samples for an *in vivo* mutation assay. The mutant frequencies (MFs) of the reporter genes were examined as an indication of tumor initiation. A single intraperitoneal (ip) injection of 10 mg/kg DEN was administered to rats 18 h after the PH to initiate hepatocytes. Tumor-promoting activity was evaluated based on the development of glutathione S-transferase placental form (GST-P)-positive foci at week 10. The genotoxic carcinogens 2-acetylaminofluorene (2-AAF), 2-amino-3-methylimidazo [4,5-f] quinolone (IQ) and safrole (SF), the non-genotoxic carcinogens piperonyl butoxide (PBO) and phenytoin (PHE), the non-carcinogen acetaminophen (APAP) and the genotoxic non-hepatocarcinogen aristolochic acid (AA) were tested to validate the GPG46 model. The validation results indicate that the GPG46 model could be a powerful tool in understanding chemical carcinogenesis and provide valuable information regarding human risk hazards.
Keywords: medium-term animal model, *gpt* delta rats, *in vivo* genotoxicity

* Kagoshima University

Fujimoto H, Woo GH, Morita R*, Itahashi M*, Akane H*, Nishikawa A, Shibutani M*: Increased cellular

distribution of vimentin and Ret in the cingulum of rat offspring after developmental exposure to decabromodiphenyl ether or 1,2,5,6,9,10-hexabromocyclododecane.

J Toxicol Pathol. 2013;26:119-29.

To determine effects of developmental exposure to brominated flame retardants (BFRs), weak thyroid hormone disruptors, on white matter development, white matter-specific global gene expression analysis was performed using microdissection techniques and microarrays in male rats exposed maternally to decabromodiphenyl ether (DBDE), one of the representative BFRs, at 10, 100 or 1000 ppm. Based on previous gene expression profiles of developmental hypothyroidism and DBDE-exposed cases, vimentin$^+$ immature astrocytes and ret proto-oncogene (Ret)$^+$ oligodendrocytes were immunohistochemically examined after developmental exposure to representative BFRs, i.e., DBDE, 1,2,5,6,9,10-hexabromocyclododecane (HBCD; 100, 1000 or 10,000 ppm) and tetrabromobisphenol A (TBBPA; 100, 1000 or 10,000 ppm). Vimentin$^+$ and Ret$^+$ cell populations increased at ≥ 100 ppm and ≥ 10 ppm DBDE, respectively. Vimentin$^+$ and Ret$^+$ cells increased at ≥ 1000 ppm HBCD, with no effect of TBBPA. The highest dose of DBDE and HBCD revealed subtle fluctuations in serum thyroid-related hormone concentrations. Thus, DBDE and HBCD may exert direct effects on glial cell development at ≥ middle doses. At high doses, hypothyroidism may additionally be an inducing mechanism, although its contribution is rather minor.

Keywords: BFRs, glial development, hypothyroidism

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Toyoda T, Akagi J, Cho YM, Mizuta Y, Onami S, Suzuki I, Ogawa K: Detection of γ-H2AX, a biomarker for DNA double-strand breaks, in urinary bladders of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN)-treated rats.

J Toxicol Pathol. 2013;26:215-21.

To evaluate the potential role of DNA repair in bladder carcinogenesis, we performed an immunohistochemical analysis of expression of various DNA repair enzymes and γ-H2AX, a high-sensitivity marker of DNA double-strand breaks, in the urothelium of male F344 rats treated with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN), a bladder-specific carcinogen. Our results clearly demonstrated that γ-H2AX aggregation was foci were specifically generated in nuclei of bladder epithelial cells of BBN-treated rats, not being found in untreated controls or mesenchymal cells. γ-H2AX-positive cells were detected not only in hyperplastic and neoplastic areas but also in normal-like urothelium after BBN treatment. These data indicate that γ-H2AX has potential as a useful biomarker for early detection of genotoxicity in the rat urinary bladder. To the best of our knowledge, this is the first report demonstrating expression of γ-H2AX during bladder carcinogenesis.

Keywords: urinary bladder, γ-H2AX, DNA repair

Ohmachi Y*, Yoshida M, Ogiu T*: Two cases of metastatic parathyroid carcinoma in male C3H mice following irradiation.

J Toxicol Pathol. 2013;26:413-7.

White nodules were observed in the thyroid in two male C3H mice (at 99 and 122 weeks of age) exposed to fast neutrons at the age of 8 weeks. Histopathologically, in both cases, tumors were developed in the region corresponding to the parathyroid gland, and the tumor cells were arranged in a solid sheet or nest-like structures. Necrosis, cell debris and/or hemorrhage were sometimes seen in the center of the tumor structures. Tumor cells were small and uniform with scanty cytoplasm, cell margins were indistinct, and basally located tumor cells were aligned along the vascular stroma. Mitotic figures were frequently observed. Metastasis to the renal cortex was observed in both cases. These cases were diagnosed as parathyroid carcinoma. A parathyroid tumor is an extremely rare endocrine tumor in mice, regardless of whether the tumor is spontaneous or experimentally induced. These cases may have been induced by neutron-exposure; however, how radiation induces parathyroid carcinoma in mice is not clear.

Keywords: parathyroid carcinoma, neutron, metastasis

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Okamura T, Umemura T, Inoue T, Tasaki M, Ishii Y, Nakamura Y*1, Park EY*1, Sato K*1, Matsuo T*2, Okamoto S*2, Nishikawa A, Ogawa K: Chemopreventive effects of 4-methylthio-3-butenyl

isothiocyanate (raphasatin) but not curcumin against pancreatic carcinogenesis in hamsters.
J Agric Food Chem. 2013;61:2103-8.

The modifying effects of 4-methylthio-3-butenyl isothiocyanate (MTBITC) and curcumin were investigated in N-nitrosobis(2-oxopropyl)amine (BOP)-initiated hamsters. Male 6-week-old Syrian hamsters were subcutaneously injected with 10 mg/kg body weight (b.w.) of BOP four times a week, and fed a diet supplemented with 80 mg/kg diet of MTBITC, equivalent to 4.6 mg/kg b.w./day for the initiation stage or 3.8 mg/kg b.w./day for the post-initiation stage administration, respectively or 2000 mg/kg diet of curcumin, equivalent to 118.8 mg/kg b.w./day for the initiation stage or 100.8 mg/kg b.w./day for the post-initiation stage administration, respectively. The incidence of combined pancreatic lesions, including atypical hyperplasias and adenocarcinomas, was significantly decreased to 55% ($P<0.05$) by the 80 mg/kg diet MTBITC given during the initiation stage as compared to the BOP alone group (80%) but not by the curcumin administration at 16 weeks after the BOP-treatment. In the second study, the multiplicity of combined pancreatic lesions was also significantly decreased to 0.50 ± 0.51 ($P<0.05$) by 700 mg/kg diet MTBITC given in the initiation stage (equivalent to 59.0 mg/kg b.w./day) as compared to the BOP alone group (1.10 ± 1.02). Our results indicate that the naturally occurring isothiocyanate MTBITC may exert preventive effects against BOP-initiation of hamster pancreatic carcinogenesis.
Keywords: isothiocyanate, chemoprevention, pancreatic cancer

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Imai T*, Cho YM, Takahashi M*, Kitahashi T*, Takami S, Nishikawa A, Ogawa K: High susceptibility of heterozygous (+/fa) lean Zucker rats to 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis.
Oncol Rep. 2013;29:1914-22.

Susceptibility to 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis was investigated in lean Zucker (+/fa) rats carrying one mutated leptin receptor gene and wild-type controls (+/+). Rats with both genotypes were given a single DMBA administration and divided into two groups, one group was fed on basal diet mixed with 10% corn oil and the other was fed on basal diet alone. The minimum latency period of palpable carcinomas in +/fa rats of both groups was 8 weeks following DMBA treatment, in contrast to the 11-12 weeks in +/+. The incidence and multiplicity of carcinomas increased or showed a tendency for increase in the early stages in +/fa rats of both groups as compared to the +/+ counterparts. The volumes of carcinomas showed a tendency to increase in the corn oil diet groups of both genotypes. The major histopathological phenotype of carcinomas in all groups was well-differentiated without distinct atypia (multiplicity, 0.69-1.09/rat), but moderately/poorly differentiated carcinomas with atypia were also found, predominantly in +/fa rats (0.09-0.21). These latter tumors were characterized by elevated ERK activity but not estrogen receptor expression. Serum leptin concentrations in +/fa rats at 7 weeks of age were higher than those in +/+ and were elevated by the corn oil diet; however, no obvious change was detected in other serum parameters examined. In conclusion, +/fa rats proved more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, and hyperleptinemia was suggested to contribute to tumor growth as well as to susceptibility to tumorigenesis and more aggressive phenotypes in Zucker lean rats.
Keywords: Zucker rat, DMBA, mammary carcinogenesis

* National Cancer Center Research Institute

Toyoda T, Takami S*1, Imai T*2, Cho YM, Hasumura M, Mizuta Y, Onami S, Suzuki I, Hirose M, Nishikawa A, Ogawa K: A 13-week subchronic toxicity study of garden balsam extract in F344 rats.
Jpn J Food Chem Safety. 2013;20:52-60.

A subchronic toxicity study of garden balsam (*Impatiens balsamina* L.) extract (GBE) was performed in male and female F344 rats with oral administration in their drinking water at concentrations of 0%, 1.25%, 2.5%, and 5.0% for 13 weeks. No chemical-related clinical signs and changes of body weights, food intake, and water consumption were observed in any groups during the experiment.

Regarding serum biochemistry, in males, significant increase of Na was observed in 2.5% and 5.0% group and that of Cl was seen in all treated groups. In females, significant increase of Cl and decrease of inorganic phosphorus (IP) were detected at 2.5% and 5.0%. However, no related histopathological lesions were observed in the kidney, intestine and bone tissue. Therefore, it is considered that the changes in serum electrolyte levels were not associated with any meaningful toxicological effects. There were no significant differences in hematological data, organ weights and histopathological findings among the groups. Based on the results, the no-observed-adverse-effect level (NOAEL) for GBE in male and female F344 rats was estimated to be more than 5.0% (3997 and 4577 mg/kg bw/day, respectively).

