Miyahara M, Sugi E[*1], Katoh T[*2], Hironiwa T[*3], Sunaga H[*1], Luo LZ[*4]: Study of effective factors in detection of irradiated food using thermoluminescence based on the models of reference minerals.
Radiation Physics and Chemistry 2012;81:705-11.

In the thermoluminescence (TL) detection method for irradiated foods, accurate standards have been developed for detecting irradiated foods. The standard method describes that emission maximum temperature (T1*i*) and TL ratio for non-heated or non-mixed sample can be in the range of 150−250 °C and more than 0.1, respectively, when it was irradiated food. But when irradiated food is heated up to 200 °C, or mixed up with non-irradiated stuffs, T1*i* and TL ratio would not drop in the range. Here we examined the effects of the two processes, heating and mixing with non-irradiated food, on T1*i* and G1/G1k ratio (ratio of G1 and average G1 for 1-kGy-irradiated JF2, this value is modeled after TL ratio) using a model consisting of irradiated and non-irradiated geochemical standards of feldspar (JF1, JF2, PF, etc.). T1*i* temperatures for irradiated JF1, JF2, and PF ranged from 163 to 175 °C, while those for the non-irradiated JF2 ranged from 253 to 263 °C. T1*i* temperatures for 5-kGy-irradiated and preheated JF2 for 10 s, 20 s, and 30 s at 180 °C were 215, 225, and 231 °C, respectively. When JF2 was irradiated from 100 Gy to 5 kGy, the T1*i* was almost constant at any doses. G1/G1k ratios at 100, 200, and 500 Gy were 0.15, 0.23, and 0.60, respectively. G1/G1k ratio was proportional to the given dose at the integration temperature ranges. The TS sample, which originated from farm soil in Tanegashima Island, gave the same results as JF2. T1*i*s for 5-kGy-irradiated and preheated JF2 for 20 s at 150, 180, and 200 °C were 197, 225, and 246 °C, respectively. Longer and higher preheating resulted in higher T1*i*. Longer and higher preheating extremely reduced the G1/G1k ratio, and in some cases the ratio was less than 0.1. This means TL ratio is useless in determination of the standard for irradiated food. Peak temperatures for JF2 in mixture of 5-kGy-irradiated to non-irradiated (1.25 − 5%) were 261 − 263 °C (non-irradiated portion, T1*n*) and 177−180 °C (irradiated portion T1*i*). The peak positions are almost the same as those of original components and would not be affected by the mixing ratio. But TL ratio could not be used to determine irradiated food because mixing would reduce it remarkably. Some of the glow curves were simulated by a computer program. In conclusion, T1*i*/*n* is a key factor in an irradiated food determination practice for sample containing feldspar, rather than TL ratio.
Keywords: Irradiated geochemical reference feldspar, Preheating, Irradiated food detection

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Shibata H, Saito H, Yomota C, Kawanishi T, Okuda H: Alterations in the detergent-induced membrane permeability and solubilization of saturated phosphatidylcholine/cholesterol liposomes: effects of poly (ethylene glycol)-conjugated lipid.
Chem Pharm Bull. 2012;60:1105-11.

We have investigated the effects of two bile salts, chenodeoxycholate (CDC) and ursodeoxycholate (UDC), and a widely used detergent, Triton X-100, on normal and poly(ethylene glycol) -modified liposomes (PEGylated liposomes). We found that the effect of detergents on the lipid bilayer of liposomes depends on both the kind of detergent and the lipid composition, including the presence or absence of PEG-lipid. Moreover, the effects of T(X-100) on the lipid bilayers of the PEGylated liposomes significantly differed from those on the lipid bilayers of the normal liposomes.
Keywords: liposome, release, detergent

Shibata H, Saito H, Kawanishi T, Okuda H, Yomota C: Comparison of particle size and dispersion state among commercial cyclosporine formulations and their effects on pharmacokinetics in rats.
Chem Pharm Bull. 2012;60:967-75.

Generic versions of Neoral, a microemulsion capsule formulation of cyclosporine, have been approved worldwide. In this study, we measured the physicochemical properties of both the innovator and the generic formulations, and compared their bioavailability in rats. Our results suggest that the physicochemical differences between the innovator and the generics will not have a significant effect on C(max) or AUC, which is necessary to ensure bioequivalence.

Keywords: microemulsion, biorelevant medium, generic

Shibata H, Yomota C, Kawanishi T, Okuda H: Polyethylene glycol prevents in vitro aggregation of slightly negatively-charged liposomes induced by heparin in the presence of bivalent ions.
Biol Pharm Bull. 2012;35:2081-7.

In a previous study, we found that the slightly negatively-charged liposomes aggregate only in the culture medium of human umbilical vein endothelial cells, whereas the liposomes modified with polyethylene glycol (PEG) (PEGylated) did not aggregate. In the present study, we investigated the underlying mechanism of this phenomenon. In the presence of heparin, higher concentrations of Ca(2+) or Mg(2+) increased the particle size of the non-PEGylated liposomes, although no change in the particle size of PEGylated liposomes was observed.
Keywords: liposome, polyethylene glycol, aggregation

Yoshida H, Nishikawa M*, Yasuda S*, Toyota H*, Kiyota T*, Takahashi Y*, Takakura Y*: Fibronectin inhibits cytokine production induced by CpG DNA in macrophages without direct binding to DNA.
Cytokine 2012;60:162-70.

The expression of fibronectin (FN) was significantly higher in primary macrophages than in a macrophage-like cell line, RAW264.7, suggesting that abundant FN might suppress the responsiveness in the primary macrophages. However, electrophoretic analysis revealed that FN did not bind to plasmid DNA (pDNA) in the presence of a physiological concentration of divalent cations. Surprisingly, marked tumor necrosis factor-α production from murine macrophages upon CpG DNA stimulation was significantly reduced by exogenously added FN in a concentration-dependent manner but not by BSA, laminin or collagen. The results of the present study has revealed a novel effect of FN whereby the glycoprotein modulates cytokine signal transduction via CpG-DNA/TLR9 interaction in macrophages without direct binding to DNA.
Keywords: fibronectin, TLR9, inflammatory response

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梶村計志*，川口正美*，四方田千佳子：難水溶性製剤の溶出試験に界面活性剤として使用されるポリソルベート80の品質に関する研究.
医薬品医療機器レギュラトリーサイエンス 2012:43:650-5.

難溶性製剤の溶出試験に用いられる界面活性剤の試薬特性が溶出試験結果に及ぼす影響を検討した．ポリソルベート80の市販試薬10種類につき，pH，酸価，けん化価，よう素価，HPLCクロマト形状などを検討したところ，若干の差が認められた．しかし，ナブメトン錠，リボフラビン酪酸錠，アリルエストレノール錠の溶出挙動では，試薬間で差が認められず，ポリソルベート80では試薬による溶出試験結果への影響は少ないと考えられた，
Keywords: ポリソルベート80，赤外吸収スペクトル，酸価

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北山裕貴*，新村航*，四方田千佳子，斎藤博幸*：ポストインサーション法によって調製したPEG修飾リポソームの表面物性に関する研究.
膜 2013:38:50-6.

PEG修飾リポソームの固定水和層長の変化をリポソーム膜表面に存在するPEGリン脂質濃度当たりで比較したところ，リポソーム膜最外層におけるPEGリン脂質被覆率と固定水和層長との相関はpre-mixed法とpost-insertion法でほぼ同じであることが示され，修飾法の違いにかかわらずPEGリポソームの表面物性がPEG鎖の表面被覆率によって制御されていることが明らかとなった．さらに，PEG修飾リポソームの固定水和層長の増大はPEG鎖のコンフォメーション変化に起因することが示唆された.
Keywords: リポソーム，PEG，表面特性

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Yamaki T*, Ohdate R*, Nakadai E*, Yoshihashi Y*, Yonemochi E*, Terada K*, Moriyama H*, Izutsu K, Yomota C, Okuda H, Kawanishi T: Component crystallization and physical collapse during freeze-drying of l-arginine-citric acid mixtures.
Chem Pharm Bull. 2012;60:1176-81.

Component crystallization and physical collapse during freeze-drying of aqueous solutions containing protein-stabilizing L-arginine and citric acid mixtures were studied. Freeze-drying microscopy (FDM) and

thermal analysis of the solute-mixture frozen solutions showed collapse onset at temperatures (T(c)) approximately 10°C higher than their T(g)'s (glass transition temperatures of the maximally freeze-concentrated solute phase). Experimental freeze-drying of these solutions at a low chamber pressure showed the occurrence of physical collapse at shelf temperatures close to or slightly higher than the T(c). Slower ice sublimation at higher chamber pressures induced the physical collapse from lower shelf temperatures. The large effect of chamber pressures on the collapse-inducing shelf temperatures confirmed significance of the sublimation-related heat loss on the sublimation interface temperature during the primary drying. Drying of the single-solute L-arginine solution resulted in cake-structure solids composed of its anhydrous crystal. Thermal and powder X-ray diffraction (PXRD) analysis suggested slow crystal nucleation of L-arginine dihydrate in the frozen solutions. Characterization of the frozen solutions and freeze-dried solids should enable rational formulation design and process control of amino acid-containing lyophilized pharmaceuticals.

Keywords: freeze-drying, crystallization, collapse

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阿曽幸男，宮辻恵，宮崎玉樹，川西徹：医薬品添加剤等の結晶化度測定法に関する研究（その２）．
医薬品医療機器レギュラトリーサイエンス 2012;43:955-60.

マルトース水和物を用いた検討により，(1)等温ミクロ熱量計を用い，非晶質化した成分が結晶化するときに発生する結晶化熱をもとに測定する方法，(2)示差走査熱量計を用い，ガラス転移温度における比熱の変化量をもとに測定する方法，(3)^{13}C-固体高分解能NMRにより，結晶のシグナル強度をもとに測定する方法が結晶化度測定法として有用であることを明らかにした．

Keywords：結晶化度，^{13}C-固体高分解能NMR，等温ミクロ熱量計

宮崎玉樹，阿曽幸男，奥田晴宏：コムギデンプンの『総たん白質含量』試験法に関する研究．
医薬品医療機器レギュラトリーサイエンス 2013;44:94-101.

Wheat starch is a pharmaceutical excipient whose pharmacopeia monograph has been harmonized by Japanese, the United States and European pharmacopeias. In the monograph, total protein in wheat starch is determined by the Kjeldahl method in which selenium is used as a catalyst for sulphuric acid digestion. Since selenium is toxic, alternative catalyst is preferable. In this study, feasibility of titanium dioxide as a catalyst was studied. By using experimental design method, the composition and the amount of the catalyst were determined on the basis of the recovery of nitrogen and time required for digestion of the sample. Using 4g of a catalyst mixture containing potassium sulphate, cupric sulphate pentahydrate and titanium dioxide (100:3:3) and 25mL of sulphuric acid, 6.0g of a starch sample was satisfactory digested. Continuous heating for more than 30min was needed after a clear solution was obtained to get higher recovery of nitrogen. The recovery of nitrogen obtained by the present method was equivalent to that obtained by the method described in the harmonized monograph. Therefore, titanium dioxide can be used as a catalyst instead of selenium.

Keywords: Kjeldahl method, wheat starch, catalyst

米山智城[*1]，井上則子[*2]，立木秀尚[*3]，富樫一天[*4]，中山聡[*5]，工藤喬[*1]，清水久夫[*1]，香取典子：日本におけるバイオアナリシス分析法バリデーションの実施に関する指針（バイオアナリシスフォーラム素案）について．
医薬品医療機器レギュラトリーサイエンス 2012;43:750-60.

This is a report to propose the draft guideline on Bioanalytical Method Validation (BMV) in Japan prepared by Japan Bioanalysis Forum (JBF). The preparation of the draft BMV guideline by JBF is based on the request to the JBF from the working group on this subject in the Ministry of Health, Labour and Welfare (MHLW), which is led by Yasuo Ohno, Ph.D. at National Institute of Health Sciences. This is the first report on the BMV guideline in Japan since no detailed BMV guideline has yet been issued in Japan while the regulatory guidelines on BMV have been published from the Food and Drug Administration (FDA) in 2001 and the European Medical Agencies (EMA) in 2011, respectively.

This report is composed of the preface and the following seven chapters and definitions: (1) Introduction, (2) Scope, (3) Reference standard, (4) Method validation, (5) Analysis of study samples, (6) Docu-

mentation and (7) Supplement. The contents of this draft BMV guideline are not significantly different from the preceding regulatory guidelines from the FDA and the EMA while the initial scope of the draft BMV guideline in Japan is limited to the bioanalysis of small molecule compounds using chromatographic analytical methods. Some of new terminologies are proposed in this report as the proper definitions have not yet been established as the result of the lack of a detailed BMV guideline in Japan so far.

It is expected that this draft guideline prepared by JBF will contribute to further discussion on BMV in Japan.

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

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Sakamoto T, Portieri A*, Arnone D*, Taday P*, Kawanishi T, Hiyama Y: Coating and density distribution analysis of commercial ciprofloxacin hydrochloride monohydrate tablets by terahertz pulsed spectroscopy and imaging.

J Pharm Innov. 2012;7:87-93.

Terahertz pulsed spectroscopy was used to qualitatively detect ciprofloxacin hydrochloride monohydrate (CPFX・Cl・2O) in tablets, and terahertz pulsed imaging (TPI) was used to scrutinize not only the coating state but also the density distribution of tablets produced by several manufacturers. TPI was also used to evaluate distinguishability among these tablets. The same waveform, which is a unique terahertz absorption spectrum derived from pure CPFX・Cl・2O, was observed in all of the crushed tablets and in pure CPFX・Cl・2O. TPI can provide information about the physical states of coated tablets. Information about the uniformity of parameters such as a coating thickness and density can be obtained. In this study, the authors investigated the coating thickness distributions of film-coated CPFX・Cl・2O from four different manufacturers. Unique terahertz images of the density distributions in these commercial tablets were obtained. Moreover, B-scan (depth) images show the status of the coating layer in each tablet and the density map inside the tablets. These features would reflect differences resulting from different tablet-manufacturing processes.

Keywords: terahertz pulsed imaging, coating, density distribution

* TeraView

Sakamoto T, Fujimaki Y*1, Takada Y*2, Aida K*2, Terahara T*2, Kawanishi T, Hiyama Y: Non-destructive analysis of tulobuterol crystal reservoir-type transdermal tapes using near infrared spectroscopy and imaging.

J Pharm and Biomed Anal. 2013;74:14-21.

A non-destructive method for analyzing crystalline tulobuterol (TBR; a bronchodilator [β_2-blocker]) in transdermal drug delivery system tapes with a crystal reservoir system was developed. A near infrared spectroscopy (NIRS) and a near infrared spectroscopic imaging (NIRI) were used to investigate the distribution of TBR crystals in transdermal tapes. The characteristic peak derived from a first overtone of secondary amine which appears based on crystal growth was used for the detection of crystals. NIR images were composed by the integrated values of that peak at each pixel. The time-course analysis by NIRS showed that the intensity of the peaks gradually increased, and the intensity reached a plateau between day 30 and day 42 after preparation of the model tapes. The authors observed the growth and distribution of TBR crystals in small areas in several types of matrices by NIRI time-course measurement. The authors also found that a macroscopic map can provide a rough distribution map of crystalline TBR in a whole matrix. In the case in which a tape distributed from the innovator was examined, the characteristic peak was also detected through a liner or a supporting board, by transmittance-reflectance NIR measurement.

Keywords: NIR spectroscopy, transdermal drug delivery system, chemical imaging

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Koide T, Nagato T*, Kanou Y*, Matsui K*, Natsuyama S*, Kawanishi T, Hiyama Y: Detection of component segregation in granules manufactured by high shear granulation with over-granulation conditions using near-infrared chemical imaging.
Int J Pharm. 2013;441:135-45.

The objective of this study was to evaluate the high shear granulation process using near-infrared (NIR) chemical imaging technique and to make the findings available for pharmaceutical development. We prepared granules and tablets made under appropriate- and over-granulation conditions with high shear granulation and observed these granules and tablets using NIR chemical imaging system. We found an interesting phenomenon: lactose agglomeration and segregation of ingredients occurred in experimental tablets when over-granulation conditions, including greater impeller rotation speeds and longer granulation times, were employed. Granules prepared using over-granulation conditions were larger and had progressed to the consolidation stage; segregation between ethenzamide and lactose occurred within larger granules. The segregation observed here is not detectable using conventional analytical technologies such as high pressure liquid chromatography (HPLC) because the content of the granules remained uniform despite the segregation. Therefore, granule visualization using NIR chemical imaging is an effective method for investigating and evaluating the granulation process.
Keywords: image analysis, near-infrared spectroscopy, high shear granulation

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Un K, Sakai-Kato K, Oshima Y, Kawanishi T, Okuda H: Intracellular trafficking mechanism, from intracellular uptake to extracellular efflux, for phospholipid/cholesterol liposomes.
Biomaterials 2012;33:8131-41.