Keywords: garden balsam extract, *Impatiens balsamina*, subchronic toxicity

*1 Biosafety Research Center, Foods, Drugs and Pesticides
*2 National Cancer Center Research Institute

Pitchakarn P*1, Chewonarin T*1, Ogawa K, Suzuki S*2, Asamoto M*2, Takahashi S*2, Shirai T*2, Limtrakul P*1: Ellagic Acid inhibits migration and invasion by prostate cancer cell lines.
Asian Pac J Cancer Prev. 2013;14:2859-63.

Polyphenolic compounds from pomegranate fruit extracts (PFEs) have been reported to possess antiproliferative, pro-apoptotic, anti-inflammatory and anti-invasion effects in prostate and other cancers. However, the mechanisms responsible for the inhibition of cancer invasion remain to be clarified. In the present study, we investigated anti-invasive effects of ellagic acid (EA) in androgen-independent human (PC-3) and rat (PLS10) prostate cancer cell lines *in vitro*. The results indicated that non-toxic concentrations of EA significantly inhibited the motility and invasion of cells examined in migration and invasion assays. The EA treatment slightly decreased secretion of matrix metalloproteinase (MMP)-2 but not MMP-9 from both cell lines. We further found that EA significantly reduced proteolytic activity of collagenase/gelatinase secreted from the PLS-10 cell line. Collagenase IV activity was also concentration-dependently inhibited by EA. These results demonstrated that EA has an ability to inhibit invasive potential of prostate cancer cells through action on protease activity.

Keywords: ellagic acid, invasion, metastasis

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Nojiri A*1, Toyoda T, Tanaka T*2, Yoshida T*1, Tatematsu M*3, Tsukamoto T*4: Inflammation enhanced X-irradiation-induced colonic tumorigenesis in the Min mouse.
Asian Pac J Cancer Prev. 2013;14:4135-9.

Inflammation is potential risk factor of various human malignancies. Inflammatory bowel syndromes such as ulcerative colitis are well known as risk factors for colon cancer. Here, we examined enhancing effects of dextran sulfate sodium (DSS)-associated inflammation on X-irradiation induced colonic tumorigenesis in Min and wild-type (WT) mice. Animals were X-irradiated at 1.5 Gy at 5 weeks of age (at 0 experimental week) and 2% DSS in drinking water was administered at 5 or 11 experimental weeks. Mice were sacrificed at 16 weeks and incidence and multiplicity of colonic tumors were assessed. Incidence of colonic tumors in Min mouse was increased from 33.3% to 100% ($p<0.05$) with X-irradiation alone, whereas no tumors were developed in WT mice. In DSS-treated Min mice, X-irradiation increased the number of colonic tumors. Total number of colonic tumors was increased 1.57 times to 30.7 ± 3.83 tumors/mouse with X-irradiation+DSS at 5 weeks comapared to 19.6 ± 2.9 in corresponding DSS alone group ($p<0.05$). When the duration of inflammation was compared, longer period of DSS effect promoted more colonic tumorigenesis. Collectively, we conclude that X-irradiation and DSS-induced inflammation act synergistically for colonic tumorigenesis.

Keywords: Min mouse, X-irradiation, colon

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Toyoda T, Tsukamoto T*1, Yamamoto M*2, Ban H*1, Saito N*1, Takasu S, Shi L*3, Saito A*4, Ito S*5, Yamamura Y*5, Nishikawa A, Ogawa K, Tanaka T*6,

Tatematsu M[*7]: Gene expression analysis of a *Helicobacter pylori*-infected and high-salt diet-treated mouse gastric tumor model: identification of CD177 as a novel prognostic factor in patients with gastric cancer.
BMC Gastroenterol. 2013;13:122.

Helicobacter pylori（*H. pylori*）infection and excessive salt intake are known as important risk factors for stomach cancer in humans. In the present study, we investigated the global gene expression associated with stomach carcinogenesis and prognosis of human gastric cancer using a mouse model. To find candidate genes involved in stomach carcinogenesis, we firstly constructed a carcinogen-induced mouse gastric tumor model combined with *H. pylori* infection and high-salt diet. Gene expression profiles in glandular stomach of the mice were investigated by oligonucleotide microarray. In the microarray analysis, 35 and 31 more than two-fold up-regulated and down-regulated genes, respectively, were detected in the *H. pylori*-infection and high-salt diet combined group compared with the other groups. On immunohistochemical analysis of CD177, one of the up-regulated genes, in human advanced gastric cancer specimens, over-expression was evident in 33 of 55 cases, significantly correlating with a favorable prognosis. Multivariate analysis including clinicopathological factors as covariates revealed high expression of CD177 to be an independent prognostic factor for overall survival. These results suggest that our mouse model combined with *H. pylori* infection and high-salt diet is useful for gene expression profiling in gastric carcinogenesis, providing evidence that CD177 is a novel prognostic factor for stomach cancer.
Keywords: Cd177, gastric cancer, *Helicobacter pylori*

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Jin M, Kijima A, Hibi D, Ishii Y, Takasu S, Matsushita K, Kuroda K, Nohmi T, Nishikawa A, Umemura T: *In vivo* genotoxicity of methyleugenol in *gpt* delta transgenic rats following medium-term exposure.
Toxicol Sci. 2013;131:387-94.

Methyleugenol（MEG）is commonly used as a fragrance and flavoring agent, but MEG has been shown to induce hepatocellular tumors in rodents, the role of genotoxicity in the mode of action is not able to be fully understood in spite of the DNA reactive metabolite from MEG being identified. In this study, a *gpt* delta transgenic rat model was used to clarify whether genotoxic mechanisms are involved in MEG-induced hepatocarcinogenesis following medium-term exposure. F344 *gpt* delta rats were subjected to repeated oral administration of MEG at dosages of 0, 10, 30, or 100 mg/kg（a carcinogenic dose）for 13 weeks. The relative weight of the liver in the male and female rats that received 100 mg/kg and the absolute weight of the liver in the male rats that received 100 mg/kg of MEG were significantly increased. In addition, the number and area of glutathione S-transferase placental form（GST-P）positive foci and proliferating cell nuclear antigen（PCNA）positive cell ratios in the hepatocytes were significantly increased in the male and female rats that received 100 mg/kg compared to the control animals. In the *in vivo* mutation assays, a significant increase in the *gpt* and Spi^- mutant frequencies（MFs）was observed in both sexes at the carcinogenic dose. These results suggest a possible participation of genotoxic mechanisms in MEG-induced hepatocarcinogenesis.
Keywords: methyleugenol, *gpt* delta rat, medium-term exposure

Hibi D, Kijima A, Suzuki Y, Ishii Y, Jin M, Sugita-Konishi Y, Yanai T*, Nishikawa A, Umemura T: Effects of p53 knockout on ochratoxin A-induced genotoxicity in p53-deficient *gpt* delta mice.
Toxicology. 2013;304:92-9.

Ochratoxin A（OTA）is a mycotoxin produced by fungal species and is carcinogenic targeting the S3 segment of the renal proximal tubules in rodents. We previously reported that exposure of *gpt* delta rats to OTA induced both mutations in the red/gam gene（Spi^-）, suggesting large deletion mutations, and fluctuations in genes transcribed by p53 in the kidneys, which were associated with DNA double-strand break（DSB）repair, particularly homologous recombination

(HR) repair. In the present study, to investigate the effects of p53 knockout on OTA-induced mutagenicity, apoptosis, and karyomegaly in renal tubular cells, p53-proficient and p53-deficient *gpt* delta mice were given 1 and 5mg/kg of OTA for 4 weeks. Significant increases in Spi^- mutant frequencies (MFs) were observed in the kidneys of p53-deficient *gpt* delta mice given 5mg/kg of OTA, but not in the kidneys of p53-proficient *gpt* delta mice given the same dose. There were no changes in *gpt* MFs in both genotypes of mice treated with OTA. Western blotting analysis demonstrated that p53 protein levels in the kidneys of p53-proficient mice given OTA were significantly increased compared with the control. Incidences of apoptosis and karyomegaly in not only the outer stripe of outer medulla but also the cortex were significantly higher in p53-deficient at 5mg/kg than in p53-proficient *gpt* delta mice at same dose, which had no change in the cortex, the inner stripe of outer stripe, and the inner medulla. Given that p53 regulates HR repair in DSBs, these results suggest that OTA may promote large deletion mutations in the process of HR repair for DSBs. Additionally, the lower incidence of karyomegaly and apoptosis found in the p53-proficient *gpt* delta mice suggests that these phenomena may arise from OTA-induced DNA damage.

Keywords: ochratoxin, *gpt* delta mice, p53

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Fujii Y*[1], Kimura M*[1], Ishii Y, Yamamoto R*[1], Morita R*[1], Hayashi SM*[2], Suzuki K*[1], Shibutani M*[1]: Effect of enzymatically modified isoquercitrin on preneoplastic liver cell lesions induced by thioacetamide promotion in a two-stage hepatocarcinogenesis model using rats.

Toxicology. 2013;305:30-40.