In the work presented here, we investigated the trafficking processes from intracellular uptake to extracellular efflux using conventional liposomes constructed with phospholipids (DOPC) and cholesterols (Chol). Following intracellular transport of liposomes via endocytosis, DOPC was localized in the endoplasmic reticulum (ER) and Golgi apparatus after escape from the endosome/lysosome, whereas Chol was only localized in the ER. Moreover, proteins involved in the intracellular trafficking of liposomal components were identified. Additionally, we showed that DOPC was partly effluxed via ABCG1, while Chol was partly effluxed via ABCA1 or ABCB1; suggesting that each liposomal component examined in this study was effluxed through different transporters. Our findings offer valuable information regarding targeted delivery to specific intracellular organelles, and how to possibly avoid unexpected toxic effects following multiple applications of liposome formulations.
Keywords: liposome, intracellular trafficking

Un K, Kawakami S*1, Yoshida M*1, Higuchi Y *1, Suzuki R*2, Maruyama K*2, Yamashita F*1, Hashida M*1,3: Efficient suppression of murine intracellular adhesion molecule-1 using ultrasound-responsive and mannose-modified lipoplexes inhibits acute hepatic inflammation.
Hepatology 2012;56:259-69.

In this study, we developed an ICAM-1 small interfering RNA (siRNA) transfer method using ultrasound (US) -responsive and mannose-modified liposome/ICAM-1 siRNA complexes (Man-PEG_{2000} bubble lipoplexes (Man-PEG_{2000} BLs)), and achieved efficient HEC-selective ICAM-1 siRNA delivery in combination with US exposure. Moreover, the sufficient ICAM-1 suppression effects were obtained via this ICAM-1 siRNA transfer in vitro and in vivo, and potent anti-inflammatory effects were observed in various types of inflammation. In conclusion, HEC-selective and efficient ICAM-1 siRNA delivery using Man-PEG_{2000} BLs and US exposure enables suppression of various types of acute hepatic inflammation.
Keywords: bubble lipoplex, siRNA transfer, anti-inflammation

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Kuribayashi R, Hashii N, Harazono A, Kawasaki N: Rapid evaluation for heterogeneities in monoclonal antibodies by liquid chromatography/mass spectrometry with a column-switching system.

J Pharm Biomed Anal. 2012;67-68:1-9.

The development of therapeutic antibodies has grown over the last several years. Most of the recombinant monoclonal antibodies (mAbs) produced by mammalian cells are glycoproteins. Glycosylation of the mAbs can be associated with effector functions, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, as well as immunogenicity and clearance. Thus, mAb glycan heterogeneity is a significant characteristic associated with the safety and efficacy of the products. Therefore, glycan heterogeneity should be evaluated during research and development (R&D) and during development of mAbs manufacturing processes to identify the process parameters that affect glycan heterogeneity and to enhance understanding of the manufacturing process. There is an increasing need for a rapid, easy, and automated evaluation method for glycan heterogeneity. Liquid chromatography/mass spectrometry (LC/MS) is a method that can be used to analyze glycoforms. LC/MS is marked by the ability to measure the oligosaccharide composition of each glycoform, whereas other general methods, such as capillary electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and ion-exchange chromatography, cannot. However, a laborious off-line purification of mAbs is required to evaluate glycan heterogeneities. In this study, we demonstrate the use of a rapid, easy, and automated evaluation system for mAb glycoforms by LC/MS. This LC/MS system uses a column-switching system equipped with 2 columns, a protein A affinity column and a reversed-phase column (desalting column). We devised 2 column-switching systems: one that targeted intact mAbs (system 1) and one that targeted the light and heavy chains of the mAbs (system 2). Our results show that the proposed systems are applicable as a tool to evaluate the glycoforms in several situations, including the research, development, and production processes of mAbs. Additionally, we hope that our systems are useful as process analytical technology (PAT) for molecular heterogeneities containing glycoforms of mAbs in implementation of quality by design (QbD).

Keywords: process analytical technology, therapeutic antibody, glycoform

Tada M, Itoh S, Ishii-Watabe A, Suzuki T, Kawasaki N: Functional analysis of the Notch ligand Jagged1 missense mutant proteins underlying Alagille syndrome.

FEBS J. 2012;279:2096-107.

Heterozygous mutations in the JAG1 gene, encoding Notch ligand Jagged1, cause Alagille syndrome (ALGS). As most of the mutations are nonsense or frameshift mutations producing inactive truncated proteins, haploinsufficiency is considered the major pathogenic mechanism of ALGS. However, the molecular mechanisms by which the missense mutations cause ALGS remain unclear. Here we analyzed the functional properties of four ALGS missense mutant proteins, P163L, R184H, G386R and C714Y, using transfected mammalian cells. P163L and R184H showed Notch-binding activities similar to that of the wild-type when assessed by immunoprecipitation. However, their trans-activation and cis-inhibition activities were almost completely impaired. These mutant proteins localized mainly to the endoplasmic reticulum (ER), suggesting that the mutations induced improper protein folding. Furthermore, the mutant proteins bound more strongly to the ER chaperone proteins calnexin and calreticulin than the wild-type did. C714Y also localized to the ER, but possessed significant trans-activation activity and lacked enhanced binding to the chaperones, indicating a less severe phenotype. The properties of G386R were the same as those of the wild-type. Dominant-negative effects were not detected for any mutant protein. These results indicate that accumulation in the ER and binding to the chaperones correlate with the impaired signal-transduction activities of the missense mutant proteins, which may contribute to the pathogenic mechanism of ALGS. Our findings, which suggest the requirement for cell-surface localization of Jagged1 for cis-inhibition activities, also provide important information for understanding the molecular basis of Notch-signaling pathways.

Keywords: Jagged1, Alagille syndrome

Sato B[*1], U-Katagiri Y[*1], Iijima K[*1], Yamada H[*1], Ito S, Kawasaki N, Okita H[*1], Fujimoto J[*2], Kiyokawa N[*1]: The human CD10 lacking an N-glycan at Asn628 is deficient in surface expression and neutral endopeptidase activity.

Biochim Biophys Acta 2012;1820:1715-23.

BACKGROUND: CD10, also known as neprilysin or

enkephalinase exhibiting neutral endopeptidase (NEP) activity, is expressed by B-lineage hematopoietic cells as well as a variety of cells from normal tissues. It cleaves peptides such as cytokines to act for terminating inflammatory responses. Although CD10 molecules of the human pre-B-cell line NALM-6 have 6 consensus N-glycosylation sites, three of them are known to be N-glycosylated by X-ray crystallography.

METHODS: In order to investigate the role of N-glycans in the full expression of NEP activity, we modified N-glycans by treatment of NALM6 cells with various glycosidases or alter each of the consensus N-glycosylation sites by generating site-directed mutagenesis and compared the NEP activities of the sugar-altered CD10 with those of intact CD10.

RESULTS: CD10 of the human B-cell line NALM-6 was dominantly localized in raft microdomains and heterogeneously N-glycosylated. Although neither desialylation nor further degalactosylation caused defective NEP activity, removal of only a small part of N-glycans by treatment with glycopeptidase F under non-denaturing conditions decreased NEP activity completely. All of the three consensus sites of CD10 in HEK293 cells introduced with wild type-CD10 were confirmed to be N-glycosylated. Surface expression of N-glycan at Asn(628)-deleted CD10 by HEK293 cells was greatly decreased as well as it lost entire NEP activities.

CONCLUSIONS: N-glycosylation at Asn(628) is essential not only for NEP activities, but also for surface expression.

GENERAL SIGNIFICANCE: Quality control system does not allow dysfunctional ecto-type proteases to express on plasma membrane.

Keywords: CD10, NALM-6, N-glycosylation

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The leukocyte cell-surface antigen CD38 is the major nicotinamide adenide dinucleotide glycohydrolase in mammals, and its ectoenzyme activity is involved in calcium mobilization. CD38 is also a raft-dependent signaling molecule. CD38 forms a tetramer on the cell surface, but the structural basis and the functional significance of tetramerization have remained unexplored. We identified the interfaces contributing to the homophilic interaction of mouse CD38 by site-specific cross-linking on the cell surface with an expanded genetic code, based on a crystallographic analysis. A combination of the three interfaces enables CD38 to tetramerize: one interface involving the juxtamembrane α-helix is responsible for the formation of the core dimer, which is further dimerized via the other two interfaces. This dimerization of dimers is required for the catalytic activity and the localization of CD38 in membrane rafts. The glycosylation prevents further self-association of the tetramer. Accordingly, the tetrameric interaction underlies the multifaceted actions of CD38.

Keywords: CD38, tetrameric interaction, glycosylation

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Asanuma-Date K*1, Hirano Y*1, Le N*1, Sano K*1, Kawasaki N, Hashii N, Hiruta Y, Nakayama K*2, Umemura M*2, Ishikawa K*2, Sakagami H*1, Ogawa H*1: Functional Regulation of Sugar Assimilation by N-Glycan-specific Interaction of Pancreatic α-Amylase with Glycoproteins of Duodenal Brush Border Membrane.

J Biol Chem. 2012;287:23104-18.

Porcine pancreatic α-amylase (PPA) binds to N-linked glycans of glycoproteins (Matsushita, H., Takenaka, M., and Ogawa, H. (2002) J. Biol Chem., 277, 4680-4686). Immunostaining revealed that PPA is located at the brush-border membrane (BBM) of enterocytes in the duodenum and that the binding is inhibited by mannan but not galactan, indicating that PPA binds carbohydrate-specifically to BBM. The ligands for PPA in BBM were identified as glycoprotein N-glycans that are significantly involved in the assimilation of glucose, including sucrase-isomaltase (SI) and Na(+)/Glc cotransporter 1 (SGLT1). Binding of SI and SGLT1 in BBM to PPA was dose-dependent and inhibited by mannan. Using BBM vesicles, we found functional changes in PPA and its ligands in BBM due to the N-glycan-specific interaction. The starch-degrading activity of PPA and maltose-degrading activity of SI were enhanced to 240 and 175%, respectively, while Glc uptake by SGLT1 was markedly inhibited by PPA at high but physiologically possible concentrations, and the binding was attenuated by the addition of mannose-specific lectins, especially from Galanthus nivalis. Additionally, recombinant human pancreatic α-amylases expressed in yeast and purified by single-step affinity chromatography exhibited the same carbohydrate binding specificity as PPA in binding assays with sugar-biotinyl polymer probes. The results indicate that mammalian pancreatic α-amylases share a common carbohydrate binding activity and specifically bind to the intestinal BBM. Interaction with N-glycans in the BBM activated PPA and SI to produce much Glc on the one hand and to inhibit Glc absorption by enterocytes via SGLT1 in order to prevent a rapid increase in blood sugar on the other.

Keywords: N-glycans, porcin pancreatic α-amylase, brush-border membrane

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Watanabe T*, Ito Y*, Sato A*, Hosono T*, Niimi S, Ariga T*, Seki T*: Annexin A3 as a negative regulator of adipocyte differentiation.

J Biochem. 2012;152:355-63.

Annexin A3 is a protein belonging to the annexin family, and it is mainly present in cellular membranes as a phospholipid-binding protein that binds via the calcium ion. However, its physiological function remains to be clarified. We examined the expression of annexin A3 in mouse tissues and found for the first time that annexin A3 mRNA and its protein were expressed more strongly in adipose tissues than in other tissues. In adipose tissues, annexin A3-expressing cells were present in the stromal vascular fraction, and precisely identical to Pref-1-positive preadipocytes, Pref-1 being an epidermal growth factor repeat-containing transmembrane protein that inhibits adipogenesis. In 3T3-L1 cells, used as a model of adipogenesis, annexin A3 was down-regulated at an early phase of adipocyte differentiation, and this pattern paralleled that of Pref-1. Suppression of annexin A3 in these cells with siRNA caused elevation of the PPARγ2 mRNA level and lipid droplet accumulation. In conclusion, our data suggest that annexin A3 is a negative regulator of adipocyte differentiation.

Keywords: Annexin A3, adipocyte, differentiation

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Hyuga S*, Shiraishi M*, Hori A*, Hyuga M, Hanawa T*: Effects of Kampo Medicines on MDR-1-Mediated Multidrug Resistance in Human Hepatocellular Carcinoma HuH-7/PTX Cells.

Biol Pharm Bull. 2012;35:1729-39.

Paclitaxel-resistant HuH-7 (HuH-7/PTX) cells were established by one-week exposure of HuH-7 cells to paclitaxel to analyze the effects of Kampo medicines on MDR-1-mediated multidrug resistance. HuH-7/PTX cells expressed high levels of MDR-1 and efficiently exported calcein-acetoxymethylester (calcein-AM), which is a substrate of MDR-1, suggesting that HuH-7/PTX cells resist paclitaxel by overexpressing MDR-1. We assessed the effects of 26 kinds of Kampo medicine on MDR-1 by calcein-AM efflux assay using HuH-7/

PTX cells, and the results revealed that takushato and goreisan are potential inhibitors of drug efflux by MDR-1. Additionally, the sensitivity of HuH-7/PTX cells to paclitaxel was increased in combination with these Kampo medicines, indicating that takushato and goreisan overcame paclitaxel resistance in the cells by suppressing drug export by MDR-1. We further clarified that Alismatis Rhizoma contained in both takushato and goreisan reversed paclitaxel resistance by preventing drug efflux by MDR-1 without affecting the expression levels of MDR-1. Moreover, the principal components of Alismatis Rhizoma, Alisol A, Alisol B, and Alisol B acetate, were found to increase the sensitivity to paclitaxel in HuH-7/PTX by inhibiting drug export by MDR-1 without affecting the expression levels of MDR-1. These results suggested that the reversal effects of takushato and goreisan on paclitaxel resistance are derived from these principal components in Alismatis Rhizoma. Accordingly, Kampo medicines containing Alismatis Rhizoma such as takushato and goreisan may be useful as MDR-1 inhibitors.

Keywords: multidrug resistance, Kampo Medicines, paclitaxel

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医薬品医療機器レギュラトリーサイエンス 2012;43:1050-8.

Heparin sodium and heparin calcium are used throughout the world as anticoagulants for the treatment of venous thrombosis and prophylaxis of clotting during extracorporeal circulation. Since the heparin contamination problem that occurred in early 2008, the Japanese Pharmacopoeia (JP) monographs for heparin sodium and heparin calcium have been continually revised to ensure the safety and quality of heparin products. It has been suggested that contaminated heparin is characterized by high concentrations of protein and nucleotidic impurities. The currently available protein limiting tests have poor sensitivity, and the current JP heparin monographs do not include a limiting test for nucleotidic impurities. Therefore, there is an urgent need for additional revision of the limiting test for protein and for addition of a limiting test for nucleotidic impurities in order to ensure the quality of heparin products. In this study, we developed limiting tests for protein and nucleotidic impurities in heparin by referring to the US Pharmacopeia and European Pharmacopoeia. Furthermore, the suitability of the tests for the JP heparin monographs was evaluated in a collaborative study with Japanese heparin manufacturers and suppliers.

Keywords: heparin, limiting test for protein impurities, limiting test for nucleotidic impurities

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Tada M, Ishii-Watabe A, Maekawa K, Fukushima-Uesaka H, Kurose K, Suzuki T, Kaniwa N, Sawada J, Kawasaki N, Nakajima T.E[*1], Kato K[*1], Yamada Y[*2], Shimada Y[*1], Yoshida T[*2], Ura T[*3], Saito M[*3], Muro K[*3], Doi T[*4], Fuse N[*1], Yoshino T[*4], Ohtsu A[*4], Saijo N[*4], Okuda H, Hamaguchi T[*1], Saito Y, Matsumura Y[*4]: Genetic polymorphisms of FCGR2A encoding Fcγ receptor IIa in a Japanese population and functional analysis of the L273P variant.

Immunogenetics 2012;64:869-77.

Fcγ receptor IIa (FcγRIIa) plays an important role in antibody-dependent cellular cytotoxicity and inflammation. Changes in FcγRIIa expression levels or activity caused by genetic polymorphisms in FCGR2A, the gene encoding FcγRIIa, may lead to differences in disease progression as well as efficacy of antibody therapeutics between individuals. In this study, we sequenced the 5'-flanking region along with all exons and their flanking regions of FCGR2A from 111 Japanese subjects. Forty-eight genetic variations were

found including 12 novel ones. Beside the well-known functional 497A>G（H166R）polymorphism, we detected 818T>C（L273P）at 0.005 frequency. Since the functional significance of this polymorphism has not been revealed, we next assessed the functions of the L273P substitution by expressing wild-type and the variant proteins in human Jurkat cells. The L273P variant protein showed similar cell surface expression and IgG-binding properties as the wild-type, but it exhibited a stronger signal transduction activity based on the approximately 2-fold enhancement of tyrosine phosphorylation of FcγRIIa itself. The current results suggest that L273P could have functional significance in the antibody-dependent clinical responses through FcγRIIa.
Keywords: FcγRIIa, genetic polymorphisms

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Harashima M*, Hyuga M, Nagaoka Y*, Saito C*, Furukawa M*, Seki T*, Ariga T*, Kawasaki N, Niimi S: 26S proteasome inhibitors inhibit dexamethasone-dependent increase of tyrosine aminotransferase and tryptophan 2,3-dioxygenase mRNA levels in promary cultured rat hepatocytes.
J Biophys Chem. 2012;3:348-56.

Dexamethasone（Dex）, a ligand for transcriptional enhancement of tyrosine aminotransferase（TAT）and tryptophan 2,3-dioxygenase（TO）genes,（100 nM）maximally increased these mRNA levels at 12 h and 7 h in primary cultured rat hepatocytes and the nuclear fraction, respectively. Lactacystin（5 μM）and epoxomicin（0.5 μM）, 26S proteasome inhibitors, significantly suppressed the Dex-dependent maximum increase of TAT and TO mRNA levels in the cells and the nuclear fraction. Electrophoretic mobility shift assay demonstrated that lactacystin did not affect binding of glucocorticoid receptor to glucocorticoid responsive element. Furthermore, lactacystin did not affect the activation of GRE luciferase reporter by Dex transfected to the cells. The results demonstrate that 26S proteasome is positively involved in the Dex-dependent increase of TAT and TO mRNA levels in the cells and suggest that the mechanism of action of 26S proteasome may be degradation of some RNase(s), which breaks down TAT and TO mRNAs.
Keywords: 26S proteasome inhibitors, dexamethasone, mRNA level

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栗林亮佑，村上真紀*：抗コリン薬の過活動膀胱における臨床評価
レギュラトリーサイエンス学会誌 2012;2:187-201.

抗コリン薬を有効成分とするトルテロジン，ソリフェナシン，イミダフェナシン及びプロピベリンの効能・効果である過活動膀胱について，新薬の承認審査時のポイントをまとめた.
Keywords: 抗コリン薬，臨床評価，レギュラトリーサイエンス

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Kawabe K*1,2, Tateyama D*2, Toyoda H*1, Kawasaki N, Hashii N, Nakano H*1, Matsumoto S*1, Nonaka M*1, Matsumura H*2, Hirose Y*1, Morita A*1, Katayama M*3, Sakuma M*3, Kawasaki N*1, Kusuda-Furue M*2, Kawasaki T*1: A novel antibody for human induced pluripotent stem cells and embryonic stem cells recognizes a type of keratan sulfate lacking oversulfated structures.
Glycobiology 2013;23:322-6.

We have generated a monoclonal antibody（R-10G）specific to human induced pluripotent stem（hiPS）/embryonic stem（hES）cells by using hiPS cells（Tic）as an antigen, followed by differential screening of mouse hybridomas with hiPS and human embryonal carcinoma（hEC）cells. Upon western blotting with R-10G, hiPS/ES cell lysates gave a single but an unusually diffuse band at a position corresponding to >250 kDa. The antigen protein was isolated from the induced pluripotent stem（iPS）cell lysates with an affinity column of R-10G. The R-10G positive band was resistant to digestion with peptide N-glycanase F（PNGase F）, neuraminidase, fucosidase, chondrotinase ABC and heparinase mix, but it disappeared almost completely on digestion with keratanase, keratanase II and endo-β-galactosidase, indicating that the R-10G epitope is a keratan sulfate. The carrier protein of the R-10G epitope was identified as podocalyxin by liquid chromatography/mass spectrometry（LC/MS/MS）analysis of the R-10G positive-protein band material ob-

tained on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The R-10G epitope is a type of keratan sulfate with some unique properties. (1) The epitope is expressed only on hiPS/ES cells, i.e. not on hEC cells, unlike those recognized by the conventional hiPS/ES marker antibodies. (2) The epitope is a type of keratan sulfate lacking oversulfated structures and is not immunologically cross-reactive with high-sulfated keratan sulfate. (3) The R-10G epitope is distributed heterogeneously on hiPS cells, suggesting that a single colony of undifferentiated hiPS cells consists of different cell subtypes. Thus, R-10G is a novel antibody recognizing hiPS/ES cells, and should be a new molecular probe for disclosing the roles of glycans on these cells.

Keywords: R-10G, iPS, keratin

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Anjiki N*1,2, Fushimi H*3, Hosoe J, Fushimi N*4, Komatsu K*3, Cai S-Q*5, Ikezaki H*1, Mikage M*2, Kawahara N*6, Goda Y: Use of a taste-sensing system to discriminate Kasseki (Aluminum Silicate Hydrate with Silicon Dioxide) in The Japanese Pharmacopoeia and Huashi (Talc) in Pharmacopoeia of The People's Republic of China.

J Trad Med. 2013;30:34-40.

'Kasseki' in Japanese or 'Huashi' in Chinese are highly similar crude mineral drugs. Though almost the same Chinese characters are used for both, the definition of the former in The Japanese Pharmacopoeia (JP) is different from that of the latter in Pharmacopoeia of The People's Republic of China (CP). Namely, Kasseki is defined as "a mineral substance, mainly composed of aluminum silicate hydrate and silicon dioxide" in JP, while Huashi is defined as "mainly hydrated magnesium silicate" in CP. Since the Kasseki used in Japan is imported from China, discrimination of these two is important from the viewpoint of regulatory science. In this report we applied a taste-sensing system having artificial lipid membrane sensors to discriminate between Kasseki and Huashi. First, seven types of sensors were tested on serial concentrations of water extracts of Kasseki and Huashi. The results suggested that the AC0 and AAE sensors were appropriate for our purpose when 1% (w/w) water extracts of samples were used. Next, we tested ten each of Kasseki and Huashi samples in this condition. For the Kasseki samples, both sensors showed specifically localized output values ranging from 0 to -5 mV. By contrast, for the Huashi samples, AC0 characteristically showed output values deviating from the range within ±5 mV and AAE showed a wide range of output values, from -22 to 1 mV. These data suggest that the taste-sensing system can discriminate Kasseki from Huashi when their 1% (w/w) water extracts are measured by AC0 and AAE sensors.

Keywords: Kasseki, Huashi, taste-sensing system

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Takeda A, Wakana D, Yokokura T*1, Kamiya H*1, Asama H*1, Kondo S*1, Wada A*1, Ukita K*1, Wakabayashi K*1, Takahashi K*1, Tomitsuka H*1, Sasaki H*1, Kikuchi Y*2, Yamamoto Y*3, Shimada Y*3, Goda Y: Studies on the identification test for JUNCI HERBA.

Pharm and Med Dev Regulatory Sci. 2012;43:1116-20.

JUNCI HERBA is the crude drug derived from the aerial part or the pith of *Juncus effusus* (Juncaceae). The drug is mainly used for the treatment of various eye disorders as a component of Kampo formulas, such as Jijinmeimokuto. The formula, Jijinmeimokuto, has been added to the standards of approval for OTC Kampo formulations as of April, 2011, but its component, JUNCI HERBA, has no disclosed official std. yet, even though it is used under a letter of approval. Therefore, we investigated the identification test of the drug in prepn. for listing of the drug in The Japanese Standards for non-Pharmacopoeial Crude Drugs 2012 (non-JPS 2012). We have developed a test based on the detection of Iuteolin and its 3',5-dimethylether derivative by TLC. The test clearly distinguished the drug from EQUISETI or EPHEDRAE HERBA, both of

which have similar appearance to JUNCI HERBA. The established TLC conditions were as follows: chromatographic support, silica gel; developing solvent, EtOAc/2-butanone/water/formic acid (25/3/1/1) : developing length, 7 cm; visualization, UV (365 nm) and ferric chloride-methanol reagent: R_f values, 0.75 for luteolin and 0.4 for luteolin 3',5-dimethylether.

Keywords: JUNCI HERBA, luteolin 3',5-dimethylether, Non-JPS 2012

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Anjiki N*1, 2, Hosoe J, Fuchino H*3, Ikezaki H*1, Mikage M*2, Kawahara N*3, Goda Y: Quality evaluation of essential oils by a taste-sensing system.
Jpn J Food Chem Safety 2012;19:32-7.

Recently, it has been recognized effectiveness and functionality of aromatherapy, a natural holistic approach to therapy using essential oils and other plant extracts. Many common essential oils have been used for such as perfume materials, flavor ingredients and antiseptic purposes since ancient times and are still widely used today. Essential oils are registered in "The Japan's Specifications and Standards for Food Additives" mainly used as bitter substances and anti-oxidants, and also seven essential oils are registered in "The Japanese Pharmacopoeia Sixteenth Edition". In this study for development of a new method for the quality evaluation of essential oils, we investigated the profile analysis of 16 kinds of essential oils by a taste-sensing system. As the results, 16 kinds of essential oils were classified mainly into 5 types by the taste distributions. Furthermore, we purchased com. clove and thyme oils, both of which showed high taste intensities in "anionic bitterness" and investigated the relationship between their anionic bitterness intensity and the amts. of the main constituents, namely eugenol and thymol for clove and thyme oils, resp. In consequence, as clove oils, the "anionic bitterness" intensities of eight samples were approx. the same as those of the corresponding std. samples of eugenol. As for the remaining three samples, more than 70% of the "anionic bitterness" intensity was attributed to eugenol content. These data strongly suggest that the "anionic bitterness" taste of clove oil is mostly derived from eugenol. Meanwhile, as thyme oils, no correlation was obsd. between the "anionic bitterness" intensity and thymol content. This finding suggests that constituents other than thymol may have a larger effect on the anionic bitterness intensity of thyme oil.

Keywords: essential oils, taste-sensing system, taste classification

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Amakura Y*1, Fuchino H*2, Yoshimura M*1, Yamakami S*1, Yoshida T*1, Goda Y, Kawahara N*2: High-performance TLC comparison of components in the Crude Drugs "Scutellariae Radix" available in Japan.
Pharm and Med Dev Regulatory Sci. 2012;43:644-9.

As part of a project to construct a database of official crude drugs, constituents in fifteen samples of "Scutellariae Radix" (the root of *Scutellaria baicalensis*) on the market in Japan were compared by means of high-performance TLC (HPTLC) according to the crude drug identification test in the Japanese Pharmacopoeia. A main spot corresponding to std. baicalin was detected in all of the HPTLC chromatograms, together with spots of wogonin, wogonoside, and chrysin 6-C-arabinoside 8-C-glucoside. These data provide a characteristic pattern that should be useful as an indicator for quality inspection of crude drugs available on the market. In addition, HPLC analysis was performed, confirming the presence of baicalin as the main component of these crude drugs. Peaks of wogonin, wogonoside, baicalein, and chrysin 6-C-arabinoside-8-C-glucoside were also detected.

Keywords: crude drug, Scutellaria Root, HPTLC

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Daikonya A*1, Fuchino H*1, Takahashi Y*2, Goda Y, Kawahara N*1: Inhibitory effect of ginger (*Zingiber officinale* Roscoe) from the Japanaese market on nitrilenoxide production, and metabolome analysis

based on LC/MS.

Jpn J Pharmacog. 2013;67:1-6.

As a part of construction of "Comprehensive Medicinal Plant Database" mainly used for Kampo medicine, ginger rhizomes (Zingiber officinale Roscoe) were collected from the Japanese market, and extracted with hot water. We evaluated their inhibitory activity on nitric oxide (NO) production by LPS/IFN-・activated macrophages. The ginger extracts (GEs) showed weak inhibitory activity against NO production. Furthermore, we measured the total ion chromatogram (TIC) of the GEs using LC/MS to perform metabolome analysis. The result of principal component analysis (PCA) revealed that the GEs were classified into two groups. Next, orthogonal partial least squares (OPLS) were performed on the basis of NO inhibitory activity. As a result, the GEs were divided into two groups according to the strength of activity. It was shown that [6]-gingerol, main component of ginger, was contributed the distinction of two groups. These results suggested that [6]-gingerol might be useful as a biomarker to evaluate an anti-inflammatory effect of ginger.

Keywords: *Zingiber officinale*, Comprehensive Medicinal Plant Database, LC/MS metablomics

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Abbaskhan A*1, Choudhary MI*1, Ghayur MN*2, Parween Z*1, Shaheen F*1, Gilani A*1, Maruyama T, Iqbal K*1, Tsuda Y*1: Biological activities of Indian celery, *Seseli diffusum* (Roxb. ex Sm.) Sant. & Wagh.

Phytother Res. 2012;26:783-6.

In continuation of our work on Indian celery (*Seseli diffusum* (Roxb. ex Sm.) Santapau & Wagh; Umbelliferae), the fractionation of the 80% MeOH−H_2O extract of the seeds was performed to identify the principles responsible for its folk use as an antispasmodic and diuretic. Several compounds were isolated as active components: seselin (1) and anthriscinol methyl ether (4) showed a selective cytotoxicity to some yeast strains. Compound 1 also showed spasmolytic activity. On the other hand, isopimpinellin (3) and isorutarin (5) exhibited a spasmogenic effect on the smooth muscle preparations. Compound 5 was also found to have antioxidant activity. Among them, compound 4 was isolated for the first time from this plant.

Keywords: *Seseli diffusum*, cytotoxicity, spasmolytic activity

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日本食品化学学会誌 2012;19:111-8.

During the course of our study on the borderline of pharmaceuticals to non-pharmaceuticals, the morphological features and the internal transcribed spacer (ITS) sequences in the nuclear rDNA of *Sida* plants and the crude drugs/health foods so called *Sida* products were investigated. As the results, we revealed that 7 of 11 products tested contained *Sida* plants and 3 products among them included the other plant material (s) together with *Sida*. The ITS sequences of *Sida* plants observed in this study were classified into 6 genotypes. One of them is identical with that of *Sida fallax* whereas the others had no identical sequence on the international nucleotide sequence databases. On the other hand, other species including *Urena*, *Malva* and *Triumfetta* plants of the family, Malvaceae were detected from 7 products. In field survey on Oahu Island, the state of Hawaii, USA, Malvaceus plants possessing a *Sida* like flower were observed at the same place together with *Sida* plant. This growing environment in field is likely to be one of the reasons for the contamination in the products. Simultaneously, our field survey suggests that the appearances of the flowers were not critical points for the identification of *Sida* plants. Based on microscopic observations, we found that the stellate hair on leaves and the features of mericarps were suitable for the purpose. In conclusion, the exact identification of their botanical origin is important for regulation of *Sida* products on the borderline of pharmaceuticals to non-pharmaceuticals.

Keywords: *Sida*属植物，DNA配列解析，形態観察

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Kumeta Y, Maruyama T, Wakana D, Kamakura H, Goda Y: Chemical analysis reveals the botanical origin of shatavari products and confirms the absence of alkaloid asparagamine A in Asparagus racemosus. *J Nat Med*. 2013;67:168-73.

Shatavari－a famous Ayurveda materia medica used mainly as a tonic for women－is distributed in health food products all over the world. The Ayurvedic Pharmacopoeia of India identifies the botanical origin of shatavari as the tuberous root of *Asparagus racemosus*. We recently investigated by DNA analysis the botanical origin of shatavari products on the Japanese market. The results suggested that their botanical origin was *Asparagus*; however, species identification was difficult. In this study, we analyzed steroidal saponins, including those specific to this plant, in these products and confirmed their origin as *A. racemosu*s. Next, alkaloid analyses of an authentic *A. racemosus* plant and these products were performed, because several papers have reported the isolation of a pyrrolo[1,2-a]azepine alkaloid, asparagamine A, from this plant. Our results suggested that neither plant material nor products contained asparagamine A. It has been pointed out that *Stemona* plants are sometimes mistaken for shatavari, because their tuberous roots have a similar shape to that of *A. racemosus,* and pyrrolo[1,2-a]azepine alkaloids are thought to be *Stemona*-specific. These data strongly suggest that *A. racemosus* does not contain asparagamine A, and that previous isolation of asparagamine A from materials claimed as originating from *A. racemosus* was likely caused by misidentification of *Stemona* plants as *A. racemosus*.

Keywords: *Asparagus racemosus*, *Stemona* plants, asparagamine A

Kakigi Y*, Hakamatsuka T, Icho T*, Goda Y, Mochizuki N*: Comprehensive Analysis of Flavonols in Ginkgo biloba Products by Ultra-High-Performance Liquid Chromatography Coupled with Ultra-Violet Detection and Time-of-Flight Mass Spectrometry. *Biosci Biotech Biochem*. 2012;76:1003-7.

The aim of this study was to confirm the flavonol compositions of commercial ginkgo leaf products on the Japanese food market. A total of 22 commercial ginkgo leaf products and 3 ginkgo raw leaves were examined by ultra-high-performance liquid chromatography coupled with ultra-violet detection and time-of-flight mass spectrometry (UHPLC－UV－TOFMS), and then applied to multivariate data analysis. Using this method, 11 flavonol glycosides, 3 biflavones and quercetin were identified and 22 ginkgo leaf products tested were classified into 4 groups. Most ginkgo leaf products contained high percentages of flavonol glycosides. On the other hand, we revealed that some products contained high percentages of queretin and biflavones inspite of flavonol glycosides.