To investigate the protective effect of enzymatically modified isoquercitrin (EMIQ) on the hepatocarcinogenic process, we used a two-stage hepatocarcinogenesis model in *N*-diethylnitrosamine-initiated and thioacetamide (TAA)-promoted rats. We examined the modifying effect of co-administration with EMIQ on the liver tissue environment including hepatic macrophages and lymphocytes and on the induction mechanism of preneoplastic cell apoptosis during early stages of hepatocellular tumor promotion. TAA increased the number and area of glutathione *S*-transferase placental form $(GST\text{-}P)^+$ liver cell foci and the numbers of proliferating and apoptotic cells in randomly selected areas in liver sections. Co-administration with EMIQ suppressed these effects. TAA also increased the numbers of $ED2^+$, $cyclooxygenase\text{-}2^+$, and heme $oxygenase\text{-}1^+$ liver cells, as well as the number of $CD3^+$ lymphocytes. These effects were also suppressed by EMIQ. EMIQ increased liver levels of thiobarbituric acid-reactive substance and 8-hydroxydeoxyguanosine, and $TUNEL^+$ apoptotic cells, death receptor 5 $(DR5)^+$ cells and $4\text{-hydroxy-2-nonenal}^+$ cells within $GST\text{-}P^+$ foci. Outside the $GST\text{-}P^+$ foci, EMIQ decreased the numbers of apoptotic cells and $DR5^+$ cells. These results suggest that TAA-induced tumor promotion involves activation of hepatic macrophages producing proinflammatory factors. EMIQ may suppress the TAA-induced tumor-promoting activity by an anti-inflammatory mechanism mediated by suppressing the activation of these macrophages. Furthermore, EMIQ may suppress tumor-promoting activity differentially between the inside and outside of $GST\text{-}P^+$ foci. Within $GST\text{-}P^+$ foci, EMIQ facilitates the apoptosis of preneoplastic cells through the upregulation of DR5. Outside the $GST\text{-}P^+$ foci, EMIQ suppresses apoptosis and the subsequent regeneration of non-transformed liver cells.

Keywords: death receptor 5, enzymatically modified isoquercitrin, thioacetamide

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Suzuki S*, Pitchakarn P*, Ogawa K, Naiki-Ito A*, Chewonarin T*, Punfa W*, Asamoto M*, Shirai T*, Takahashi S*: Expression of glutathione peroxidase 2 is associated with not only early hepatocarcinogenesis but also late stage metastasis.

Toxicology. 2013;311:115-23.

Understanding of mechanisms of cancer progression is very important for reduction of cancer mortality. Of six rat hepatocellular carcinoma (HCC) cell lines, differing in their metastatic potential to the lung after inoculation into the tail vein of nude mice, the most metastatic featured particular overexpression of glutathione peroxidase 2 (GPX2). Therefore, we analyzed the influence of interference in highly

metastatic L2 cells by siRNA transfection. Gpx2 siRNA significantly inhibited cell proliferation at 24 and 48h time points with induction of apoptosis but not cell cycle arrest. High expression of mutated p53 was detected in all HCC cell lines, with reduction in Gpx2 siRNA-transfected cells. Migration and invasion *in vitro* were also suppressed as compared to control siRNA-transfected cells and secretion of matrix metalloproteinase 9 was reduced. *In vivo*, the numbers and areas of metastatic nodules per area in the lungs were significantly reduced in the mice inoculated with Gpx2 siRNA-transfected cells as compared to control siRNA-transfected cells. In conclusion, expression of GPX2 is associated with cancer metastasis from rat HCCs both *in vitro* and *in vivo*. Together with immunohistochemical findings of elevated expression in rat and also human liver lesions, the results point to important roles in hepatocarcinogenesis.

Keywords: glutathione peroxidase 2, hepatocellular carcinoma, carcinogenesis

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Kuroda K, Ishii Y, Takasu S, Kijima A, Matsushita K, Watanabe M, Takahashi H, Sugita-Konishi Y, Sakai H*, Yanai T*, Nohmi T, Ogawa K, Umemura T: Cell cycle progression, but not genotoxic activity, mainly contributes to citrinin-induced renal carcinogenesis. *Toxicology*. 2013;311:216-24.

Citrinin (CTN) is a food-contaminating mycotoxin that efficiently induces renal tumors in rats. However, the modes of carcinogenic action are still unknown, preventing assessment of the risks of CTN in humans. In the present study, the proliferative effects of CTN and its causal factors were investigated in the kidneys of *gpt* delta rats. In addition, three *in vivo* genotoxicity assays (reporter gene mutation using *gpt* delta rats and comet and micronucleus assays using F344 rats) were performed to clarify whether CTN was genotoxic *in vivo*. CTN was administrated at 20 and 40mg/kg/day, the higher dose being the maximal tolerated dose and a nearly carcinogenic dose. In the kidney cortex of *gpt* delta rats, significant increases in the labeling indices of proliferating cell nuclear antigen (PCNA)-positive cells were observed at all doses of CTN. Increases in the mRNA expression levels of Ccna2, Ccnb1, Ccne1, and its transcription factor E2f1 were also detected, suggesting induction of cell cycle progression at all tested doses of CTN. However, histopathological changes were found only in rats treated with the higher dose of CTN, which was consistent with increases in the mRNA expression levels of mitogenic factors associated with tissue damage/regeneration, such as Hgf and Lcn2, at the same dose. Thus, the proliferative effects of CTN may result not only from compensatory reactions, but also from direct mitogenic action. Western blot analysis showed that ERK phosphorylation was increased at all doses, implying that cell cycle progression may be mediated by activation of the ERK pathway. On the other hand, *in vivo* genotoxicity analyses were negative, implying that CTN did not have the potential for inducing DNA damage, gene mutations, or chromosomal aberrations. The overall data clearly demonstrated the molecular events underlying CTN-induced cell cycle progression, which could be helpful to understand CTN-induced renal carcinogenesis.

Keywords: citrinin, cell proliferation, renal carcinogenesis

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Yamamoto R*, Shimamoto K*, Ishii Y, Kimura M*, Fujii Y*, Morita R*, Suzuki K*, Shibutani M*, Mitsumori K*: Involvement of PTEN/Akt signaling and oxidative stress on indole-3-carbinol (I3C)-induced hepatocarcinogenesis in rats. *Exp Toxicol Pathol*. 2013;65:845-52.

We previously reported that indole-3-carbinol (I3C) had hepatocellular tumor-promoting activity in a short-term (8 weeks) two-stage liver carcinogenesis model in rats. It was suggested that this effect was related to the production of reactive oxygen species (ROS) caused by cytochrome P450 1A (CYP1A) induction. In the present study, 0.5% I3C was administered to DEN-initiated rats for 26 weeks to examine the effect of prolonged administration of I3C and to clarify the possible mechanisms of I3C-induced hepatocarcinogenesis. The number and area of GST-P positive foci, ROS production, TBARS level, 8-OHdG content and mRNA levels of Ahr and Nrf2 gene batteries significantly increased in the DEN-I3C group compared with the DEN-alone group. Furthermore, some GST-P positive preneoplastic foci progressed to

hepatocellular adenomas with the prolongation of I3C administration. Lack of PTEN and phospho-Smad2/3 expression and translocations of PDPK1 and phospho-Akt substrates to underneath the cell membrane were observed in the majority of hepatocellular adenomas. In addition, the number of Ki-67 positive cells increased in adenomas compared with the preneoplastic foci. These results suggest that the administration of I3C for 26 weeks in DEN-initiated rats induces tumor progression from hepatocellular altered foci to hepatocellular adenomas by ROS-mediated Akt activation that inhibits the TGF-β/Smad signaling and results in the increased cell proliferation.

Keywords: indole-3-carbinol, hepatocarcinogenesis, reactive oxygen species

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Matsuo S, Takahashi M, Inoue K, Tamura K, Irie K, Kodama Y, Nishikawa A, Yoshida M: Thickened area of external granular layer and Ki-67 positive focus are early events of medulloblastoma in Ptch1(+/-) mice.

Exp Toxicol Pathol. 2013;65:863-73.

Patched1 (Ptch1) encodes a receptor for Sonic hedgehog (Shh) and is major gene related to human medulloblastoma (MB) in the Shh subgroup. MB is thought to arise from residual granule cell precursors (GCPs) located in the external granular layer (EGL) of the developing cerebellum. As the detailed preneoplastic changes of MB remain obscure, we immunohistochemically clarified the derived cell, early events of MBs, and the cerebellar developmental processes of Ptch1(+/-) (Ptch1) mice, an animal model of human MB of the Shh subgroup. In Ptch1 mice, the earliest proliferative lesions were detected at PND10 as focal thickened areas of outer layer of the EGL. This area was composed of GCP-like cells with atypia and nuclei disarrangement. In the latter cerebellar developmental period, GCP-like cell foci were detected at high incidence in the outermost area of the cerebellum. Their localization and morphological similarities indicated that the foci were derived from GCPs in the EGL. There were two types of the foci. A Ki-67-positive focus was found in Ptch1 mice only. This type resembled the GCPs in the outer layer of EGL characterized by having proliferating activity and a lack of neuronal differentiation. Another type of focus, Ki-67-negative, was observed in both genotypes and exhibited many of the same features of mature internal granule cells, suggesting that the focus had no preneoplastic potential. Due to morphological, immunohistochemical characteristics, our results indicate that the focal thickened area of EGL and Ki-67-positive foci are preneoplastic lesions of MB.

Keywords: cerebellar development, medulloblastoma, sonic hedgehog

Tasaki M, Kuroiwa Y, Inoue T, Hibi D, Matsushita K, Kijima A, Maruyama S*, Nishikawa A, Umemura T: Lack of *nrf2* results in progression of proliferative lesions to neoplasms induced by long-term exposure to non-genotoxic hepatocarcinogens involving oxidative stress.

Exp Toxicol Pathol. 2014;66:19-26.