Keywords: *Ginkgo biloba*, flavonols, principal component analysis

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Jin JS*[1,2], Tobo T*[1], Chung MH, Ma CM*[1,3], Hattori M*[1]: Transformation of trachelogenin, an aglycone of tracheloside from safflower seeds, to phytoestrogenic (-)-enterolactone by human intestinal bacteria. *Food Chemistry* 2012;134:74-80.

For the purpose of surveying naturally occurring precursors of oestrogenic substances, and their metabolic processes, to mammalian lignans such as enterodiol (END) and enterolactone (ENL), many plant lignans have been studied. Trachelogenin, an aglycone of tracheloside, occurring in the seeds of *Carthamus tinctorius* L. (safflower), was demonstrated to transform to seven metabolites, including (-)-ENL, by anaerobic incubation with a human faecal bacterial mixture, when the reaction was monitored by LC/MS. The structures of the metabolites were determined by spectroscopic means after a large-scale incubation and purification of the respective metabolites. Moreover, the ligand-binding affinity of these metabolites to oestrogen receptors (ERs) α and β was measured in comparison with that of (+)-ENL. (-)- and (+)-ENL were found to significantly bind to both ERα and β, in which an appreciable difference in affinity was observed between (+)- and (-)-ENL for ERβ, but not for ERα.

Keywords: trachelogenin, bacterial transformation, enterolactone

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Kikura-Hanajiri R, Uchiyama N, Kawamura M, Goda Y: Changes in the prevalence of synthetic cannabinoids and cathinone derivatives in Japan until early 2012.

Forensic Toxicol. 2013;31:44-53.

The changes in the prevalence of designer drugs and their legal status in Japan were investigated on the basis of the analyses of 686 different products containing synthetic cannabinoids and/or cathinone derivatives obtained from 2009 to February 2012. In the early stages of distribution of herbal-type products containing synthetic cannabinoids, cyclohexylphenols and naphthoylindoles were mostly found in the products. In November 2009, however, cannabicyclohexanol, CP-47,497 and JWH-018 were controlled as "designated substances" under the Pharmaceutical Affairs Law in Japan, and the cyclohexylphenols have since disappeared from the illegal drug market and been replaced by various analogs of the naphthoylindoles, phenylacetylindoles and benzoylindoles. These compounds, which have high affinities for the cannabinoid CB1 receptor, have become very popular, and the number of emergency hospitalizations associated with their use has dramatically increased from 2011. Other synthetic compounds with different structures and pharmacological effects, such as cathinone derivatives, have been detected together with the synthetic cannabinoids in herbal-type products since 2011. Moreover, many new types of synthetic cannabinoids, different from the four typical structures described, have also begun to appear since 2011. In addition to the synthetic cannabinoids, liquid or powdery-type products containing cathinone derivatives have been widely distributed recently. In 2009, the most popular cathinone derivative was 4-methylmethcathinone. After this compound was controlled as a designated substance in November 2009, cathinone derivatives, which have a pyrrolidine structure at the nitrogen atom and a 3,4-methylenedioxy structure, or analogs of 4-methylmethcathinone, became popular. In the present analysis, tryptamines were also detected in 31 % of the products containing cathinone derivatives. Local anesthetics such as procaine, lidocaine, benzocaine and dimethocaine were also frequently detected. In total, we identified at least 35 synthetic cannabinoids and 22 cathinone derivatives during this survey.

Keywords: designer drugs, synthetic cannabinoids, cathinone derivatives

Takahashi K*, Uchiyama N, Fukiwake T*, Hasegawa T*, Saijou M*, Motoki Y*, Kikura-Hanajiri R, Goda Y: Identification and quantitation of JWH-213, a cannabimimetic indole, as a designer drug in an herbal product.

Forensic Toxicol. 2013;31:145-50.

In our survey of designer drugs in the Japanese market, a cannabimimetic indole was identified as a new active compound in a herbal product. The structure of this compound was elucidated by liquid chromatography − photodiode array − mass spectrometry (LC − PDA − MS), gas chromatography − mass spectrometry (GC − MS), high-resolution MS, and nuclear magnetic resonance (NMR) analyses. The compound was finally identified as (4-ethyl-1-naphthalenyl)(2-methyl-1-pentyl-1*H*-indol-3yl)methanone (JWH-213), an indole-based cannabinoid receptor ligand. To our knowledge, this is the first finding of JWH-213 as a designer drug in a herbal product. The quantitative LC − PDA analysis showed that the JWH-213 content in the product was 252 mg/pack.

Keyword: JWH-213, designer drug, synthetic cannabinoid

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Kitajima M*, Iwai M*, Kogure N*, Kikura-Hanajiri R, Goda Y, Takayama H*: Aspidosperma-aspidosperma-type bisindole alkaloids from Voacanga Africana.

Tetrahedron 2013;69:796-801.

Four new aspidosperma − aspidosperma-type bisindole alkaloids 1 − 4 were isolated from the seeds and root bark of *Voacanga africana* (Apocynaceae) and their structures were determined by spectroscopic analysis. Among them, voacandimine A (2) featured a linkage mode new to dimeric monoterpenoid indole alkaloids.

Keywords: indole alkaloid, *Voacanga africana*, structure elucidation

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Inagaki S*, Hirashima H*, Taniguchi S*, Higashi T*, Min JZ*, Kikura-Hanajiri R, Goda Y, Toyo'oka T*:

Rapid enantiomeric separation and simultaneous determination of phenethylamines by ultra high performance liquid chromatography with fluorescence and mass spectrometric detection: application to the analysis of illicit drugs distributed in the Japanese market and biological samples.

Drug Test Anal. 2012;4:1001-8.

A rapid enantiomeric separation and simultaneous determination method based on ultra high performance liquid chromatography (UHPLC) was developed for phenethylamine-type abused drugs using (*R*)-(-)-4-(N,N-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-(-)-DBD-Py-NCS) as the chiral fluorescent derivatization reagent. The derivatives were rapidly enantiomerically separated by reversed-phase UHPLC using a column of 2.3-μm octadecylsilica (ODS) particles by isocratic elution with water-methanol or water-acetonitrile systems as the mobile phase. The proposed method was applied to the analysis of products containing illicit drugs distributed in the Japanese market. Among the products, 1-(3,4-methylenedioxyphenyl)butan-2-amine (BDB) and 1-(2-methoxy4,5-methylenedioxyphenyl)propan-2-amine (MMDA-2) were detected in racemic form. Furthermore, the method was successfully applied to the analysis of hair specimens from rats that were continuously dosed with diphenyl(pyrrolidin-2-yl)methanol (D2PM). Using UHPLC-fluorescence (FL) detection, (*R*)- and (*S*)-D2PM from hair specimens were enantiomerically separated and detected with high sensitivity. The detection limits of (*R*)- and (*S*)-D2PM were 0.12 and 0.21 ng/mg hair, respectively (signal-to-noise ratio (S/N) = 3).

Keywords: phenethylamines, diphenyl(pyrrolidin-2-yl)methanol, chiral derivatization method

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Wada M*, Yamahara K*, Ikeda R*, Kikura-Hanajiri R, Kuroda N*, Nakashima K*: Simultaneous determination of N-benzylpiperazine and 1-(3-trifluoromethylphenyl) piperazine in rat plasma by HPLC-fluorescence detection and its application to monitoring of these drugs.

Biomed Chromatogr. 2012;6:21-5.

An HPLC-fluorescence detection method for simultaneous determination of N-benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP) labeled with 4-(4,5-diphenyl-1 *H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) was described. DIB-BZP and -TFMPP were well separated within 13 min without interference of peaks from plasma components. The lower detection limits of BZP and TFMPP at a signal-to-noise ratio of 3 were 0.9 and 4.6 ng/mL, respectively. Precisions of the proposed method for intra- and inter-day assays were less than 4.8 and 9.1% as %RSD (n = 5). Furthermore, the method could be successfully applied to monitor both compounds in plasma after their sole or co-administration to rats (each dose, 2 mg/kg). Clearance of TFMPP was significantly different under the conditions (P = 0.047).

Keywords: benzylpiperazine, 1-(3-Trifluoromethylphenyl) piperazine, HPLC-fluorescence detection

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Uchiyama N, Matsuda S, Wakana D, Kikura-Hanajiri R, Goda, Y: New cannabimimetic indazole derivatives, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA) and *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA), identified as designer drugs in illegal products.

Forensic Toxicol. 2013;31:93-100.

Two new cannabimimetic indazole derivatives, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA, 1) and *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA, 2), have been identified as designer drugs in illegal products. These identifications were based on liquid chromatography − mass spectrometry, gas chromatography − mass spectrometry, high-resolution mass spectrometry, and nuclear magnetic resonance spectroscopy. Because there have been neither chemical nor pharmacological data about compound 1 until now, this is the first report of this compound. Compound 2 was reported as a potent cannabinoid CB1 receptor modulator when synthesized by Pfizer in 2009; but this is the first report of its detection in illegal products.

Keywords: *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA), *N*-

(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide（AB-FUBINACA), synthetic cannabinoid

Uchiyama N, Kawamura M, Kikura-Hanajiri R, Goda, Y: Identification of two new-type synthetic cannabinoids, *N*-(1-adamantyl)-1-pentyl-1*H*-indole-3-carboxamide（APICA）and *N*-(1-adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide（APINACA), and detection of five synthetic cannabinoids, AM-1220, AM-2233, AM-1241, CB-13（CRA-13), and AM-1248, as designer drugs in illegal products.
Forensic Toxicol. 2012;30:114-25.

Two new-type synthetic cannabinoids, *N*-(1-adamantyl)-1-pentyl-1*H*-indole-3-carboxamide（APICA, 1）and *N*-(1-adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide（APINACA, 2), have been identified as designer drugs in illegal products being sold in Japan. The identification was based on liquid chromatography－mass spectrometry（LC-MS), gas chromatography－mass spectrometry（GC-MS), high-resolution MS and nuclear magnetic resonance（NMR）analyses. Both mass and NMR spectrometric data revealed that 1 was 1-pentyl-*N*-tricyclo[3.3.1.1^{3,7}]dec-1-yl-1*H*-indole-3-carboxamide, and 2 was 1-pentyl-*N*-tricyclo[3.3.3.1.^{3,7}]dec-1-yl)-1*H*-indazole-3-carboxamide. Though many of the synthetic cannabinoids detected in illegal products, such as JWH-018, have a 3-carbonyl indole moiety, compounds 1 and 2 are a new type of synthetic cannabinoids having an amide and an adamantyl group, and 2 also has an indazole group in place of an indole group. There has been no synthetic, chemical or biological information about 1 and 2 until now, making this the first report of these cannabimimetic compounds（1 and 2）as designer drugs. In addition, five synthetic cannabinoids, AM-1220, AM-2233, AM-1241, CB-13（CRA-13）and AM-1248, are also described herein as newly distributed designer drugs in Japan.
Keywords: *N*-(1-adamantyl)-1-pentyl-1*H*-indole-3-carboxamide, *N*-(1-adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide, AM-1220

Hirasawa Y[*1], Kato Y[*1], Wong CP[*1], Uchiyama N, Goda Y, Hadi HA[*2], Morita H[*1]: Huperminone A, a novel C_{16}N-type *Lycopodium* alkaloid from *Huperzia phlegmaria*.
Tetrahedron Lett. 2013;54:1593-5.

A novel C_{16}N-type *Lycopodium* alkaloid consisting of a decahydroquinoline and a cyclohexanone, huperminone 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. This unique C_{16}N-type skeleton lacking in a nitrogen atom may be generated from $C_{16}N_2$-type phlegmarane skeleton.
Keywords: huperminone A, *Huperzia phlegmaria*, *Lycopodium* alkaloid

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Hashimoto M[*1], Seshime Y[*1], Kitamoto K[*2], Uchiyama N, Goda Y, Fujii I[*1]: Identification of csypyrone B2 and B3 as the minor products of *Aspergillus oryzae* type III polyketide synthase CsyB.
Bioorg Med Chem Lett. 2013;23:650-3.

Since our first report on the identification of the fungal type III polyketide synthase（PKS）genes *csyA*～*D* in *Aspergillus oryzae* RIB40, type III PKS homologues have also been found in other fungal species. We previously reported the isolation and structural determination of csypyrone B1 as the main product of CsyB when inductively expressed in *Aspergillus oryzae*. Herein we report the isolation and identification of the two minor products of the *csyB* transformant in addition to csypyrone B1 as 4-(3-acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)butyric acid and 5-(3-acetyl-4-hydroxy-2-oxo-2*H*-pyran-6-yl)pentanoic acid. These compounds were named csypyrone B2 and B3, respectively, and both are homologues of main product csypyrone B1 with different side chain lengths. This result suggests that the carbon skeleton of the csypyrone B precursor is constructed by the condensation of fatty acyl-CoA and acetylmalonyl-CoA followed by pyrone formation. The alkyl side chain of the precursor may be oxidatively cleaved by enzyme(s) in the host fungus to give variations of csypyrone B with propanoic acid, butyric acid, or pentanoic acid side chains.
Keywords: *Aspergillus oryzae*, Type III polyketide synthase, csypyrone B2

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Hirasawa Y[*1], Matsuya R[*1], Shaari K[*2], Lajis NH[*2], Uchiyama N, Goda Y, Morita H[*1]: Lycobelines A－C, novel $C_{16}N_2$-type *Lycopodium* alkaloids from *Huperzia goebelii*.

Tetrahedron Lett. 2012;53:3971-3.

Novel $C_{16}N_2$-type *Lycopodium* alkaloids consisting of a decahydroquinoline with an aminohexyl side chain, lycobelines A－C (1－3), were isolated from the club moss of *Huperzia goebelii*, and their structures and relative stereochemistry were elucidated on the basis of spectroscopic data and chemical correlations.

Keywords: *Lycopodium* alkaloid, lycobelines A－C, *Huperzia goebelii*

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Ogawa Y[*1], Uchiyama N, Konishi T[*1], Urade Y[*2]: Oxypinnatanine promotes non-rapid eye movement sleep in mice.

Sleep and Biological Rhythms 2013;11:40-5.

The flowers and leaves of *Hemerocallis fulva* var. *sempervirens*, Akinowauregusa in Japanese, were eaten to cure insomnia in Okinawa, Japan. We found that *H. fulva* var. *sempervirens* contained oxypinnatanine, a unique derivative of glutamic acid or glutamine with a furfuryl group isolated from only a few plants. In this study, we demonstrated by electroencephalographic analyses that an oral administration of oxypinnatanine (5, 15 and 30 mg/kg) to mice at light-off time increased non-rapid eye movement (non-REM, NREM) sleep in a dose-dependent manner. During the 3-h period after the administration, oxypinnatanine (30 mg/kg) increased the total time of NREM sleep by 84%, by increasing the number of stage transitions from wakefulness to NREM sleep by 76% and the number of NREM sleep bouts twofold, and by decreasing the mean episode duration of wakefulness by 54% without changing the mean episode duration of NREM sleep, the amount of rapid eye movement sleep or rebound insomnia after the induction of NREM sleep. Therefore, oxypinnatanine is an effective sleep-promoting substance of *H. fulva* var. *sempervirens*

Keywords: *Hemerocallis fulva* var. *sempervirens*, non-rapid eye movement sleep, oxypinnatanine

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Lee HC[*1], Inoue T, Sasaki J[*2], Nakasaki Y[*1], Hattori M[*3], Kono N[*1], Itoh T[*4], Ogiso H[*5], Taguchi R[*5], Arita M[*1], Sasaki T[*2], Arai H[*1]: LPIAT1 regulates arachidonic acid content in phosphatidylinositol and is required for cortical lamination in mice.

Mol Biol Cell 2012;23:4689-700.

Dietary arachidonic acid (AA) has roles in neuronal development and cognitive function in infants. AA is remarkably enriched in phosphatidylinositol (PI), a constituent of biological membranes; however, physiological significance of AA-containing PI remains unknown. Here we show that lysoPI acyltransferase 1 (LPIAT1) plays a crucial role in brain development in mice. Lpiat1(-/-) mice show no LPIAT activity with AA as an acyl donor and show reduced AA contents in PI. Lpiat1(-/-) mice show atrophy of the cerebral cortex. Immunohistochemical analysis reveals disordered cortical lamination and delayed neuronal migration in the cortex of Lpiat1(-/-) mice. These results demonstrate that AA-containing PI play an important role in normal cortical lamination during brain development in mice.

Keywords: Arachidonic acid, Phosphatidylinositol

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Lee HC[*1], Kubo T[*1], Kono N[*1], Kage-Nakadai E[*2], Gengyo-Ando K[*2], Mitani S[*2], Inoue T, Arai H[*1]: Depletion of mboa-7, an enzyme that incorporates polyunsaturated fatty acids into phosphatidylinositol (PI), impairs PI 3-phosphate signaling in Caenorhabditis elegans.

Genes Cells 2012;17:748-57.

Phosphatidylinositol (PI) is a constituent of biomembranes and a precursor of all phosphoinositides (PIPs). A prominent characteristic of PI is that its sn-2 position is highly enriched in polyunsaturated fatty acids (PUFAs). However, the biological significance of PUFA-containing PI remains unknown. We previously identified C. elegans mboa-7 as an acyltransferase that

incorporates PUFAs into PI. In this study, we performed an RNAi enhancer screen against PI kinases and phosphatases using mboa-7 mutants that have a reduced PUFA content in PI. Among the genes tested, knockdown of vps-34, a catalytic subunit of class III PI 3-kinase that produces PI 3-phosphate (PI3P) from PI, caused severe growth defects in mboa-7 mutants. We also found that, like knockdown of vps-34, knockdown of autophagy-related genes caused severe growth defects in mboa-7 mutants. Finally, we showed that autophagic clearance of protein aggregates is impaired in mboa-7 mutants. Taken together, these results suggest that the PUFA chain in PI has a role in some PI3P signaling.

Keywords: Phosphatidylinositol, PUFA

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Kuroda T, Yasuda S, Kusakawa S, Hirata N, Kanda Y, Suzuki K, Takahashi M*1, Nishikawa S*2, Kawamata S*2, Sato Y: Highly sensitive in vitro methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPS cells.

PLoS One 2012;7:e37342.

Human induced pluripotent stem cells (hiPSCs) possess the capabilities of self-renewal and differentiation into multiple cell types, and they are free of the ethical problems associated with human embryonic stem cells (hESCs). These characteristics make hiPSCs a promising choice for future regenerative medicine research. There are significant obstacles, however, preventing the clinical use of hiPSCs. One of the most obvious safety issues is the presence of residual undifferentiated cells that have tumorigenic potential. To locate residual undifferentiated cells, in vivo teratoma formation assays have been performed with immunodeficient animals, which is both costly and time-consuming. Here, we examined three in vitro assay methods to detect undifferentiated cells (designated an in vitro tumorigenicity assay) : soft agar colony formation assay, flow cytometry assay and quantitative real-time polymerase chain reaction assay (qRT-PCR). Although the soft agar colony formation assay was unable to detect hiPSCs even in the presence of a ROCK inhibitor that permits survival of dissociated hiPSCs/hESCs, the flow cytometry assay using anti-TRA-1-60 antibody detected 0.1% undifferentiated hiPSCs that were spiked in primary retinal pigment epithelial (RPE) cells. Moreover, qRT-PCR with a specific probe and primers was found to detect a trace amount of Lin28 mRNA, which is equivalent to that present in a mixture of a single hiPSC and 5.0×10^4 RPE cells. Our findings provide highly sensitive and quantitative in vitro assays essential for facilitating safety profiling of hiPSC-derived products for future regenerative medicine research.

Keywords: iPS cell, Lin 28, Regenerative medicine

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Nakaya M*1, Chikura S*1, Watari K*1, Mizuno N*1, Mochinaga K*1, Mangmool S*1, Koyanagi S*1, Ohdo S*1, Sato Y, Ide T*2, Nishida M*1, Kurose H*1: Induction of cardiac fibrosis by β-blocker in G protein-independent and GRK5/β-arrestin2-dependent signaling pathways.

J Biol Chem. 2012;287:35669-77.

G-protein coupled receptors (GPCRs) have long been known as receptors that activate G protein-dependent cellular signaling pathways. In addition to the G protein-dependent pathways, recent reports have revealed that several ligands called "biased ligands" elicit G protein-independent and β-arrestin-dependent signaling through GPCRs (biased agonism). Several β-blockers are known as biased ligands. All β-blockers inhibit the binding of agonists to the β-adrenergic receptors. In addition to β-blocking action, some β-blockers are reported to induce cellular responses through G protein-independent and β-arrestin-dependent signaling pathways. However, the physiological significance induced by the β-arrestin-dependent pathway remains much to be clarified in vivo. Here, we demonstrate that metoprolol, a β_1-adrenergic receptor-selective blocker, could induce cardiac fibrosis through a G protein-independent and β-arrestin2-dependent pathway. Metoprolol, a β-blocker, increased the expression of fibrotic genes responsible for cardiac fibrosis in cardiomyocytes. Furthermore, metoprolol induced the interaction between β_1-adrenergic receptor and β-arrestin2, but not β-arrestin1. The interaction between β_1-adrenergic receptor and β-arrestin2 by metoprolol was impaired in the G protein-coupled receptor kinase 5 (GRK5)

-knockdown cells. Metoprolol-induced cardiac fibrosis led to cardiac dysfunction. However, the metoprolol-induced fibrosis and cardiac dysfunction were not evoked in β-arrestin2- or GRK5-knock-out mice. Thus, metoprolol is a biased ligand that selectively activates a G protein-independent and GRK5/β-arrestin2-dependent pathway, and induces cardiac fibrosis. This study demonstrates the physiological importance of biased agonism, and suggests that G protein-independent and β-arrestin-dependent signaling is a reason for the diversity of the effectiveness of β-blockers.

Keywords: β-blocker, cardiac fibrosis, biased agonism

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Nakaya M*1, Tajima M*1, Kosako H*2, Nakaya T*3, Hashimoto A*1, Watari K*1, Nishihara H*1, Ohba M*1, Komiya S*1, Tani N*2, Nishida M*1, Taniguchi H*2, Sato Y, Matsumoto M*2, Tsuda M*1, Kuroda M*3, Inoue K*1, Kurose H*1: GKR6 deficiency in mice causes autoimmune disease due to impaired apoptotic cell clearance.

Nat Commun. 2013;4:1532.

Efficient engulfment of apoptotic cells is critical for maintaining tissue homoeostasis. When phagocytes recognize ‘eat me’ signals presented on the surface of apoptotic cells, this subsequently induces cytoskeletal rearrangement of phagocytes for the engulfment through Rac1 activation. However, the intracellular signalling cascades that result in Rac1 activation remain largely unknown. Here we show that G-protein-coupled receptor kinase 6 (GRK6) is involved in apoptotic cell clearance. GRK6 cooperates with GIT1 to activate Rac1, which promotes apoptotic engulfment independently from the two known DOCK180/ELMO/Rac1 and GULP1/Rac1 engulfment pathways. As a consequence, GRK6-deficient mice develop an autoimmune disease. GRK6-deficient mice also have increased iron stores in splenic red pulp in which F4/80+ macrophages are responsible for senescent red blood cell clearance. Our results reveal previously unrecognized roles for GRK6 in regulating apoptotic engulfment and its fundamental importance in immune and iron homoeostasis.

Keywords: GRK6, apoptotic cell clearance, autoimmune disease

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Wu Y*, Qi X*, Gong L*, Xing G*, Chen M*, Miao L*, Yao J*, Suzuki T, Furihata C, Luan Y*, Ren J*: Identification of BC005512 as a DNA damage responsive murine endogenous retrovirus of GLN family involved in cell growth regulation.

PLoS One 2012;7:e35010.

By using oligonucleotide microarray, we identified an unknown gene BC00551, whose expression in mouse liver was specifically induced by seven well-known genotoxins (GTXs), but not by non-genotoxins. Bioinformatics revealed that BC00551 was a member of the GLN family of murine endogenous retrovirus (ERV). BC00551 expression was specifically induced by another seven GTXs, covering diverse genotoxicity mechanisms. While in p53 deficient L5178Y cells, GTXs could not induce BC00551 expression. RNA interference revealed that down-regulation of BC00551 expression induced G1/S phase arrest, inhibited cell proliferation and thus suppressed cell growth in NIH/3T3 cells. Together, our results provide the first evidence that BC005512, was responsive to DNA damage and involved in cell growth regulation.

Keywords: Genotoxicity, GLN family of murine endogenous retrovirus (ERV), Cell growth regulation

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Watanabe T*, Suzuki T, Natsume M*, Nakajima M*, Narumi K*, Hamada S*, Sakuma T*, Koeda A*, Oshida K*, Miyamoto Y*, Maeda A*, Hirayama M*, Sanada H*, Honda H*, Ohyama W*, Okada E*, Fujiishi Y*, Sutou S*, Tadakuma A*, Ishikawa Y*, Kido M*, Minamiguchi R*, Hanahara I*, Furihata C*: Discrimination of genotoxic and non-genotoxic hepatocarcinogens by statistical analysis based on gene expression profiling in the mouse liver as determined by quantitative real-time PCR.

Mutat Res. 2012;747:164-75.

qPCR was conducted on liver samples from B6C3F1 mice, at 4 and 48h following a single intraperitoneal administration of 12 different chemicals: 8 genotoxic hepatocarcinogens and 4 non-genotoxic hepatocarcinogens. We quantified 35 genes selected from our previous DNA microarray studies. The current findings demonstrate a successful discrimination between genotoxic and non-genotoxic hepatocarcinogens, using qPCR and PCA, on 12 genes associated with a Trp53-mediated signaling pathway for DNA damage response at 4 and 48 h after a single administration of chemicals.

Keywords: Genotoxic hepatocarcinogens, Gene expression, qPCR

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Suenaga K[*1], Takasawa H[*2], Watanabe T[*1], Wako Y[*2], Suzuki T, Hamada S[*2], Furihata C[*1]: Differential gene expression profiling between genotoxic and non-genotoxic hepatocarcinogens in young rat liver determined by quantitative real-time PCR and principal component analysis.

Mutat Res. 2013;751:73-83.

We applied the candidate marker genes for discrimination of genotoxic/non-genotoxic carcinogens in mice to rat hepatocarcinogens in the rat liver. qPCR analysis of 33 genes was conducted on liver samples from F344 rats at 4 and 48 h after a single oral administration of 2 genotoxic hepatocarcinogens, a non-genotoxic hepatocarcinogen, and a non-genotoxic non-hepatocarcinogen. Thirty-two genes exhibited significant changes in their gene expression ratios. The changes appeared to be greater at 4h than at 48 h. Finally, statistical analysis via PCA successfully differentiated the genotoxic hepatocarcinogens from the non-genotoxic hepatocarcinogen and the non-genotoxic non-hepatocarcinogen at 4h based on 16 genes and at 48 h based on 10 genes.

Keywords: Genotoxic hepatocarcinogens, Gene expression, qPCR

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迫田秀行，松岡厚子：デラミネーション破壊の再現と内部クラック観察.

臨床バイオメカニクス 2012;33:303-9.

人工関節に使用される超高分子量ポリエチレン製コンポーネントのデラミネーション破壊は人工関節の不具合の主要因である．そこで我々は，臨床で発生するデラミネーションを再現可能な新規試験法を開発してきた．本研究では，三次元レーザー顕微鏡を用いて，試料内部に発生した内部クラックの観察法を開発した．その結果，デラミネーションの発生に先立って内部クラックが発生することが示唆された．今後，デラミネーション発生の機構の解明につながる可能性が期待された．

Keywords: artificial joint, delamination, UHMWPE

迫田秀行，京本政之*，井上祐貴*，石原一彦*，松岡厚子：人工関節摺動面材料の形状変化による摩耗量評価の可能性の検討.

臨床バイオメカニクス 2012;33:311-5.

人工関節の摺動面における摩耗は人工関節の不具合の主要因であるため，新材料の開発においては摩耗試験による摩耗特性評価が必須である．しかし，重量変化から摩耗量を評価する現在の摩耗量評価法では，含水量の影響で新規材料の低摩耗を適切に評価できていない可能性が高い．現在使用されている超高分子量ポリエチレン（UHMWPE）と，新規材料として期待されているポリエーテンルエーテルケトン（PEEK）について，含水量の変化と形状変化を評価したところ，UHMWPEに比べPEEKでは含水量が30倍でかつ計量中の安定性にも欠けるのに対し，荷重下でも形状の変化がほとんどないことから，PEEKのような材料では重量変化から摩耗量を評価するより形状変化から摩耗量を評価する方が適切であると考えられた．

Keywords: artificial joint, wear, PEEK

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迫田秀行，松岡厚子：人工関節用超高分子量ポリエチレンのデラミネーション破壊特性評価.

日本人工関節学会誌 2012;42:723-4.

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）のデラミネーション破壊は，摩耗と並び人工関節の主な不具合要因の一つである．しかし，その評価法は確立していないため，新規評価法を開発し，様々な材料について評価を行った．その結果，UHMWPEに酸化劣化が生じるとデラミネーション特性は大幅に低下することがわかった．また，滅菌を目的とした低線量のガンマ線照射や，高度架橋UHMWPEでは，Virginよりデラミネーション特性が向上していることが示唆された．本評価法を新材料に適用し評価することで，今後の

不具合低減への貢献が期待できる．
Keywords: artificial joint, delamination, UHMWPE

小関弘展[*1]，志田崇之[*2]，依田周[*2]，堀内英彦[*2]，迫田秀行，尾崎誠[*2]：生体人工材料表面におけるバイオフィルム形成．
関節外科 2013;32:101-5.

埋植用生体材料は，その表面に細菌が付着・増殖することにより，難治性の感染症の温床となる危険性がある．材料により細菌付着性に違いがあるという報告があることから，本研究では整形外科領域で使用されるコバルトクロム合金，チタン合金，純チタンの各試料を，インプラント関連感染症の代表的病原菌である表皮ブドウ球菌の菌液に３分間浸漬した．試料をリンスした後，２～６時間培養し，各時点でのバイオフィルム占拠率を評価することで，細菌の付着性について検討した．その結果，バイオフィルム占拠率はいずれの試料においても継時的に増加したが，コバルトクロム合金のそれは他の試料のそれに比べ有意に低かった．表面の粗さが細菌の付着・増殖の足場となることが報告されており，コバルトクロム合金の表面粗さが他の試料より小さかったことが，コバルトクロム合金において細胞の付着性が低かった原因であると考えられた．
Keywords: biofilm, biomaterial, implant-related infection

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Nomaguchi M[*1], Yokoyama M[*2], Kono K, Nakayama E E[*3], Shioda T[*3], Saito A[*4], Akari H[*4], Yasutomi Y[*5], Matano T[*6], Sato H[*2], Adachi A[*1]: Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells
Microbes and Infection 2013;15:56-65.

HIV-1 is strictly adapted to humans, and cause disease-inducing persistent infection only in humans. We have generated a series of macaque-tropic HIV-1 (HIV-1mt) to establish non-human primate models for basic and clinical studies. HIV-1mt clones available to date grow poorly in macaque cells relative to SIVmac239. In this study, viral adaptive mutation in macaque cells, G114E in capsid (CA) helix 6 of HIV-1mt, that enhances viral replication was identified. Computer-assisted structural analysis predicted that another Q110D mutation in CA helix 6 would also increase viral growth potential. A new proviral construct MN4Rh-3 carrying CA-Q110D exhibited exquisitely enhanced growth property specifically in macaque cells. Susceptibility of MN4Rh-3 to macaque TRIM5α/TRIMCyp proteins was examined by their expression systems. HIV-1mt clones so far constructed already completely evaded TRIMCyp restriction, and further enhancement of TRIMCyp resistance by Q110D was not observed. In addition, Q110D did not contribute to evasion from TRIM5α restriction. However, the single-cycle infectivity of MN4Rh-3 in macaque cells was enhanced relative to the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells irrelevant to TRIM5 proteins restriction.
Keywords: HIV-1, Macaque cells, Virus growth

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田原麻衣子，小林憲弘，久保田領志，塚本多矩[*1]，杉本直樹，西村哲治[*2]：陰イオン存在下における水道水中のハロ酢酸類のLC/MSおよびLC/MS/MS分析の定量精度の検証．
水道協会雑誌 2012;81(4):20-7.

Haloacetic acids (HAAs) are disinfection byproducts (DBPs) of the chlorination of tap water, and derivatization GC/MS analysis of monochloroacetic acid (MCAA), dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA) are officially set on Water Quality Standard in Japan. As the present official standard method not only consumes time but also obliges any analyst to require the careful cautions for the precision enhancement, we had established direct injection LC/MS analysis that can be one of alternative methods to determine HAAs in tap water sample without pretreatments by carcinogenic solvent extraction, concentration process and derivatization. However, there is still concern that the precision of quantitative values is not reproductive because the anionic species in real

tap water sample might lead to the ion suppression of HAAs. In this report, to set this LC/MS and LC/MS/MS analysis as the official standard method on Water Quality Standard in Japan in future, we evaluated the precision of LC/MS and LC/MS/MS analysis, the adaptability of various analysis columns, and the ion suppression of haloacetic acids by anionic species (Cl^-, Br^-, F^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}). Our study resulted that the direct injection LC/MS and/or LC/MS/MS could analyze haloacetic acids in tap water with sufficient precision.