To explore the role of oxidative stress in chemical carcinogenesis driven by non-genotoxic mechanisms, *nrf2*-deficient (*nrf2*$^{-/-}$) and nrf2-wild-type (*nrf2*$^{+/+}$) mice were exposed to pentachlorophenol (PCP) at concentrations of 600 or 1200ppm for 60 weeks, or piperonyl butoxide (PBO) at concentrations of 3000 or 6000ppm in the diet for 52 weeks, respectively. Additional studies were performed to examine 8-hydroxydeoxyguanosine (8-OHdG) levels in liver DNA and hepatotoxicological parameters in serum following 8 weeks of exposure of each group to PBO at the same doses as in the long-term study. Exposure to 600ppm PCP caused cholangiofibrosis (CF) only in *nrf2*$^{-/-}$ mice, while 1200ppm PCP induced CF in both genotypes. Moreover, cholangiocarcinomas were found with significant incidence only in *nrf2*$^{-/-}$ mice treated with 1200ppm PCP. Short-term exposure to 6000ppm PBO caused significant elevation of 8-OHdG levels in both genotypes, while exposure to 3000ppm caused a significant increase in 8-OHdG only in *nrf2*$^{-/-}$ mice. There were no inter-genotype changes in the incidences of regenerative hepatocellular hyperplasia (RHH) following long-term exposure to PBO. However, the incidence and multiplicity of hepatocellular adenomas, especially those observed in RHH, were much higher in *nrf2*$^{-/-}$ mice treated with 6000ppm PBO than in *nrf2*$^{+/+}$ mice treated with 6000ppm PBO. Therefore, oxidative stress generated through PCP or PBO metabolism may promote the proliferation and

progression of preneoplastic lesions to neoplasms.
Keywords: *nrf2*-deficient mice, cholangiofibrosis, regenerative hepatocellular hyperplasia

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Hibi D, Kijima A, Kuroda K, Suzuki Y, Ishii Y, Jin M, Nakajima M*, Sugita-Konishi Y, Yanai T*, Nohmi T, Nishikawa A, Umemura T: Molecular mechanisms underlying ochratoxin A-induced genotoxicity: global gene expression analysis suggests induction of DNA double-strand breaks and cell cycle progression.
J Toxicol Sci. 2013;38:57-69.

Ochratoxin A (OTA) is a renal carcinogen primarily affecting the S3 segment of proximal tubules in rodents. In our previous study, we reported that OTA induces reporter gene mutations, primarily deletion mutations, in the renal outer medulla (OM), specifically in the S3 segment. In the present study, to identify genes involved in OTA-induced genotoxicity, we conducted a comparative analysis of global gene expression in the renal cortex (COR) and OM of kidneys from *gpt* delta rats administered OTA at a carcinogenic dose for 4 weeks. Genes associated with DNA damage and DNA damage repair, and cell cycle regulation were site-specifically changed in the OM. Interestingly, genes that were deregulated in the OM possessed molecular functions such as DNA double-strand break (DSB) repair (Rad18, Brip1, and Brcc3), cell cycle progression (Cyce1, Ccna2, and Ccnb1), G(2)/M arrest in response to DNA damage (Chek1 and Wee1), and p53-associated factors (Phlda3 and Ccng1). Significant increases in the mRNA levels of many of these genes were observed in the OM using real-time RT-PCR. However, genes related to oxidative stress exhibited no differences in either the number or function of altered genes in both the OM and COR. These results suggested that OTA induced DSB and cell cycle progression at the target site. These events other than oxidative stress could trigger genotoxicity leading to OTA-induced renal tumorigenicity.
Keywords: DNA damage, karyomegaly, mycotoxin

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Yoshida M, Suzuki D, Matsumoto K*[1], Shirota M*[2], Inoue K, Takahashi M, Morita T, Ono A: Simulation of acute reference dose (ARfD) settings for pesticides in Japan.
J Toxicol Sci. 2013;38:205-14.

In order to develop guidelines for setting acute reference doses (ARfDs) for pesticides in Japan, we conducted simulations of ARfD settings based on evaluation reports for 201 pesticides assessed by the Food Safety Commission (FSC) in Japan over the last 8 years. Our conceptual principles were based on the concepts written by Solecki et al. (2005) and were adapted for toxicological data required in Japan. Through this process, we were able to set the ARfDs for over 90% of the 201 pesticides tested. The studies that provided the rationale for ARfD setting were primarily reproductive and developmental toxicity studies, acute neurotoxicity studies, and pharmacology studies. For approximately 30% of the pesticides simulated in the present study, it was not necessary to establish ARfDs. Some of the simulated ARfDs resulting from their endpoints may be conservative estimates, because the evaluation reports were written for acceptable daily intake settings. Thus, it was sometimes difficult to distinguish acute toxic alerts from repeated toxicities. We were unable to set an ARfD for 14 pesticides because of insufficient data on acute toxicities. This could be improved by more complete recordkeeping. Furthermore, we categorized the 201 pesticides by mechanism of action or chemical structure. Our simulation indicates that the conceptual framework presented here can be used as a basis for the development of guidelines on ARfD settings for pesticides in Japan.
Keywords: acute reference dose, pesticide, evaluation report

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Morita R*, Yafune A*, Shiraki A*, Itahashi M*, Ishii Y, Akane H*, Nakane F*, Suzuki K*, Shibutani M*, Mitsumori K*: Liver tumor promoting effect of orphenadrine in rats and its possible mechanism of action including CAR activation and oxidative stress.
J Toxicol Sci. 2013;38:403-13.

Orphenadrine (ORPH), an anticholinergic agent, is a cytochrome P450 (CYP) 2B inducer. CYP2B inducers are known to have liver tumor-promoting effects in

rats. In this study, we performed a rat two-stage liver carcinogenesis bioassay to examine the tumor-promoting effect of ORPH and to clarify its possible mechanism of action. Male rats were given a single intraperitoneal injection of N-diethylnitrosamine (DEN) as an initiation treatment. Two weeks after DEN administration, rats were fed a diet containing ORPH (0, 750, or 1,500 ppm) for 6 weeks. One week after the ORPH-administration rats were subjected to two-thirds partial hepatectomy for the acceleration of hepatocellular proliferation. The number and area of glutathione S-transferase placental form-positive foci significantly increased in the DEN-ORPH groups. Real-time RT-PCR revealed increased mRNA expression levels of Cyp2b1/2, Mrp2 and Cyclin D1 in the DEN-ORPH groups and of Gpx2 and Gstm3 in the DEN-High ORPH group. Microsomal reactive oxygen species (ROS) production and oxidative stress markers such as thiobarbituric acid-reactive substances and 8-hydroxydeoxyguanosine were increased in the DEN-High ORPH group. Immunohistochemically, constitutively active/androstane receptor (CAR) were clearly localized in the nuclei of hepatocytes in the DEN-ORPH groups. These results suggest that ORPH causes nuclear translocation of CAR resulting in the induction of the liver tumor-promoting activity. Furthermore, oxidative stress resulting from ROS production is also involved in the liver tumor-promoting activity of ORPH.

Keywords: orphenadrine, constitutive active/androstane receptor, reactive oxygen species

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Inoue K, Morikawa T, Takahashi M, Yoshida M, Ogawa K: A 13-week subchronic toxicity study of grape skin extract in F344 rats.
J Toxicol Sci. 2013;38:559-70.

A 13-week repeated oral dose toxicity study of grape skin extract (GSE) was performed using F344 rats. Four groups of animals, each consisting of ten males and ten females, were fed a diet containing 0%, 0.2%, 1.0% or 5.0% GSE for 13 weeks. Throughout the experiment, there were no treatment-related changes in clinical signs, body weight or mean food intake in any of the treated groups of either gender. Hematological studies and serum biochemical analyses revealed no treatment-related changes in all groups in both genders. In the glandular epithelial cells of the parotid glands, diffuse hypertrophy and basophilia was observed in all animals in both 5.0% groups. Hypertrophy of the parotid glands was not detected in the 0.2% or the 1.0% dose groups. In female kidneys, slight calcification in the renal proximal tubules of the cortex and medulla was observed in all groups including controls. This is a common spontaneous change in female rats, and the incidence was comparable between controls and treated groups. However, the number of tubules with calcification was higher in the 5.0% group based on a semi-morphometric analysis. Based on the histopathology of the parotid glands and the minor change in the kidneys, the no observed adverse effect level (NOAEL) of GSE in the present study was a 1.0% treatment dose in both genders (males: 0.6 ± 0.2 g/kg body weight/day; females: 0.7 ± 0.1 g/kg body weight/day).

Keywords: grape skin extract, subchronic toxicity, F344 rats

Nemoto K[*1], Ikeda A[*1], Tanaka T[*1], Inoue K, Yoshida M, Nishikawa A, Gamou T[*2], Habano W[*2], Ozawa S[*2], Degawa M[*1]: Change in the gene expression of the N-methyl-D-aspartate receptor 2C subunit by dietary β-naphthoflavone, indole-3-carbinol, or acetaminophen in the rat liver.
J Toxicol Sci. 2013;38:611-7.

We have previously demonstrated super-induced expression of the Grin2c gene encoding the N-methyl-D-aspartate receptor 2C subunit during the process of liver enlargement induced by phenobarbital, clofibrate, piperonyl butoxide, or lead nitrate. In the present study, hepatic Grin2c gene expression levels were assessed by real-time RT-PCR in male F344 rats fed for 3 days, 4 weeks, and 13 weeks a diet containing either β-naphthoflavone (BNF) (5,000 ppm), indole-3-carbinol (I3C) (2,000 ppm), or acetaminophen (AA) (12,500 ppm until the first 14 days; 10,000 ppm from 15 days on), each of which is capable of inducing hepatocellular hypertrophy. Especially, either the 4-week or the 13-week treatment with each chemical, except for BNF, resulted in a drastic increase in the expression level of the Grin2c gene. DNA microarray analysis using RNAs of 13-week-treated rats showed

that in the I3C- and AA-treated rats, the fold-increase rates of the Grin2c gene ranked second and first, respectively, among the genes analyzed. Histopathological analyses indicated that the slight hepatocellular hypertrophy in the periportal area and the hepatocellular necrosis in a portion of the centrilobular area developed in the BNF-treated and AA-treated rats, respectively. In addition, relative liver weight was significantly higher in the rats treated with BNF and I3C than in the control rats. The present findings suggest the possibility that the induction of Grin2c gene expression is not necessarily dependent on only the development of liver enlargement, although the significance of this induction remains unclear.

Keywords: hepatocellular hypertrophy, NMDA receptor, gene expression

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Hayashi S, Taketa Y, Inoue K, Takahashi M, Matsuo S, Irie K, Watanabe G*, Yoshida M: Effects of pyperonyl butoxide on the female reproductive tract in rats.

J Toxicol Sci. 2013;38:891-902.

This study was investigated the effects of piperonyl butoxide (PBO) on the female reproductive tract. Female Crj:Donryu rats were fed a basal diet containing 5,000, 10,000 or 20,000 ppm PBO for 28 days, and compared with food-restricted rats of comparable body weights to those in the PBO 10,000 or 20,000 ppm groups. Although treatment with 20,000 ppm PBO for 28 days depressed body weight gain, the abnormal estrous cyclicity, mainly prolonged diestrus, was also induced by the PBO treatment which was not correlated with body weight change. 20,000 ppm PBO treatment markedly decreased uterine weights and slightly decreased ovarian weights. 10,000 and 20,000 ppm PBO treatment increased liver weights. These cycle and organ weight changes were linked to atrophic uterus and increased atretic follicles in the ovary. In hormone assays, PBO at both doses reduced serum E2 levels, but did not affect corticosterone levels. An anti-uterotrophic assay showed a slight but significant decrease in absolute uterine weight and a reduction of endometrial epithelium height in the 20,000 ppm group. PBO was positive in an ER α antagonist reporter gene assay, although the activity was much weaker than that of 4-hydroxytamoxifen. These results indicate that high-dose PBO treatment directly induces atrophic changes in the female reproductive tract in rats, and these effects are likely the result of a hypoestrogenic state and the anti-estrogenic activity of PBO.

Keywords: piperonyl butoxide, female reproductive tract, anti-uterotrophic assay

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Toyoda T, Cho YM, Mizuta Y, Akagi J, Nishikawa A, Ogawa K: A 13-week subchronic toxicity study of sodium iron chlorophyllin in F344 rats.

J Toxicol Sci. 2014;39:109-19.

Sodium iron chlorophyllin (SIC), a water-soluble chlorophyll derivative, has been used as a food additive for green coloration. In the present study, a subchronic toxicity study of SIC was performed in male and female F344 rats with oral administration in diet at concentrations of 0%, 0.2%, 1.0%, and 5.0% for 13 weeks. No mortalities, abnormal clinical signs, and hematological changes were observed in any of the groups during the experiment. Significant reduction of body weight gain was noted in 5.0% males. In serum biochemistry, serum transferrin levels were significantly increased in 5.0% males and females. Relative spleen weights of both sexes were markedly reduced with 5.0% SIC as compared to the controls, and absolute weights of spleen were also significantly decreased in males. On histopathological assessment, diffuse hypertrophy of acinar cells in the parotid gland was observed in all examined 5.0% males and females, but not in the other groups. Based on the histopathology of the parotid glands, the no-observed-adverse-effect level (NOAEL) of SIC in the present study was estimated to be 1.0% (609 mg/kg bw/day for males and 678 mg/kg bw/day for females).

Keywords: sodium iron chlorophyllin, subchronic toxicity, salivary glands

Fujii Y*, Segawa R*, Kimura M*, Wang L*, Ishii Y, Yamamoto R*, Morita R*, Mitsumori K*, Shibutani M*: Inhibitory effect of α-lipoic acid on thioacetamide-induced tumor promotion through suppression of inflammatory cell responses in a two-

stage hepatocarcinogenesis model in rats.
Chem Biol Interact. 2013;205:108-18.

To investigate the protective effect of α-lipoic acid (a-LA) on the hepatocarcinogenic process promoted by thioacetamide (TAA), we used a two-stage liver carcinogenesis model in N-diethylnitrosamine (DEN)-initiated and TAA-promoted rats. We examined the modifying effect of co-administered a-LA on the liver tissue environment surrounding preneoplastic hepatocellular lesions, with particular focus on hepatic macrophages and the mechanism behind the decrease in apoptosis of cells surrounding preneoplastic hepatocellular lesions during the early stages of hepatocellular tumor promotion. TAA increased the number and area of glutathione S-transferase placental form (GST-P)$^+$ liver cell foci and the numbers of proliferating and apoptotic cells in the liver. Co-administration with a-LA suppressed these effects. TAA also increased the numbers of ED2$^+$, cyclooxygenase-2$^+$, and heme oxygenase-1$^+$ hepatic macrophages as well as the number of CD3$^+$ lymphocytes. These effects were also suppressed by a-LA. Transcript levels of some inflammation-related genes were upregulated by TAA and downregulated by a-LA in real-time RT-PCR analysis. Outside the GST-P$^+$ foci, a-LA reduced the numbers of apoptotic cells, active caspase-8$^+$ cells and death receptor (DR)-5$^+$ cells. These results suggest that hepatic macrophages producing proinflammatory factors may be activated in TAA-induced tumor promotion. a-LA may suppress tumor-promoting activity by suppressing the activation of these macrophages and the subsequent inflammatory responses. Furthermore, a-LA may suppress tumor-promoting activity by suppressing the DR5-mediated extrinsic pathway of apoptosis and the subsequent regeneration of liver cells outside GST-P$^+$ foci.
Keywords: α-lipoic acid, thioacetamide, hepatocarcinogenesis

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Kuroda K, Kijima A, Jin M, Ishii Y, Takasu S, Matsushita K, Nishikawa A, Umemura T: The effects of long-term exposure to ozokerite mainly consisting of an aliphatic series of hydrocarbons using F344 rats.
Food Chem Toxicol. 2013;55:476-83.

Combined chronic toxicity and carcinogenicity studies of ozokerite (OZK), a natural wax substance used as a food additive for a gum base, were performed in male and female F344 rats. Dietary concentrations of 0, 0.05, 0.1 and 0.2% OZK were applied in a 52-week chronic toxicity study and 0, 0.1 and 0.2% in a 104-week carcinogenicity study. In the chronic toxicity study, treatment with OZK caused a xenobiotic reaction against absorbed OZK, including formation of histiocytosis and granulomas with crystalline material in many organs in all of the treated males and females. Particularly in the liver, granulomatous inflammation was accompanied by hepatocellular vacuolation and changes in the serum biochemical parameters indicative of hepatic disorder. The number and area of glutathione S-transferase placental form (GST-P) positive foci were increased in all of the treated groups of both sexes, suggesting the proliferative effect of OZK. In the carcinogenicity study, the incidence of hepatocellular adenoma and the total tumor incidence in the liver of all of the treated males were significantly increased compared with the controls. In conclusion, long-term exposure to OZK caused systemic chronic inflammation due to a foreign body response. OZK was weakly carcinogenic in the liver of male F344 rats.
Keywords: ozokerite, chronic toxicity and carcinogenicity studies, food additive

Taketa Y, Inoue K, Takahashi M, Yamate J*, Yoshida M: Differential morphological effects in rat corpora lutea among ethylene glycol monomethyl ether, atrazine, and bromocriptine.
Toxicol Pathol. 2013;41:736-43.

Ethylene glycol monomethyl ether (EGME) or atrazine induces luteal cell hypertrophy in rats. Our previous study suggested that EGME stimulates both new and old corpora lutea (CL), while atrazine stimulates new CL. Bromocriptine (BRC) is known to suppress the luteolysis in rats. This study investigated the light- and electron-microscopic luteal changes induced by EGME, atrazine, or BRC. Female rats were treated with EGME (300 mg/kg/day), BRC (2 mg/kg/day), EGME and BRC (EGME + BRC), or atrazine (300 mg/kg/day) for 7 days. Luteal cell hypertrophy induced by EGME, EGME + BRC, and atrazine was

subclassified into the following two types: CL hypertrophy, vacuolated type (CL-V) characterized by intracytoplasmic fine vacuoles, and CL hypertrophy, eosinophilic type (CL-E) characterized by eosinophilic and abundant cytoplasm. The proportions of CL-V and CL-E were different among the treatments. BRC-treated old CL showed lower proportion of endothelial cells and fibroblasts than normal old CL. Ultrastructural observation revealed that the luteal cells of CL-V contained abundant lipid droplets, whereas those of CL-E in EGME and EGME + BRC groups showed uniformly well-developed smooth endoplasmic reticulum. No clear ultrastructural difference was observed between the control CL and atrazine-treated CL-E. These results indicate that EGME, atrazine, and BRC have differential luteal morphological effects.

Keywords: ethylene glycol monomethyl ether, atrazine, bromocriptine

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Sakamoto Y, Inoue K, Takahashi M, Taketa Y, Kodama Y, Nemoto K*[1], Degawa M*[1], Gamou T*[2], Ozawa S*[2], Nishikawa A, Yoshida M: Different pathways of constitutive androstane receptor-mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether.

Toxicol Pathol. 2013;41:1078-92.

The constitutive androstane receptor (CAR) is essential for Cyp2b induction, liver hypertrophy, and hepatocarcinogenesis in response to phenobarbital (PB). Liver hypertrophy with Cyp2b induction is a major mode of action of hepatocarcinogenesis in rodents. However, it remains unclear whether CAR is involved in the response to many other nongenotoxic hepatocarcinogens besides PB. In this study, we investigated CAR involvement in liver hypertrophy and hepatocarcinogenesis of Cyp2b-inducing nongenotoxic hepatocarcinogens, piperonyl butoxide (PBO), and decabromodiphenyl ether (DBDE), using wild-type and CAR knockout (CARKO) male mice. PB was used as the positive control. In the wild-type mice, 4-week treatment with PBO, DBDE, or PB induced hepatocellular hypertrophy with increased Cyp2b10 messenger RNA and Cyp2b protein expression. In CARKO mice, only PBO showed liver hypertrophy with Cyp2b10 and Cyp3a11 induction. After 27-week treatment following diethylnitrosamine initiation, PBO and PB generated many eosinophilic altered foci/adenomas in wild-type mice; however, the lesions were far less frequent in CARKO mice. DBDE increased the multiplicity of basophilic altered foci/adenomas in wild-type and CARKO mice. Our findings indicate that murine CAR plays major roles in hepatocarcinogenesis but not in liver hypertrophy of PBO. DBDE may act via CAR-independent pathways during hepatocarcinogenesis.

Keywords: hepatocarcinogenesis, piperonyl butoxide, decabromodiphenyl ether

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Takahashi M, Inoue K, Morikawa T, Matsuo S, Hayashi S, Tamura K, Watanabe G*, Taya K*, Yoshida M: Delayed effects of neonatal exposure to 17alpha-ethynylestradiol on the estrous cycle and uterine carcinogenesis in Wistar Hannover GALAS rats.