Keywords: ハロ酢酸類，LC/MS，水道水

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Kanno A*, Kawakami T, Takahashi Y*, Onodera S*: Enhancement of anti-cholinesterase activity of aqueous samples by hypochlorite oxidation for monitoring traces of organophosphorus pesticides in water.
J Toxicol Sci. 2012;37:389-400.

A reproducible method for monitoring traces of cholinesterase (ChE) inhibitors in aqueous samples is described: the method is based on chemical oxidation and a ChE inhibition assay. Chlorine was tested as an oxidizing reagent for conversion of various thiophosphorus pesticides (P=S compounds) into their P=O analogs, which have higher ChE-inhibiting activity. After treating buffered pesticide solutions (pH 6.0) with chlorine (final concentration less than 10 mg/l) of at 25°C for 15 min, the ChE-inhibiting activities and detection limits for each pesticide were determined. Greater ChE-inhibiting activities, leading to lower detection limits (ppb levels), were observed for the chlorine-treated solutions fortified azinphos methyl, diazinon, isoxathion and ronnel etc. No changes in the ChE-inhibiting activities were observed for carbamate pesticide solutions tested before and after chlorination, but an additive effect showed against ChE when these compounds were mixed with paraoxon in water. This combination of oxidative derivatization and ChE inhibition assay was applied successfully to the detection and determination of ChE inhibitors in natural and drinking water samples.

Keywords: cholinesterase-inhibition assay, hypochlorite oxidation, pesticide

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田原麻衣子，中島晋也*1,2，杉本直樹，有薗幸司*1，西村哲治*3：水道水質試験の標準液調製における不確かさと定量精度に影響を及ぼす要因.
水道協会雑誌 2012;81(5):10-6.

The obtained data on quantitative analysis of target compounds for water quality examination have unevenness. It is thought that uncertainty is caused by the operations of a chain to instrumental analysis from making standard solutions. However, the concrete evaluation method of uncertainty in each process for instrumental analysis is not shown definitely. Therefore, the uncertainty caused in making standard solutions for the quantitative analysis was verified by the model experiment that assumed the water quality examination of pesticide butamifos in this study. As the result of extraction of factors, we thought that the uncertainty caused by all of the electronic scale, the apparatus, the experimenter and the measuring instrument in a chain of making standard solutions. We calculated the combined standard uncertainty which accompanied the result of a measurement of butamifos 0.05 mg/L was 1.63%. Furthermore, the purities of butamifos standards (three companies, three products) that were measured by qNMR were 90.3, 94.7 and 94.8%. The gap with true value and the uncertainty between manufacturers that influenced on the accuracy of intra-laboratory became clear. In this study, we proved that error of intra-laboratory was influenced on the purity of standard and the accuracy of quantitative analysis is not secued under the present conditions. The analysis engineers have always to be recognizing current state in order to secure the reliability of quantitative accuracy.

Keywords：不確かさ，信頼性，精度管理

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小林憲弘，杉本直樹，久保田領志，野本雅彦*，五十嵐良明：利根川水系の浄水場におけるホルムアルデヒド水質汚染の原因物質の特定.
水道協会雑誌 2012;81:63-8.

利根川水系の浄水場においてホルムアルデヒドが高濃度で検出され，浄水場の取水停止によって広範囲で断水

が発生した．本水質汚染は，河川に流入した何らかの有機物質が浄水処理過程で反応してホルムアルデヒドが生成したものと考えられた．そこで，汚染発生時の水道原水をLC/MS/MSおよびLC/IT-TOF-MSにより分析し，水質汚染の原因物質を探索した．その結果，全ての検体からヘキサメチレンテトラミンが検出され，試料中ホルムアルデヒド生成能との間に高い相関関係が認められた．さらに，試料中ヘキサメチレンテトラミン濃度から理論上生成するホルムアルデヒド濃度を算出したところ，同試料のホルムアルデヒド生成能とほぼ一致した．以上のことから，今回の水質汚染の主な原因物質は，ヘキサメチレンテトラミンであると結論した．

Keywords：ヘキサメチレンテトラミン，ホルムアルデヒド，水質汚染事故

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伊佐間和郎，河上強志，西村哲治*：乳幼児が誤飲する可能性のある金属製アクセサリーからの有害８元素の溶出．

薬学雑誌 2012;132:959-68.

The International Standard ISO 8124-3:2010 “Safety of toys－Part 3: Migration of certain elements” controls the levels of migrated eight harmful elements (antimony, arsenic, barium, cadmium, chromium, lead, mercury and selenium) from infants toys. Moreover, the Japanese Food Sanitation Law controls the levels of migrated lead from metal accessory toys. However, the levels of migrated harmful elements from metal accessories that are not infants toys are not controlled, since they are not covered by the ISO Standard or the Food Sanitation Law. Therefore, we investigated the level of eight harmful elements migrated from metal accessories that infants may swallow by mistake. The extraction test of ISO 8124-3: 2010 was executed in 117 products (total 184 specimens), and the concentration of these eight elements was measured by inductively coupled plasma mass spectroscopy (ICP-MS). As a result, 28 and one products released lead and cadmium beyond the maximum acceptable levels of the ISO standard, respectively. Metal accessories that infants may swallow by mistake should ideally not release harmful elements such as lead and cadmium.

Keywords: lead, metal accessory, ISO-8124-3:2010

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Ohkawara S*, Tanaka-Kagawa T, Furukawa Y, Jinno H: Methylglyoxal ativates the human transient receptor potential ankyrin 1 channel.

J Toxicol Sci. 2012;37:831-5.

Methylglyoxal (MG) is an endogenous carbonyl compound that is produced in large quantity under hyperglycemic conditions, which are believed to contribute to the development of diabetic neuropathy. However, the mechanism by which this occurs and the molecular targets of MG are unclear. In the present study, we investigated the effect of MG on transient receptor potential ankyrin 1 (TRPA1) activation in human TRPA1-expressing HEK293 cells. MG activated TRPA1-expressing HEK293 cells, but failed to activate human capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1)-expressing HEK293 cells or mock-transfected HEK293 cells. MG also induced calcium (Ca^{2+}) influx in a concentration-dependent manner, and the concentration-response curve indicates that the effect of MG has an EC_{50} of 343.1 ± 17.3 μM. Interestingly, the time course in the intracellular Ca^{2+} concentration ($[Ca^{2+}](i)$) in human TRPA1-expressing HEK293 showed considerable differences in response to MG and cinnamaldehyde. Furthermore, we examined four endogenous carbonyl compounds, including MG, glyceraldehyde, glycolaldehyde, and glyoxal; only MG notably activated TRPA1-expressing HEK293 cells. These results may provide insight into the TRPA1-mediated effects of MG on diabetic neuropathy.

Keywords: transient receptor potential ankyrin 1 (TRPA1), methylglyoxal, diabetic neuropathy

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Kimura E*, Kawano Y*, Todo H*, Ikarashi Y, Sugibayashi K*: Measurement of skin permeation/penetration of nanoparticles for their safety evaluation.

Biol Pharm Bull. 2012;35:1476-86.

The aim of the present study was to quantitatively evaluate the skin permeation/penetration of nanomaterials and to consider their penetration pathway through skin. Firstly, penetration/permeation of a model fluorescent nanoparticle, Fluoresbrite®, was determined through intact rat skin and several damaged skins. Fluoresbrite® permeated through only needle-punctured skin. The permeation profiles of soluble

high molecular compounds, fluorescein isothiocyanate-dextrans (FITC-dextrans, FDs), with different molecular weights were also measured for comparison. The effects of molecular sizes and different skin pretreatments on the skin barrier were determined on the skin penetration/permeation of Fluoresbrite® and FDs. Fluoresbrite® was not permeated the intact skin, but FDs were permeated the skin. The skin distribution of titanium dioxide and zinc oxide nanoparticles was also observed after topical application of commercial cosmetics. Nanoparticles in sunscreen cosmetics were easily distributed into the groove and hair follicles after their topical application, but seldom migrated from the groove or follicles to viable epidermis and dermis. The obtained results suggested that nanoparticles did not permeate intact skin, but permeated pore-created skin. No or little permeation was observed for these nanomaterials through the stratum corneum.

Keywords: nanoparticles, titanium dioxide, skin permeation

* Josai University

小林憲弘，久保田領志，田原麻衣子，清水久美子，杉本直樹，西村哲治*：水道水質管理目標設定項目の候補とされている農薬のGC/MS一斉分析法の開発.

環境科学会誌 2012;25:378-90.

水道水質管理目標設定項目の候補とされている農薬135物質のうち60物質のGC/MS一斉分析法を検討した．農薬混合標準液を用いた分析結果では，いずれの農薬についても，検量線の直線性および繰り返し分析の再現性は概ね良好であった．また，一日許容摂取量（ADI）に基づいて目標値を推定可能な59物質全ての定量下限値は各目標値よりも低い濃度となり，そのうち56物質については目標値の1/100よりも低い濃度まで定量可能であった．さらに，精製水および水道水を用いて添加回収試験を行ったところ，$LogP_{ow}$あるいは水溶解度によって，固相抽出カラムによって濃縮できるかどうかを判断することが概ね可能であり，43物質については，回収率の平均値が70～120%の範囲の良好な結果を得ることができた．なお，本研究において開発したGC/MS一斉分析法は，最終的には水道水質検査法の新しい標準検査法に発展させることを目的としているが，そのためには，複数機関による分析法バリデーションを行い，本法の分析精度を検証する必要がある．今後は，本法の妥当性評価を早急に実施したい.

Keywords: 水道水，農薬，GC/MS

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田原麻衣子，末松孝子*1，早川昌子*2，合田幸広，小西良子，杉本直樹：定量NMRによるトリコテセン系マイコトキシン類市販試薬の純度決定.

Mycotoxins 2012;62:111-9.

定量NMR（qNMR）は測定対象化合物とは異なる基準物質との水素の原子数比から，あらゆる化合物の絶対量が算出可能である．計量学的に信頼性の高い純度が値付けられた1,4-BTMSB-d_4を基準物質とし，トリコテセン系マイコトキシン類の市販試薬5 種7 製品の純度を求めた結果，82.9–98.7%となり，製品表示値より10%前後下回るものが認められた．本研究より，マイコトキシン等の天然毒の定量用標品とされる希少な市販試薬の純度測定にqNMR が有効な手段となり得ることが示唆された．また，qNMRによる純度を試薬に標記することにより，定量値の国際整合性の確保が間接的に可能となると考えられる.

Keywords: トリコテセン，qNMR，純度

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Wang L*1, Ohishi T*1, Shiraki A*1, Morita R*1,2, Akane H*1,2, Ikarashi Y, Mitsumori K*1, Shibutani M*1: Developmental exposure to manganese chloride induces sustained aberration of neurogenesis in the hippocampal dentate gyrus of mice.

Toxicol Sci. 2012;127:508-21.

The effect of exogenously administered manganese (Mn) on developmental neurogenesis in the hippocampal dentate gyrus was examined in male mice after maternal exposure to MnCl2 (0, 32, 160, or 800 ppm as Mn in diet) from gestational day 10 to day 21 after delivery on weaning. Immunohistochemistry was performed to monitor neurogenesis and interneuron subpopulations on postnatal days (PNDs) 21 and 77 (adult stage). Reelin-synthesizing γ-aminobutyric acid (GABA) ergic interneurons increased in the hilus with ≥ 160 ppm on weaning to sustain to PND 77 at 800 ppm. Apoptosis in the neuroblast-producing subgranular zone increased with 800 ppm and TUC4-expressing immature granule cells decreased with 800 ppm on weaning, whereas at the adult stage, immature granule cells increased. On PND 21, transcript levels increased with Reln and its receptor gene Lrp8 and

decreased with Dpysl3 coding TUC4 in the dentate gyrus, confirming immunohistochemical results. Double immunohistochemistry revealed a sustained increase of reelin-expressing and NeuN-lacking or weakly positive immature interneurons and NeuN-expressing mature neurons in the hilus through to the adult stage as examined at 800 ppm. Brain Mn concentrations increased at both PNDs 21 and 77 in all MnCl2-exposed groups. These results suggest that Mn targets immature granule cells causing apoptosis and neuronal mismigration. Sustained increases in immature reelin-synthesizing GABAergic interneurons may represent continued aberration in neurogenesis and following migration to cause an excessive response for overproduction of immature granule cells through to the adult stage. Sustained high concentration of Mn in the brain may be responsible for these changes.

Keywords: manganese chloride, hippocampal dentate gyrus, γ-aminobutyric acid（GABA）ergic interneurons

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Hirose R*, Miura T*, Sha R*, Shinkai Y*, Tanaka-Kagawa T, Kumagai Y*: A method for detecting covalent modification of sensor proteins associated with 1,4-naphthoquinone-induced activation of electrophilic signal transduction pathways.
J Toxicol Sci. 2012;37:891-8.

While metabolic activation of naphthalene, yielding 1,2-naphthoquinone（1,2-NQ）and 1,4-NQ that can covalently bind to cellular proteins, has been recognized to be associated with its toxicity, the current consensus is that such electrophile-mediated covalent modification of sensor proteins with thiolate ions is also involved in activation of cellular signal transduction pathways for cellular protection against reactive materials. In the present study, we developed an immunochemical assay to detect cellular proteins adducted by 1,4-NQ. Dot blot analysis indicated that the antibody prepared against 1,4-NQ recognized the naphthalene moiety with the para-dicarbonyl group, rather than with the ortho-dicarbonyl group. Furthermore, little cross-reactivity of para-quinones with either a different number of aromatic rings（n = 1）or substituent groups was observed. With this specific antibody against 1,4-NQ, we identified nine target proteins of 1,4-NQ following exposure of human epithelial carcinoma cell line A431 to 1,4-NQ. Among them, heat shock protein 90（HSP90）and HSP70 are of interest because covalent modification of these chaperones causes activation of heat shock factor-1, which plays a role in the cellular response against electrophiles such as 1,4-NQ. Thus, our method, which does not use radiolabeled compounds, would be applicable for exploring activation of electrophilic signal transduction pathways coupled to covalent modification of sensor proteins during exposure to naphthalene as well as 1,4-NQ.

Keywords: naphthalene, covalent modification, electrophilic signal transduction

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河上強志，伊佐間和郎，中島晴信*1，吉田仁*1，大嶋智子*2，大野浩之*3，上村仁*4，塩田寛子*5，菊地洋子*5，松岡厚子，西村哲治*6：有害物質を含有する家庭用品の規制に関する法律（有害物質含有家庭用品規制法）におけるトリフェニル錫（TPT）及びトリブチル錫（TBT）の試験法改定に係わる検討.
薬学雑誌 2012;132:1197-208.

The use of triphenyltin（TPT）and tributyltin（TBT）in some household products is banned by “Act on the Control of Household Products Containing Harmful Substances” in Japan. To revise the official analytical method, the method for detecting these organotin compounds was examined in six laboratories using a textile product, water-based adhesive, oil-based paint, which contained known amounts of TPT and TBT（0.1, 1.0, 10 μg/g）. TPT and TBT were measured by GC-MS after ethyl-derivation with sodium tetraethylborate. The TBT recoveries in the samples were 70-120%. The TPT recoveries in the water-based adhesive samples were 80-110%, while its concentrations in the textile product and oil-based paint samples decreased because of dephenylation during storage. However, the precision of the method examined was satisfactory because most coefficients of variation for TPT and TBT in the samples were less than 10%. Furthermore, the revised method was able to detect concentrations lower than the officially regulated value. However, the sample matrix and the condition of analytical instrument might affect the estimated TPT and TBT concentrations. Therefore, the revised method may not

be suitable for quantitative tests; rather, it can be employed to judge the acceptable levels of these organotin compounds by comparing the values of control sample containing regulated amounts of TPT and TBT with those for an unknown sample, with deuterated TPT and TBT as surrogate substances. It is desirable that TPT in textile and oil-based paint samples are analyzed immediately after the samples obtained because of the decomposition of TPT.

Keywords: organotin compounds, household products, GC-MS

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Ohishi T*1,2, Wang L*1, Akane H*1, Shiraki A*1, Goto K*2, Ikarashi Y, Suzuki K*1, Mitsumori K*1, Shibutani M*1: Reversible aberration of neurogenesis affecting late-stage differentiation in the hippocampal dentate gyrus of rat offspring after maternal exposure to manganese chloride.
Reprod Toxicol. 2012;34:408-19.

To examine the effects of developmental manganese (Mn)-exposure on hippocampal neurogenesis, pregnant rats were treated with $MnCl_2$ $4H_2O$ in the diet at 32, 160 or 800 ppm from gestation day 10 to day 21 after delivery. Serum concentrations of thyroid-related hormones were examined in offspring exposed to $MnCl_2$ $4H_2O$ at 800 or 1600 ppm. Immunohistochemical analysis revealed increased doublecortin-positive cells in the subgranular zone of the dentate gyrus on postnatal day (PND) 21 following exposure to $MnCl_2$ $4H_2O$ at 800 ppm, indicating an increase of type-3 progenitor or immature granule cells. Reelin-positive cells, suggestive of γ-aminobutyric acid-ergic interneurons in the dentate hilus, also increased at 800 ppm on PND 21. Brain Mn concentrations increased in offspring on PND 21 at 160 and 800 ppm, whereas brain concentrations in the dams were unchanged. Serum concentrations of triiodothyronine and thyroxine decreased at 800 and 1600 ppm, whereas thyroid-stimulating hormone increased only after exposure at 800 ppm. All changes disappeared on PND 77. Thus, maternal exposure to $MnCl_2$ $4H_2O$ at 800 ppm mildly and reversibly affects neurogenesis targeting late-stage differentiation in the hippocampal dentate gyrus of rat offspring. Direct effects of accumulated Mn in the developing brain might be implicated in the mechanism of the development of aberrations in neurogenesis; however, indirect effects through thyroid hormone fluctuations might be rather minor.

Keywords: manganese, developmental neurotoxicity, hippocampal dentate gyrus

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Hanioka N*, Takahara Y*, Takahara Y*, Tanaka-Kagawa T, Jinno H, Narimatsu S*: Hydrolysis of di-n-butyl phthalate, butylbenzyl phthalate and di (2-ethylhexyl) phthalate in human liver microsomes.
Chemosphere 2012;89:1112-7.

Diester phthalates are industrial chemicals used primarily as plasticizers to import flexibility to polyvinylchloride plastics. In this study, we examined the hydrolysis of di-n-butyl phthalate (DBP), butylbenzyl phthalate (BBzP) and di(2-ethylhexyl) phthalate (DEHP) in human liver microsomes. These diester phthalates were hydrolyzed to monoester phthalates (mono-n-butyl phthalate (MBP) from DBP, mono-n-butyl phthalate (MBP) and monobenzyl phthalate (MBzP) from BBzP, and mono(2-ethylhexyl) phthalate (MEHP)) by human liver microsomes. DBP, BBzP and DEHP hydrolysis showed sigmoidal kinetics in V- [S] plots, and the Hill coefficient (n) ranged 1.37-1.96. The S(50), V(max) and CL(max) values for DBP hydrolysis to MBP were 99.7 μM, 17.2nmolmin(-1)mg(-1) protein and 85.6 μL min(-1)mg(-1) protein, respectively. In BBzP hydrolysis, the values of S(50) (71.7 μM), V(max) (13.0nmolmin(-1)mg(-1) protein) and CL(max) (91.3 μL min(-1)mg(-1) protein) for MBzP formation were comparable to those of DBP hydrolysis. Although the S(50) value for MBP formation was comparable to that of MBzP formation, the V(max) and CL(max) values were markedly lower (<3%) than those for MBzP formation. The S(50), V(max) and CL(max) values for DEHP hydrolysis were 8.40 μM, 0.43 nmol min(-1)mg(-1) protein and 27.5 μL min(-1)mg(-1) protein, respectively. The S(50) value was about 10% of DBP and BBzP hydrolysis, and the V(max) value was

also markedly lower (<3%) than those for DBP hydrolysis and MBzP formation for BBzP hydrolysis. The ranking order of CL(max) values for monoester phthalate formation in DBP, BBzP and DEHP hydrolysis was BBzP to MBzP≥DBP to MBP>DEHP to MEHP>BBzP to MBP. These findings suggest that the hydrolysis activities of diester phthalates by human liver microsomes depend on the chemical structure, and that the metabolism profile may relate to diester phthalate toxicities, such as hormone disruption and reproductive effects.

Keywords: Di(2-ethylhexyl) phthalate (DEHP), Hydrolysis, Human liver microsomes

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大嶋智子*，河上強志，山野哲夫*，尾崎麻子*，清水充*，伊佐間和郎：有害物質含有家庭用品規制法のトリフェニル錫（TPT）およびトリブチル錫（TBT）分析法改定過程において観察されたTPTの分解について．

大阪市立環境科学研究所報告 2012;74:17-22.

The round-robin test to evaluate the method for the simultaneous determination of triphenyltin (TPT) and tributyltin (TBT) in household products developed by National Institute of Health Sciences in Japan was performed by six laboratories. Samples of three types (textile, water-based adhesive, and oil-based paint) were prepared by addition of known amounts of TPT and TBT (0.1, 1.0, 10 μg/g) and sent to the participants. They were analyzed in our laboratory five month later by GC-MS after ethyl-derivation with sodium tetraethylborate. TBT in the samples showed acceptable recoveries of 66-120% , while TPT concentrations were considerably low in all samples. In the textile and water-based adhesive, diphenyltin (DPT), the degradation product of TPT, was detected, and the total amounts of DPT and TPT were comparable to the added TPT. Thus, it was confirmed that TPT converted to DPT by dephenylation in these samples. In the case of oil-based paint, however, DPT was not detected. It seemed that DPT was adsorbed strongly onto the silica cartridge column used for the clean-up of oil before the ethyl-derivation step.

Keywords: tributyl and triphenyltin, household products, dephenylation

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Kawakami T, Isama K, Nishimura T*: Survey of primary aromatic amines originating from azo dyes in commercial textile products in direct contact with skin and in commercial leather products in Japan.

J Environ Chem. 2012;22:197-204.

Twenty-six carcinogenic primary aromatic amines (PAAs) originating from azo dyes in commercial textile products that can potentially come into direct contact with human skin (31 products; 41 samples) and in leather products (23 products; 23 samples) in Japan were investigated. Twelve and 11 PAAs were detected in the textile and leather products, respectively, nearly all at low concentrations (below 1.0 μg/g). However, the concentrations of benzidine (45-593 μg/g) in one shawl and six sheets and covers (seven samples) exceeded European Union (EU) regulatory limits (below 30 μg/g). Concentrations of o-toluidine (430 μg/g), benzidine (31 μg/g), and 3,3'-dimethylbenzidine (40 μg/g) in leather products (hand-crafted leather) also exceeded EU regulatory limits. Shawls, sheets, and covers can come into direct contact with human skin. Thus, an exposure evaluation should be performed for benzidine in these products.

Keywords: Primary aromatic amine, azo dye, textile and leather product

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Kawakami T, Isama K, Nishimura T*: Analysis of isothiazolinones and other preservatives in gel-products used for cooling in Japan.

J Environ Chem. 2012;22:205-11.

Recently, two cases of contact dermatitis caused by 2-n-octyl-4-isothiazolin-3-one (OIT) used as a preservative in cooling gel-products has been reported in Japan, and one of the cases was declared a serious product accident based on the "Consumer Safety Product Act." In this study, the concentrations of three isothiazolinone preservatives (OIT, 2-methyl-4-isothiazolin-3-one [MIT], and 5-chloro-2-methyl-4-isothiazolin-3-one [Cl-MIT]), seven different parabens, carbendazim (MBC), and tebuconazole (Teb) in 24 cooling gel-products were investigated. OIT was detected in two samples (0.14 μg/g and 2.2 μg/g). MIT was detected in 11

samples at concentrations in the range of 0.12-115 μg/g and Cl-MIT was detected in six samples in concentrations ranging from a trace amount to 16 μg/g. The EU cosmetic limits were used to consider the risk of skin sensitization and the concentrations of MIT and Cl-MIT detected in several samples were over these limit. It is possible for the gel to cause contact dermatitis when the consumer's weight presses on the gel-product because OIT might penetrate from the gel to the textile surface in the case of a serious product accident. Furthermore, it is possible that using the gel product for the forehead or neck has a similar risk of skin sensitization if the gel-product's surface tears and the gel containing isothiazolinone preservative leaks out. It is advisable to replace the preservatives in cooling gel-products with non-sensitizing preservatives. All parabens were detected in the gel-products, except benzylparaben, and their concentrations were 12-696 μg/g. MBC and Teb were detected in three samples with concentrations in the ranges of 0.82-54 μg/g and 1.5-25 μg/g, respectively.

Keywords: Gel-product for cooling, isothiazolinone preservatives, contact dermatitis

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Akiyama T, Sekiguchi W, Yamazaki T, Akiyama H: Assessment of three methods for the identification of enzymatically hydrolyzed guar gum.

Food Hyg Saf Sci. 2013;54:71-4.

Enzymatically hydrolyzed guar gum (EHGG), which is used as a thickener or a soluble dietary fiber, is produced by partial hydrolysis of the guar gum (GG) backbone using mannan endo-β-1,4-mannosidase. In this study, we compared and evaluated 3 methods to distinguish EHGG from other polysaccharides used as food additives or monosaccharides. The first method is based on cross-linking reaction of saccharide hydroxyl groups mediated by borate ions. EHGG showed gelation and was distinguished from some of soluble polysaccharides, which did not form gels, and also from polysaccharides with low solubility in water. The second method is based on co-gelation with xanthan gum. It was applicable to GG, but not to EHGG. The third method is based on the alcohol precipitation of hydrophilic polymers. EHGG, some soluble polysaccharides and monosaccharides were dissolved in water at the concentration of 10%, while GG and some polysaccharides were not. The 10% solutions thus obtained were mixed with 2-propanol at the ratio of 1:1 (v/v). A white precipitate was formed in the EHGG solutions and the tested soluble polysaccharide solutions, while it was not produced in the monosaccharide solutions. This result demonstrated that soluble polysaccharides including EHGG can be distinguished from polysaccharides with low solubility or monosaccharides by the third method.

Keywords: Enzymatically hydrolyzed guar gum, Polysaccharide, Alcohol precipitation

久保田領志，田原麻衣子，小林憲弘，清水久美子，阿部晃文[*1]，中町眞美[*2]，灘重樹[*3]，服部晋也[*4]，丸岡強[*5]，杉本直樹，西村哲治[*6]：固相抽出－誘導体化GC/MS法を用いたEDTAの分析法の開発および水道原水・浄水・給水栓水中の存在実態.

水道協会雑誌 2013;82:2-9.

上水試験方法のEDTA試験法は，前処理が煩雑で長時間を要する．そこで，簡便な固相抽出による試験法を開発し，水道原水，浄水等における存在実態を評価した．3種試料水において固相カートリッジに強陰イオン交換体を用いた添加回収試験の結果，良好な回収率が得られ，本方法により前処理の時間の短縮化と操作の簡便化が可能となった．さらに，本方法を用い，浄水場等の試料水中存在実態を評価した結果，EDTAは殆どの試料から検出された．採水時期や採水地点によって濃度差が認められたが，全ての検出濃度は目標値の1/20未満であった．また，都市河川を水道原水とする浄水場試料水でEDTAは高濃度で検出されていることから，下水処理場放流水等による水道原水への影響が考えられる．

Keywords: EDTA，固相抽出，分析方法

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Miyake Y*, Mayumi K*, Jinno H, Tanaka-Kagawa T, Narimatsu S*, Hanioka N*: cDNA Cloning and Functional Analysis of Minipig Uridine Diphosphate-Glucuronosyltransferase 1A1.

Biol Pharm Bull. 2013;36:452-61.

Uridine diphosphate (UDP)-glucuronosyltransferase

1A1（UGT1A1）plays important roles in the glucuronidation of various drugs and endogenous substances. Minipigs have been used as experimental animals in pharmacological and toxicological studies, because many of their physiological characteristics are similar to those of humans. In this study, the similarities and differences in enzymatic properties of UGT1A1 between humans and minipigs were precisely identified. Minipig UGT1A1（mpUGT1A1）cDNA was firstly cloned by the rapid amplification of cDNA ends（RACE）method, and the corresponding protein as well as human UGT1A1（hUGT1A1）enzyme was expressed in insect cells. Then the kinetics of estradiol at 3-hydroxy position（E-3OH）and 7-ethyl-10-hydroxycamptothecin（SN-38）glucuronidation by recombinant UGT1A1s as well as human and minipig liver microsomes were analyzed. The homology between mpUGT1A1 and hUGT1A1 at the amino acid level was 80.9%. E-3OH and SN-38 glucuronidation by recombinant hUGT1A1 and mpUGT1A1 showed allosteric sigmoidal kinetics. The CL value（29.1 μL/min/mg protein）for E-3OH glucuronidation of mpUGT1A1 was significantly higher（1.4-fold）than that of hUGT1A1, whereas the CL value（0.83 μL/min/mg protein）for SN-38 glucuronidation was significantly lower（27%）than that of hUGT1A1; however, the kinetic models and parameter levels for E-3OH and SN-38 glucuronidation by human and minipig liver microsomes did not parallel those in the respective species. These findings suggest that the enzymatic properties of UGT1A1 are considerably different between humans and minipigs. The information on species differences in UGT1A1 function gained in this study should help with in vivo extrapolation of xenobiotic metabolism and toxicity.

Keywords: uridine diphosphate-glucuronosyltransferase 1A1（UGT1A1）, minipig, estradiol at 3-hydroxy position

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Morimoto Y*1, Horie M*1, Kobayashi N, Shinohara N*2, Shimada M*3: Inhalation Toxicity Assessment of Carbon-Based Nanoparticles.

Acc Chem Res. 2013;46:770-81.

Although the demand for nanomaterials has grown, researchers have not conclusively determined the effects of nanomaterials on the human body. To understand the effects of nanomaterials on occupational health, we need to estimate the respiratory toxicity of nanomaterials through inhalation studies, intratracheal instillation studies, and pharyngeal aspiration studies. The discrepancies observed among these studies tend to result from differences in the physiochemical properties of nanomaterials, such as aggregation and dispersion. Therefore, in all toxicity studies, identification of the physicochemical properties of nanomaterials is essential. This Account reviews the inhalation toxicity of manufactured nanomaterials and compares them with inhalation and intratracheal instillation studies of well-characterized fullerene and carbon nanotubes. In many reports, pulmonary inflammation and injury served as pulmonary endpoints for the inhalation toxicity. To assess pulmonary inflammation, we examined neutrophil and macrophage infiltration in the alveolar and/or interstitial space, and the expression of the neutrophil and/or monocyte chemokines. We also reported the release of lactate dehydrogenase（LDH）and alkaline phosphatase（ALP）in the bronchoalveolar lavage fluid（BALF）, the expression of oxidative stress-related genes characteristic of lung injury, and the presence of granulomatous lesion and pulmonary fibrosis. In the inhalation and intratracheal instillation studies of well-characterized fullerenes, exposure to fullerene did not induce pulmonary inflammation or transient inflammation. By contrast, in an inhalation study, a high concentration of multiwall carbon nanotubes（MWCNTs）and single-wall carbon nanotubes（SWCNTs）induced neutrophil inflammation or granulomatous formations in the lung, and intratracheal instillation of MWCNTs and SWCNTs induced persistent inflammation in the lung. Among the physicochemical properties of carbon nanotubes, the increased surface area is associated with inflammatory activity as measured by the increase in the rate of neutrophils measured in bronchoalveolar lavage fluid. Metal impurities such as iron and nickel enhanced the pulmonary toxicity of carbon nanotubes, and SWCNTs that included an amorphous carbon induced multifocal granulomas in the lung while purer SWCNTs did not. The aggregation state also affects pulmonary response: Exposure to well-dispersed carbon nanotubes led to the thickening of the alveolar wall and fewer granulomatous lesions in the lung, while agglomerated carbon nano-

tubes produced granulomatous inflammation. The values of the acceptable exposure concentration in some countries were based on the data of subacute and subchronic inhalation and intratracheal instillation studies of well-characterized fullerene and carbon nanotubes. In Japan, the acceptable exposure concentration of fullerene is 0.39 mg/m^3. In Europe, the proposal concentration is 44.4 μg/m^3 for acute toxicity and 0.27 μg/m^3 for chronic toxicity. The proposal acceptable exposure concentrations of carbon nanotubes are 0.03, 0.05, and 0.007 mg/m^3 in Japan, Europe, and the United States, respectively.

Keywords: fullerene, carbon nanotube, inhalation toxicity

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菊地博之，堤智昭，松田りえ子：フルオレスカミン誘導体化HPLC法による魚および水産加工品中のヒスタミン分析の性能評価.
食品衛生学雑誌 2012;53:121-7.