Reprod Toxicol. 2013;40:16-23.

We investigated the delayed effects of neonatal exposure to 17α-ethynylestradiol (EE) on the female reproductive tract using Wistar Hannover GALAS rats. Female pups received single injections of EE (0, 0.02, 0.2, 2, 20, or 200 μg/kg) within 24hours after birth and estrous cyclicity was observed until 10 months of age. All animals were treated at 9 weeks of age with the uterine carcinogen, N-ethyl-N›-nitro-N-nitrosoguanidine. Although the vaginal opening was not affected, abnormal cycles were significantly increased from 0.2 μg/kg. Persistent estrus was prominent and the incidence increased age- and dose-dependently. Severity of atypical hyperplasia of the uterus tended to increase from 2 μg/kg. In these groups, serum progesterone level was lowered relative to estradiol level. In conclusion, estrous cyclicity was a sensitive indicator reflecting delayed effects on the female reproductive tract. Early onset of anovulation leading to prolonged estrogen exposure might be a risk factor for uterine carcinogenesis.

Keywords: 17alpha-ethynylestradiol, neonatal exposure, uterine carcinogenesis

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Shigematsu Y*[1], Niwa T*[1], Rehnberg E*[1], Toyoda T, Yoshida S*[1], Mori A*[1], Wakabayashi M*[1], Iwakura Y*[2], Ichinose M*[3], Kim YJ*[4], Ushijima T*[1]: Interleukin-1β induced by *Helicobacter pylori* infection enhances mouse gastric carcinogenesis.
Cancer Lett. 2013;340:141-7.

Interleukin-1β (*Il1b*) is considered to be involved in *Helicobacter pylori* (*HP*)-induced human gastric carcinogenesis, while the role of its polymorphisms in gastric cancer susceptibility remains controversial. Here, we aimed to clarify the role of *HP* infection-induced IL1B in gastric inflammation and carcinogenesis using *Il1b*$^{-/-}$ (*Il1b*-null) mice. In gastric mucosa of the *Il1b*$^{+/+}$ (WT) mice, *HP* infection induced *Il1b* expression and severe inflammation. In contrast, in *Il1b*-null mice, recruitment of neutrophils and macrophages by *HP* infection was markedly suppressed. In a carcinogenicity test, the multiplicity of gastric tumors was significantly suppressed in the *Il1b*-null mice (58% of WT; $P < 0.005$). Mechanistically, *HP* infection induced NF-κB activation both in the inflammatory and epithelial cells in gastric mucosae, and the activation was attenuated in the *Il1b*-null mice. Accordingly, increased proliferation and decreased apoptosis of gastric epithelial cells induced by *HP* infection in the WT mice were attenuated in the *Il1b*-null mice. These results demonstrated that the IL1B physiologically induced by *HP* infection enhanced gastric carcinogenesis by affecting both inflammatory and epithelial cells.

Keywords: interleukin-1β, gastric cancer, *Helicobacter pylori*

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Tamura K, Inoue K, Takahashi M, Matsuo S, Irie K, Kodama Y, Ozawa S*, Nishikawa A, Yoshida M: Dose-response involvement of constitutive androstane receptor in mouse liver hypertrophy induced by triazole fungicides.
Toxicol Lett. 2013;221:47-56.

To clarify the dose-response relationship between constitutive androstane receptor (CAR) activity and induction of cytochrome P450 2B (CYP2B) expression and hypertrophy by triazole fungicides in mouse liver, three dose levels of cyproconazole (Cypro), tebuconazole (Teb), fluconazole (Flu), and phenobarbital (PB), a typical CYP2B inducer, were administrated in diet to male wild-type (WT) and CAR-knockout (CARKO) mice for one week. In WT mice, all compounds dose-dependently induced liver weight increases and hepatocellular hypertrophy accompanied by CYP2B expression. In CARKO mice, these effects were not induced by PB, while Cypro or Flu induced these effects only at the highest dose. Dose-dependent liver hypertrophy was detected in CARKO mice treated with Teb, but at the lowest dose the intensity was weakened compared to WT mice. The present results indicate that Cypro and Flu mainly induced CAR-mediated liver hypertrophy, while Teb slightly involved CAR. The involvement of CAR in triazole-induced liver hypertrophy was dose-responsive. In addition, all three triazoles have non-CAR-mediated liver hypertrophy pathways, indicating that the hypertrophy induced by these triazoles differs from that of PB.

Keywords: triazole, CAR, liver hypertrophy

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Kuroda K, Kijima A, Ishii Y, Takasu S, Jin M, Matsushita K, Kodama Y, Umemura T: Flumequine enhances the *in vivo* mutagenicity of MeIQx in the mouse liver.
Arch Toxicol. 2013;87:1609-19.

The combined effects of various carcinogens found in food products are a concern for human health. In the present study, the effects of flumequine (FL) on the *in vivo* mutagenicity of 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx) in the liver were investigated. Additionally, we attempted to clarify the underlying mechanisms through comprehensive gene analysis using a cDNA microarray. Male *gpt* delta mice were fed a diet of 0.03 % MeIQx, 0.4 % FL, or 0.03 % MeIQx + 0.4 % FL for 13 weeks. The effects of cotreatment with phenobarbital (PB) were also examined. Treatment with MeIQx alone increased *gpt* and Spi$^-$ mutant frequencies, and cotreatment with FL,

but not with PB, further exacerbated these effects, despite the lack of *in vivo* genotoxicity in mice treated with FL alone. FL caused an increase in Cyp1a2 mRNA levels and a decrease in Ugt1b1 mRNA levels, suggesting that the enhancing effects of FL may be due in part to modification of MeIQx metabolism by FL. Moreover, FL induced an increase in hepatocyte proliferation accompanied by hepatocellular injury. Increases in the mRNA levels of genes encoding cytokines derived from Kupffer cells, such as Il1b and Tnf, and cell cycle-related genes, such as Ccnd1 and Ccne1, suggested that FL treatment increases compensatory cell proliferation. Thus, the present study clearly demonstrated the combined effects of 2 different types of carcinogens known as contaminants in foods.

Keywords: MeIQx, flumequine, *gpt* delta mouse

Yoshida M, Suzuki D, Matsumoto K[*1], Shirota M[*2], Inoue K, Takahashi M, Morita T, Ono A: Basic Principles for Setting Acute Reference Dose, ARfD in Japan.

Shokuhin Eiseigaku Zasshi. 2013;54:331-4.

Basic principles for simulation of acute reference dose (ARfD) setting were defined based on the work of Solecki et al. (2005). The principles are: (1) Appearance of acute toxicity within 24 h after oral administration. (2) Rationale for setting toxicity that appears or could appear after single oral administration. (3) ARfD setting is assumed to be necessary for all pesticides. (4) ARfD setting is not necessary when the value is at or above the cutoff level. (5) The setting basically applies to the general population. (6) ARfD is set based on the lowest NOAEL among all the available study data concerning endpoints for acute effects. (7) Effects of exposure during critical periods should be considered as endpoints for ARfD setting. (8) The approach for the safety coefficient is the same as that for acceptable daily intake. (9) If available, human data are acceptable as an endpoint for ARfD setting.

Keywords: ARfD, pesticide, JMPR

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Kuroda K, Hibi D, Ishii Y, Takasu S, Kijima A, Matsushita K, Masumura K, Watanabe M, Sugita-Konishi Y[*1], Sakai H[*2], Yanai T[*2], Nohmi T, Ogawa K, Umemura T: Ochratoxin A induces DNA double-strand breaks and large deletion mutations in the carcinogenic target site of *gpt* delta rats.

Mutagenesis. 2014;29:27-36.

Ochratoxin A (OTA) is a carcinogen targeting proximal tubules at the renal outer medulla (ROM) in rodents. We previously reported that OTA increased mutant frequencies of the red/gam gene (Spi^-), primarily deletion mutations. In the present study, Spi^- assays and mutation spectrum analyses in the Spi^- mutants were performed using additional samples collected in our previous study. Spi^- assay results were similar to those in our previous study, revealing large (>1kb) deletion mutations in the red/gam gene. To clarify the molecular progression from DNA damage to gene mutations, *in vivo* comet assays and analysis of DNA damage/repair-related mRNA and/or protein expression was performed using the ROM of *gpt* delta rats treated with OTA at 70, 210 or 630 μg/kg/day by gavage for 4 weeks. Western blotting and immunohistochemical staining demonstrated that OTA increased γ-H2AX expression specifically at the carcinogenic target site. In view of the results of comet assays, we suspected that OTA was capable of inducing double-strand breaks (DSBs) at the target sites. mRNA and/or protein expression levels of homologous recombination (HR) repair-related genes (Rad51, Rad18 and Brip1), but not nonhomologous end joining-related genes, were increased in response to OTA in a dose-dependent manner. Moreover, dramatic increases in the expression of genes involved in G_2/M arrest (Chek1 and Wee1) and S/G_2 phase (Ccna2 and Cdk1) were observed, suggesting that DSBs induced by OTA were repaired predominantly by HR repair, possibly due to OTA-specific cell cycle regulation, consequently producing large deletion mutations at the carcinogenic target site.

Keywords: ochratoxin A, double-strand breaks, *gpt* delta rat

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Matsuda T*, Takamune M, Matsuda Y*, Yamada M: A pilot study for the mutation assay using a

highthroughput DNA sequencer.

Genes and Environ. 2013;35:53-6.

We present here a mutation assay with little bias which incorporates high-throughput DNA sequencing technology. Our strategy is simple: 1) expose cells to a test compound, 2) isolate colonies, and 3) carry out whole-genome sequencing of the clones. In this pilot study, we used *Salmonella typhimurium* TA100 as a tester strain and successfully detected mutations induced by the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2). We believe that this new mutation assay will be a very useful tool in hazard assessment of chemicals.

Keywords: whole-genome sequencing, Ames test, mutation assay

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Bétous R*1, Pillaire MJ*1, Pierini L*1, van der Laan S*1, Recolin B*1, Ohl-Séguy E*1, Guo C*2, Niimi N, Grúz P, Nohmi T, Friedberg E*2, Cazaux C*1, Maiorano D*1, Hoffmann JS*1: DNA polymerase κ-dependent DNA synthesis at stalled replication forks is important for CHK1 activation.

EMBO J. 2013;32:2172-85.

Formation of primed single-stranded DNA at stalled replication forks triggers activation of the replication checkpoint signalling cascade resulting in the ATR-mediated phosphorylation of the Chk1 protein kinase, thus preventing genomic instability. By using siRNA-mediated depletion in human cells and immunodepletion and reconstitution experiments in Xenopus egg extracts, we report that the Y-family translesion (TLS) DNA polymerase kappa (Pol κ) contributes to the replication checkpoint response and is required for recovery after replication stress. We found that Pol κ is implicated in the synthesis of short DNA intermediates at stalled forks, facilitating the recruitment of the 9-1-1 checkpoint clamp. Furthermore, we show that Pol κ interacts with the Rad9 subunit of the 9-1-1 complex. Finally, we show that this novel checkpoint function of Pol κ is required for the maintenance of genomic stability and cell proliferation in unstressed human cells.

Keywords: DNA polymerase κ, replication checkpoint

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Kimoto T*1, Horibata K, Chikura S*1, Hashimoto K*2, Itoh S*2, Sanada H*3, Muto S*4, Uno Y*4, Yamada M, Honma M: Interlaboratory trial of the rat *Pig-a* mutation assay using an erythroidmarker HIS49 antibody.

Mutat Res. 2013;755:126-34.

The peripheral blood *Pig-a* assay has shown promise as a tool for evaluating *in vivo* mutagenicity. In this study five laboratories participated in a collaborative trial that evaluated the transferability and reproducibility of a rat *Pig-a* assay that uses a HIS49 antibody reacts with an antigen found on erythrocytes and erythroid progenitors. Four of the laboratories (the in-life labs) treated male rats with a single oral dose of *N*-nitroso-*N*-ethylurea, 7,12-dimethylbenz[*a*] anthracene,or 4-nitroquinoline-1-oxide. The results indicate that rat *Pig-a* assays using a HIS49 antibody were transferable between laboratories and that data generated by the assays were reproducible. The findings also suggest that the PIGRET assay may detect the *in vivo* mutagenicity of test compounds earlier than the RBC *Pig-a* assay.

Keywords: reticulocyte, *Pig-a* assay, *in vivo* mutagenicity

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Shah N*1, de Oca MM*1, Jover-Cobos M*1, Tanamoto K*2, Muroi M*2, Sugiyama K, Davies NA*1, Mookerjee RP*1, Dhar DK*1, Jalan R*1: Role of toll-like receptor 4 in mediating multiorgan dysfunction in mice with acetaminophen induced acute liver failure.

Liver Transpl. 2013;19:751-61.

Strategies for the prevention of multiorgan dysfunction (MOD) in acetaminophen (APAP) -induced acute liver failure (ALF) are an unmet need. Our study tested the hypothesis that sterile inflammation induced by APAP in a mouse model would activate toll-like receptor 4 (TLR4) in the liver and extrahepatic organs and lead to the progression of ALF and MOD and that the administration of the

novel TLR4 antagonist STM28 (a peptide formed of 17 amino-acids) would prevent liver injury and associated MOD. In conclusion, this study provides evidence for an important role of the TLR4 antagonist in the prevention of the progression of APAP-induced ALF and MOD.

Keywords: toll-like receptor 4, liver failure

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Kimura A*1, Miyata A*2, Honma M: A combination of *in vitro* comet assay and micronucleus test using human lymphoblastoid TK6 cells.
Mutagenesis. 2013;28:583-90.

The comet assay has been widely used as a genotoxicity test for detecting primary DNA damage in individual cells. The micronucleus (MN) test is also a well-established assay for detecting clastogenicity and aneugenicity. A combination of the comet assay (COM) and MN test is capable of detecting a variety of genotoxic potentials as an in vitro screening system. Although the in vitro MN test has a robust protocol and Organisation for Economic Co-operation and Development (OECD) test guideline, the in vitro COM does not. To establish a robust protocol for the COM and to compare its sensitivity with that of the MN, we conducted COM and MN concurrently for five genotoxic agents (ethyl methanesulfonate, methyl methanesulfonate, hydrogen peroxide, gamma-rays and mitomycin C) and one non-genotoxic agent (triton X-100), using human lymphoblastoid TK6 cells. Relative cell count (RCC), relative population doubling (RPD), relative increase in cell count (RICC) and relative cell viability determined by trypan blue dye-exclusion assay (TBDE) were employed as cytotoxic measurements. However, the relative cell viability determined by TBDE just after the treatment was not an appropriate parameter of cytotoxicity for the genotoxic agents because it remained constant even at the highest doses, which showed severe cytotoxicity by RCC, RPD and RICC. The results of the COM showed qualitative agreement (positive or negative) with those of the MN except for mitomycin C, which is an interstrand cross-linker. The COM always required higher doses than the MN to detect the genotoxic potential of the genotoxic agents under the test conditions applied here. The doses that induced a comet tail always yielded <50% RICC, and do not accord to the OECD test guideline for MN because of their high cytotoxicity. These results are helpful for interpreting the results of the COM and MN in in vitro genotoxic hazard assessments. Further investigation is required to standardise the COM.

Keywords: *in vitro* comet assay, *in vitro* micronucleus assay, TK6 cells

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Sugiyama K, Yamazaki R*1, Kinoshita M*2, Kamata Y, Tani F*1, Minai Y*2, Sugita-Konishi Y: Inhibitory effect of citrinin on lipopolisaccharide-induced nitric oxide production by mouse macrophage cells.
Mycotoxin Res. 2013;29:229-34.

The present study evaluated the immunotoxicity of citrinin (CIT), a mycotoxin produced by several Aspergillus, Penicillium, and Monascus species. Because nitric oxide (NO), a pro-inflammatory mediator, plays an important role in the protection from pathogens, we addressed the effect of CIT on NO production by a mouse macrophage-like cell line RAW264 activated with lipopolysaccharide (LPS). LPS-induced NO release from RAW264 cells was inhibited by CIT. Moreover, the transcription and expression of inducible NO synthase (iNOS) by LPS was suppressed by CIT. These results show that CIT suppressed the LPS-induced NO production and iNOS expression, which contribute to the host protection against invading pathogens. This suggests that CIT on LPS-induced NO release may exert adverse effects in macrophages, indicating immunotoxic effects of this toxin.

Keywords: citrinin, immunotoxicity

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Kawamura Y*, Hayashi H*, Kurata Y*, Hiratsuka K*, Masumura K, Nohmi T: Evaluation of the genotoxicity of tamoxifen in the liver and kidney of F344 *gpt* delta transgenic rat in 3-week and 13-week repeated dose studies.
Toxicology. 2013;312:56-62.

Transgenic rat gene mutation assays can be used to assess genotoxicity of chemicals in target organs for carcinogenicity. To examine the utility of the transgenic rat assays in repeated-dose studies, we treated female F344 *gpt* delta rats with tamoxifen (TAM) at 20 and 40 mg/kg, or toremifene (TOR) at 40 mg/kg by gavage daily for 3 weeks. We also fed *gpt* delta rats with TAM at either 250 ppm (15.4-17.6 mg/kg) or 500 ppm (30.0-32.9 mg/kg) for 13 weeks. TAM is carcinogenic in the rat liver and TOR is not carcinogenic. TAM administration significantly increased *gpt* (point mutations) and Spi(-) (deletions) mutant frequencies (MFs) in the liver, the target organ of carcinogenesis; MFs were higher after treatment for 13 weeks than after treatment for 3 weeks. The MFs in the kidney did not increase in any of the TAM treatment groups. TOR had no effect on MFs (*gpt* and Spi(-)) in either the liver or the kidney. We conclude that the *gpt* delta rat assay in the repeated-dose treatment paradigm is sensitive enough to detect gene mutations induced by TAM in the target organ for carcinogenesis. Furthermore, the assay can be integrated into a 13-week dose-finding study for a 2-year cancer bioassay.

Keywords: *gpt* delta rat, tamoxifen, genotoxicity

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Horibata K, Ukai A, Kimoto T*, Suzuki T, Kamoshita N, Masumura K, Nohmi T, Honma M: Evaluation of *in vivo* genotoxicity induced by *N*-ethyl-*N*-nitrosourea, benzo[*a*]pyrene, and 4-nitroquinoline-1-oxide in the *Pig-a* and *gpt* assays.
Environ Mol Mutagen. 2013;54:747-54.

The recently developed *Pig-a* mutation assay is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient red blood cells caused by a forward mutation in the *Pig-a* gene. Because the assay can be conducted in nontransgenic animals and the mutations accumulate with repeat dosing, we believe that the *Pig-a* assay could be integrated into repeat-dose toxicology studies and provides an alternative to transgenic rodent (TGR) mutation assays. The capacity and characteristics of the *Pig-a* assay relative to TGR mutation assays, however, are unclear. Here, using transgenic *gpt* delta mice, we compared the *in vivo* genotoxicity of single oral doses of *N*-ethyl-*N*-nitrosourea (ENU, 40 mg/kg), benzo[*a*]pyrene (BP, 100 and 200 mg/kg), and 4-nitroquinoline-1-oxide (4NQO, 50 mg/kg) in the *Pig-a* (peripheral blood) and *gpt* (bone marrow and liver) gene mutation assays. *Pig-a* assays were conducted at 2, 4, and 7 weeks after the treatment, while *gpt* assays were conducted on tissues collected at the 7-week terminal sacrifice. ENU increased both *Pig-a* and *gpt* mutant frequencies (MFs) at all sampling times, and BP increased MFs in both assays but the *Pig-a* MFs peaked at 2 weeks and then decreased. Although 4NQO increased *gpt* MFs in the liver, only weak, nonsignificant increases (two- or threefold above control) were detected in the bone marrow in both the *Pig-a* and the *gpt* assay. These findings suggest that further studies are needed to elucidate the kinetics of the *Pig-a* mutation assay in order to use it as an alternative to the TGR mutation assay.

Keywords: *Pig-a* mutation assay, transgenic rodent mutation assay, *in vivo* genotoxicity

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Grúz P, Sassa A, Hosoda A*[1], Yamagishi H*[2], Usui Y*[1], Shimizu M*[1]: Exclusive induction of G:C to A:T transitions by 3-azido-1,2-propanediol in yeast.
Mutat Res. 2014;760:73-6.

Sodium azide is a strong mutagen which has been successfully employed in mutation breeding of crop plants. In biological systems, it is metabolized to azidoalanine, but further bioactivation to a putative ultimate mutagen as well as the nature of the induced DNA modifications leading to mutations remain elusive. In this study, mutations induced in the CAN1 gene of yeast Saccharomyces cerevisiae by the representative mutagen 3-azido-1,2-propanediol (azidoglycerol, AZG) have been sequenced. Analysis of the forward mutation spectrum to canavanine resistance revealed that AZG induced nearly exclusively G:C to A:T transitions. AZG also induced reversions to tryptophan prototrophy by base-pair substitutions in a dose-dependent manner. This unusual mutational specificity may be shared by other organic azido compounds.

Keywords: azidoglycerol, mutation spectrum

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Yasui M, Kanemaru Y*1, Kamoshita N, Suzuki T, Arakawa T*2, Honma M: Tracing the fates of site-specifically introduced DNA adducts in the human genome.

DNA Repair. 2014;15;11-20.

We developed a system for tracing DNA adducts in targeted mutagenesis (TATAM) and investigated the prevalence and types of consequent mutations. Targeted mutagenesis methods site-specifically replace endogenous DNA bases with bases carrying synthetic adducts using targeting vectors. The TATAM system was enabled by introduction of site-specific DNA double strand breaks (DSB), which strongly enhanced targeting efficiency through homologous recombination (HR), and a new polymerase chain reaction-based technique, which gives high yields of the target vectors carrying DNA adducts. Human lymphoblastoid TSCER122 cells are compound heterozygous for the thymidine kinase gene (*TK*-/-), and have a homing endonuclease I-SceI site in intron 4 of the *TK* gene. The TATAM system enabled targeting of the *TK*- allele with the I-SceI site using a synthetic *TK*+ allele containing an 8-oxo-7,8-dihydroguanine (8-oxoG) adduct, a typical product of oxidative DNA damage. The targeted clones (*TK*+/-) were then isolated by drug selection. Site-specific HR for DSB induced by I-SceI improved targeted integration of the synthetic allele by five orders of magnitude (from 10^{-7} to 10^{-2}). Subsequent analyses of approximately 800 target clones revealed that 8-oxoG was restored to G in 86% clones, probably reflecting base excision repair or translesion synthesis without mutation. Lesions of the remaining clones (14%) were associated with mutations. The mutation spectrum corresponded closely with that of oxidative DNA damage inducers reported, in which G:C to T:A transversions (5.9%) were predominant. Over-expression of MutY homologs in cells, which prevents G:C to T:A transversions by removing 8-oxoG:A mispairing, significantly decreased the frequency of mutations to 2.6%, indicating that the 8-oxoG adducts introduced by the TATAM system are processed in the same manner as those generated by oxidative DNA damage.

Keywords: DNA adduct, DNA damage, gene targeting

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Sassa A, Suzuki T, Kanemaru Y*1, Niimi N, Fujimoto H*2, Katafuchi A, Grúz P, Yasui M, Gupta RC*3, Johnson F*3, Ohta T*4, Honma M, Adachi N*5, Nohmi T: *In vivo* evidence that phenylalanine 171 acts as a molecular brake for translesion DNA synthesis across benzo[*a*]pyrene DNA adducts by human DNA polymerase κ.

DNA Repair. 2014;15:21-8.

Humans possess multiple specialized DNA polymerases that continue DNA replication beyond a variety of DNA lesions. DNA polymerase kappa (Pol κ) bypasses benzo[*a*]pyrene diolepoxide-N^2-deoxyguanine (BPDE-N^2-dG) DNA adducts in an error-free manner. In the previous work, we changed the amino acids in the active site and examined the bypass efficiency. The substitution of alanine for phenylalanine 171 (F171A) enhanced by 18-fold *in vitro*, the efficiencies of dCMP incorporation opposite (-)- and (+)-*trans-anti*-BPDE-N^2-dG. In this study, we generated cells that express wild-type Pol κ (*POLK*+/-), F171A (*POLK* F171A/-) or lack expression of Pol κ (*POLK*-/-) from the human Nalm-6 pre-B cell line to examine the *in vivo* significance. Mutations were analyzed with shuttle vectors having (-)- or (+)-*trans-anti*-BPDE-N^2-dG in the *supF* gene. The frequencies of mutations were in the order of *POLK*-/->*POLK*+/->*POLK* F171A/- in BPDE-N^2-dG adducts. These results suggest that F171 may function as a molecular brake for bypass across BPDE-N^2-dG by Pol κ and raise the possibility that the cognate substrates for Pol κ are not BP adducts in DNA but may be lesions in DNA induced by endogenous mutagens.

Keywords: DNA polymerase κ, benzo[*a*]pyrene

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Matsumoto M, Yamaguchi M*1, Yoshida Y*2, Senuma M*2, Takashima H*2, Kawamura T, Kato H, Takahashi M, Hirata-Koizumi M, Ono A, Yokoyama

K*3, Hirose A: An antioxidant, N,N'-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: A 28-day repeated dose test and reproduction/developmental toxicity test.

Food Chem Toxicol. 2013;56:290-6.

A 28-day repeated dose toxicity test and reproduction/developmental toxicity test for N,N'-diphenyl-p-phenylenediamine (DPPD) were conducted in [Crl:CD(SD)] SPF rats. Male and female rats were dosed with DPPD by gavage for 28 days at 0, 100, 300, or 1000 mg/kg bw/day or for a total of 42-46 days at 0, 8, 50, or 300 mg/kg bw/day. No significant adverse effects were observed in the repeated dose toxicity study up to 1000 mg/kg bw/day in both sexes. In the reproduction/developmental toxicity study, two females showed piloerection, hypothermia, and pale skin; one died and the other showed dystocia on day 23 of pregnancy at 300 mg/kg bw/day. Another female delivered only three live pups at 300 mg/kg bw/day. A significantly prolonged gestation period was observed at 50 and 300 mg/kg bw/day. The NOAELs of repeated dose toxicity and reproduction/developmental toxicity were considered to be 1000 and 8 mg/kg bw/day, respectively.

Keywords: N,N'-diphenyl-p-phenylenediamine, Prostaglandin, Gestation period

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Takahashi M, Ishida S*, Hirata-Koizumi M, Ono A, Hirose A: Repeated dose and reproductive/developmental toxicity of perfluoroundecanoic acid in rats.

J Toxicol Sci. 2014;39:97-108.

Perfluoroalkyl acids (PFAAs) are environmental contaminants that have received attention because of their possible effects on wildlife and human health. In order to obtain initial risk information on the toxicity of perfluoroundecanoic acid (PFUA), we conducted a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD test guideline 422). PFUA was administered by gavage to rats at 0 (vehicle: corn oil), 0.1, 0.3 or 1.0 mg/kg/day. At 1.0 mg/kg/day, body weight gain was inhibited in both sexes, and there was a decrease in fibrinogen in both sexes and shortening of the activated partial thromboplastin time in males. An increase in blood urea nitrogen and a decrease in total protein in both sexes and increases in alkaline phosphatase and alanine transaminase and a decrease in albumin in males were observed at 1.0 mg/kg/day. Liver weight was increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and this change was observed after a recovery period. In both sexes, centrilobular hypertrophy of hepatocytes was observed at 0.3 mg/kg/day and above and focal necrosis was observed at 1.0 mg/kg/day. In reproductive/developmental toxicity, body weight of pups at birth was lowered and body weight gain at 4 days after birth was inhibited at 1.0 mg/kg/day, while no dose-related changes were found in the other parameters. Based on these findings, the no observed adverse effect levels (NOAELs) for the repeated dose and reproductive/developmental toxicity were considered to be 0.1 mg/kg/day and 0.3 mg/kg/day, respectively.

Keywords: Perfluoroundecanoic acid, Repeated dose toxicity, Reproductive and developmental toxicity

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Mirokuji Y*1, Abe H*2, Okamura H*1, Saito K*1, Sekiya F*1, Hayashi SM*1, Maruyama S*1, Ono A, Nakajima M*3, Degawa M*4, Ozawa S*5, Shibutani M*2, Maitani T*4: The JFFMA assessment of flavoring substances structurally related to menthol and uniquely used in Japan.

Food Chem Toxicol. 2013;64:314-21.

Using the procedure devised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), we performed safety evaluations on four flavoring substances structurally related to menthol (l-menthyl 2-methylbutyrate, dl-menthyl octanoate, dl-menthyl palmitate, and dl-menthyl stearate) uniquely used in Japan. While no genotoxicity study data were available in the literature, all four substances had no chemical structural alerts predictive of genotoxicity. Moreover, they all four are esters consisting of menthol and simple carboxylic acids that were assumed to be

immediately hydrolyzed after ingestion and metabolized into innocuous substances for excretion. As menthol and carboxylic acids have no known genotoxicity, it was judged that the JECFA procedure could be applied to these four substances. According to Cramer's classification, these substances were categorized as class I based on their chemical structures. The estimated daily intakes for all four substances were within the range of 1.54-4.71μg/person/day and 60-1250 μg/person/day, using the methods of Maximized Survey-Derived Intake and Single Portion Exposure Technique, respectively, based on the annual usage data of 2001, 2005, and 2010 in Japan. As the daily intakes of these substances were below the threshold of concern applied to class I substances viz., 1800 μg/person/day, it was concluded that all four substances raise no safety concerns when used for flavoring foods under the currently estimated intake levels.

Keywords: Japanese unique flavoring substances, Menthol, Joint FAO/WHO Expert Committee on Food Additives (JECFA)

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