我が国では，ヒスタミンを原因とする食中毒事例が毎年報告されているが，食品に含まれるヒスタミンの規格基準および公定分析法は示されていない．そこで，食品中のヒスタミンの規格試験法を開発することを目的として，既報のタンデム固相抽出を用いたヒスタミン分析法を一部改良すると共に，改良した分析法の性能を評価した．本法の妥当性を確認するために，25，50 μg/gの濃度になるようにヒスタミンを添加したマグロ試料，および50，100 μg/gの濃度となるようにヒスタミンを添加した魚醤およびいわし丸干しなどの水産加工品試料を作製し，食品中の金属に関する分析法の妥当性評価ガイドラインに従った実験計画により分析を行った．その結果，全ての検討試料および濃度において，真度は88.8～99.6％，併行精度は1.3～2.1％，室内精度は2.1～4.7％と良好な結果が得られた．さらに，本法の適用可能な食品の範囲を検証するために，一般にヒスタミン汚染が懸念される７種の切り身および水産加工品について，上記と同様の添加濃度において添加回収試験を実施したところ，全ての検討試料において，83.4～102.0％の回収率が得られた．以上の結果から，本法はヒスタミンの規格試験法として十分な性能を有しており，切り身や干物，缶詰等の多様な形態の試料についても適用することが可能な方法であると考えられる．また，本法を用いて市場流通している切り身および加工品（32検体）のヒスタミン含有濃度の実態調査を実施したところ，一部の加工品から高濃度のヒスタミンが検出された.

Keywords: histamine, analytical method, fluorescamine

坂本智徳*，赤木浩一*，渡邉敬浩，松田りえ子，樋脇弘*：食品中メチル水銀の定量分析のためのフェニル誘導体化GC-MS法の開発.
分析化学 2012;61:327-33.

フェニル誘導体化－ガスクロマトグラフィー－質量分析（GC-MS）法による食品中メチル水銀の分析法を検討した．臭化カリウム・硫酸銅（II）飽和硫酸混液によってメチル水銀を試料から分離し，トルエンに抽出したのち*L*-システイン溶液に逆抽出した．抽出したメチル水銀をテトラフェニルホウ酸ナトリウムによってフェニル誘導体化し，*n*-ヘプタンに抽出した．誘導体化したメチルフェニル水銀を，１級－２級アミン（PSA）ミニカラムを用いて精製し，GC-MS（SIM）により測定した．５種の認証標準試料（CRM-7402a，CRM-7403a，BCR-463，ERMCE-464 及びDOLT-4）を用いた分析法の性能評価の結果，真度（％）98～108，併行精度（RSD%）10未満，室内精度（RSD%）15 未満であり，厚生労働省によって示された性能基準を満たす分析法であることが確認された.

Keywords: methylmercury, GC-MS, phenylation

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片岡洋平，渡邉敬浩，白政優子，松田りえ子：タコ，イカ，ハマグリ，アサリおよびチョコレート中のカドミウム濃度の実態調査.
食品衛生学雑誌 2012;53:146-51.

市場に流通するタコ，イカ，ハマグリ，アサリおよびチョコレート中のカドミウム濃度の実態を調査した．食品中の金属に関する試験法の妥当性評価ガイドライン（食安発第0926001号）に従って妥当性を確認した方法により，分析を行った．調査した40試料の海産食品中，31試料から本調査で設定した定量下限の1/2濃度となる0.025 mg/kgを超えるカドミウムが検出されたが，Codexが定める基準値（2 mg/kg）を超過した試料はなかった．全調査試料中の最大濃度は，タコでは0.19 mg/kg，イカでは0.18 mg/kg，ハマグリでは0.38 mg/kg，アサリでは0.16 mg/kgであった．またチョコレートでは，30 試料中21 試料から0.025 mg/kg以上のカドミウムが検出され，最大値は0.54 mg/kgであった.

Keywords: cadmium, atomic absorption spectrometry,

ICP-OES

齊藤静夏，根本了，松田りえ子：LC-MS/MSによる緑茶中の残留農薬一斉試験法.
日本食品化学学会誌 2012;19:104-10.

A multiresidue method for the determination of pesticides in green tea was developed by modification of Japanese official method. In this method, a sample was allowed to swell in water before extraction with acetonitrile. After the removal of water by salting-out, the crude extract was passed through an ODS mini-column, and then purified by a tandemized graphitized carbon/primary secondary amine（PSA）mini-column and graphitized carbon mini-column, prior to the determination by LC-MS/MS. The recoveries of 135 compounds from fortified green tea after a spike at maximum residue levels（MRLs）set by Japan, were in the ranged from 70 to 106%, except for 15 compounds, and the relative standard deviations were within the required analytical performance criteria for pesticide residues in Japan. The limits of quantitation（LOQs）of all the tested compounds were below MRLs set by Japan.
Keywords: pesticide, green tea, multiresidue method

Watanabe T, Matsuda R: Effect of the distribution of analyte concentration in lot, sample size, and number of analytical runs on food-testing results.
J Agric Food Chem. 2012;60:10702-8.

In testing, it is necessary to obtain the correct measured values that reflect analyte concentrations in the lot. Control of the analytical performance and appropriate sampling are essential in order to obtain the correct values. In the present study, we estimated the distribution of the analyte concentrations in specific food product lots and examined the influence of the sample size and the number of analytical runs on the variability of the testing results. The combinations of analyte and food studied were pesticide residues in fresh vegetables, nitrate in fresh vegetables and food additives in processed meat products. The results of our study suggested the followings: increase in the sample size beyond a certain number dose not efficiently reduce the variability of the test results; the specific sample size required to maintain the variability of the testing results at an appropriate level depends on the breadth of distribution of concentrations in the lot and the precision of the analysis; increasing the number of analytical runs was more efficient in reducing the variability of the testing results than increasing the sample size, when the breadth of distribution of concentrations in the lot was narrow enough to be comparable with the analytical precision.
Keywords: Sampling, Testing, Variation

石井里枝*，高橋邦彦*，戸谷和男*，根本了，松田りえ子：LC-MS/MSによる畜水産食品中のクロメプロップおよびクロメプロップ酸分析法の開発.
食品衛生学雑誌 2012;53:217-24.

LC-MS/MSを用いた畜水産食品中のクロメプロップおよびその代謝物であるクロメプロップ酸の分析法を開発した．試料から塩酸酸性下，アセトン－*n*-ヘキサン混液で抽出し，アセトニトリル－*n*-ヘキサン分配による脱脂操作後，SAXミニカラムとPSAミニカラムで精製した．10食品（牛の筋肉，牛の脂肪，牛の肝臓，牛乳，ブリ，さけ，うなぎ，しじみ，鶏卵およびはちみつ（そば蜜））を用いて，残留基準値濃度もしくは一律基準値濃度（0.01 ppm）における添加回収試験を行った結果，真度はクロメプロップが81～97%，クロメプロップ酸93～101%であった．併行精度はクロメプロップが2.1～14%，クロメプロップ酸が1.3～7.2%であった．また，本法による定量下限値はクロメプロップが0.002 mg/kg，クロメプロップ酸が0.00154 mg/kg（クロメプロップに換算すると0.002 mg/kg）であった.
Keywords: clomeprop, clomeprop acid, LC-MS/MS

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Chen S[*1], Tsutsumi T, Takatsuki S, Matsuda R, Kameya H[*1], Saito K[*1], Furuta M[*2], Todoriki S[*1]: Identification of 2-alkylcyclobutanones in cashew nut（Anacardium Occidentale）.
Food Irradiation Japan 2012;47:19-28.

The natural existence of the irradiation markers, namely, 2-dodecylcyclobutanone（2-dDCB）, 2-tetradecenylcyclobutanone（2-tDeCB）, and 2-tetradecylcyclobutanone（2-tDCB）in cashew nut（*Anacardium occidentale*）has recently been reported. In this study, 2-dDCB, 2-tDCB and 2-tDeCB were extracted from cashew nut of 2 different origins using supercritical fluid extraction（SFE）. The irradiated samples were analyzed by gas chromatography-mass spectrometry, whereas the non-irradiated samples were analyzed with high-resolution gas chromatography-mass spec-

trometry（GC-HRMS）. 2-dDCB, 2-tDCB and 2-tDeCB were detected and identified in the irradiated samples at 1 kGy or greater. However, none of 2-ACBs was detected in the non-irradiated samples with GC-HRMS. The radioproduction yield of 2-alkylcyclobutanones（nmol/mmol precursor fatty acid/kGy）were 1.3, 1.3 and 1.7 for 2-dDCB, 2-tDCB and 2-tDeCB, respectively.
Keywords: irradiation, 2-alkylcyclobutanone, cashew nut

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堤智昭，鍋師裕美，五十嵐敦子，蜂須賀暁子，松田りえ子：マーケットバスケット方式による放射性セシウムおよび放射性カリウムの年間預託実効線量推定.
食品衛生学雑誌 2013;54:7-13.

東日本大震災に伴い発生した東京電力福島第一原子力発電所事故により，食品が放射性物質に汚染される事態が生じている．そこで，食品中の放射性物質による健康影響を評価するために，東京都，宮城県，および福島県でマーケットバスケット方式によるトータルダイエット試料を作製し，放射性セシウムおよび，放射性カリウム濃度を測定し，一年当たりの預託実効線量を推定した．放射性セシウムの年間預託実効線量は東京都が0.0021 mSv/year，宮城県が0.017 mSv/year，および福島県が0.019 mSv/yearであった．宮城県および福島県の値は東京都の8倍以上であったが，いずれも厚生労働省より示された許容線量1 mSv/yearを大きく下回っていた．一方，天然放射性核種である放射性カリウムの年間預託実効線量は，0.17～0.20 mSv/yearであり，地域間で大きな差は見られなかった.
Keywords：放射性セシウム，マーケットバスケット法，年間実効預託線量

鍋師裕美，堤智昭，蜂須賀暁子，松田りえ子：乾しいたけの水戻しおよび牛肉の加熱調理による放射性セシウム量の変化.
食品衛生学雑誌 2013;54:65-70.

食品摂取による内部被ばく状況の推定・把握，さらに食品中放射性セシウムの低減法の提案に有用となる，食品中の放射性物質量の調理変化に関する科学的データを集積することを目的に，乾しいたけおよび牛肉を用いて放射性セシウム量の調理変化を検討した．その結果，乾しいたけ中の放射性セシウム量は，水戻しにより約50%減少した．牛肉中の放射性セシウム量は，焼くことで約10%，揚げることで約12%，ゆでることで60-65%，煮ることで約80%減少した．牛肉においては加熱方法により減少率が大きく異なり，“焼く，揚げる”よりも“ゆでる，煮る”の方が，放射性セシウムの減少率を8倍程度高められるという結果が得られた.
Keywords：放射性物質汚染食品，放射性セシウム，調理変化

Saito S, Nemoto S, Matsuda R: Multi-residue analysis of pesticides in agricultural products by liquid chromatography time-of-flight mass spectrometry.
Food Hyg Saf Sci. 2012;53:255-63.

The applicability of liquid chromatography time-of-flight mass spectrometry（LC-TOF-MS）for determining pesticide residues in agricultural products was investigated. TOF-MS conditions for monitoring target ions, together with their fragment ions, were carefully optimized. The developed LC-TOF-MS method was evaluated for 154 pesticides in soybean and spinach by using matrix-matched standards. No significant matrix effect was observed for most of the tested pesticides at a concentration level of 0.01 mg/kg, where the limits of quantification were less than 0.01 mg/kg for 145 of the 154 pesticides（S/N>10）. In addition, no significant interferences were observed in the chromatograms of the blank extracts. These results indicate that LC-TOF-MS determination may become a powerful tool for multi-residue analysis of pesticides in agricultural products.
Keywords: liquid chromatography time-of-flight mass spectrometry（LC-TOF-MS）, pesticides, multi-residue analysis

高橋邦彦*，石井里枝*，根本了，松田りえ子：LC-MS/MSによる農産物および畜水産物中のジノセブおよびジノテルブの分析法.
食品衛生学雑誌 2013;54:1-6.

LC-MS/MSを用いた農産物と畜水産物中のジノセブおよびジノテルブの分析法を開発した．農産物はアセトンで抽出し，得られた抽出液にヘキサンと飽和塩化ナトリウム溶液を加えて振とうした後，その上層をPSAミニカラムによる精製に供した．一方，畜水産物はアセトン-ヘキサン-水-塩化ナトリウムで抽出し，得られた抽出液をPSAミニカラム精製に供した．測定条件として分析カラムにC18を，移動相に0.005％酢酸含有メタノール－水混液（19：1）のアイソクラテイックモードで，イオン化はESIのネガティブモードを用いた．検量線は0.0005～0.04μg/mLの範囲で直線性（r^2>0.997）を示し

た．農産物および畜水産物の計20種に基準値濃度で添加して操作したときのジノセブおよびジノテルブの平均回収率（n=5）は77～111%，相対標準偏差は2～15%，定量限界値は両成分ともに0.001μg/gであった．
Keywords: dinoseb, dinoterb, LC-MS/MS

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渡邉敬浩，石川智子，松田りえ子：GC-FIDを用いたトランス脂肪酸分析法の性能評価手法および性能基準値の検討．
食品衛生学雑誌 2013;54:31-48.

GC-FIDを用いたトランス脂肪酸分析法の性能評価手法ならびに性能基準値を検討した．測定法は，The American Oil Chemists' Society（AOCS）の公認法（Ce1h-05）を原法とした．一般食品からの脂質抽出法は，衛新第13号に記載の方法およびAOAC 996.06を原法とした．分散推定時の自由度が4以上になる実験計画に従い添加試料を分析し，得られた一群の定量値から真度および精度を推定することを性能評価手法とした．添加試料の調製には，食品に含まれる蓋然性の低いトランス脂肪酸分子種を用いた．実際に推定した真度および精度の解析結果に基づき，90～110%を真度の基準値，相対標準偏差として10%を室内精度の基準値とすることが提案される．
Keywords: *trans*-fatty acid, performance evaluation, performance criteria

Watanabe-Ishitsuka A, Akiyama H, Kondo K, Obitsu S, Kawahara N*, Teshima R, Goda Y: Determination of cyanogenic glycoside linamarin in cassava flour using liquid chromatography-tandem mass spectrometry.
Jpn J Food Chem Safety 2012;19:38-43.

A specific and reliable method was developed for determining the presence of linamarin, a cyanogenic glucoside in cassava flour and cassava starch, using liquid chromatography-electrospray ionization tandem mass spectrometry. Linamarin was extracted with acetonitrile and then purified by solid-phase clean-up using a NH_2 cartridge column. Extracts were diluted to approximate the mobile phase composition and then filtered prior to analysis. Isocratic HPLC was used to introduce samples for electrospray negative ionization tandem mass spectrometry. Residues were identified by monitoring the multiple reaction monitoring (MRM) transitions of precursor ions with mass charge *m/z* 246.1 and common product ions with *m/z* 161.0. Qualitative and quantitative confirmation data were acquired simultaneously by monitoring alternative MRM transitions. Calibration with a standard solution was linear over a working range of 0.001-0.1 ppm (r^2=0.995-0.999), which is equivalent to 0.18-18 μg/g in food samples. The mean recovery of cassava flour was approximately 92-100%. The detection limits of the proposed method in cassava flour and tapioca samples were 0.75 μg/g and 0.84 μg/g, respectively.
Keywords: cyanogenic glycoside, linamarin, liquid chromatography-tandem mass spectrometry

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Ishizaki S*1, Sakai Y*2, Yano T*3, Ishihata K*3, Nakano S*3, Yamada T*3, Nagashima Y*1, Shiomi K*1, Nakao Y*2, Akiyama H: Specific Detection by Polymerase Chain Reaction (PCR) of Potentially Allergenic Salmonid Fish Residues in Processed Food.
Biosci Biotechnol Biochem. 2012;76:980-5.

A sensitive qualitative polymerase chain reaction (PCR) method to detect salmon DNA was developed for verifying the allergen labeling of foods and for identifying hidden salmon ingredients in processed foods. In this study, a new primer pair, SKE-F/SKE-R, was designed to detect the gene encoding mitochondrial DNA cytochrome b for specific detection of *Salmonidae* species. The amplified DNA fragment (212 bp) using this primer set was specifically detected from *Oncorhynchus keta*, *O. tshawytscha*, *O. gorbuscha*, and *O. mykiss* DNA. Furthermore, 0.02 fg/μl of salmon DNA (corresponding to 10 copies) was detected using this method. When the developed PCR method was used for investigating commercial food products, salmon DNA was detected in those including salmon in the list of ingredients. This method is expected to be reliable for detecting salmon residues in processed foods and is expected to be practical for monitoring the labeling system for allergenic food materials.
Keywords: food allergy, salmon, PCR

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Tsuruda S[*1], Akaki K[*1], Hiwaki H[*1], Suzuki A[*2], Akiyama H: Multiplex Real-Time PCR Assay for Simultaneous Detection of *Omphalotus guepiniformis* and *Lentinula edodes*.

Biosci Biotechnol Biochem. 2012;76:1343-9.

A rapid multiplex real-time PCR assay was developed to achieve highly specific, simultaneous detection of two kinds of mushrooms, *Omphalotus guepiniformis* and *Lentinula edodes*. Primers and TaqMan minor groove binder probes were designed based on the internal transcribed spacers 1-5.8S region of rDNA and evaluated based on specificity for fruiting bodies of 17 *O. guepiniformis*, 16 *L. edodes* and samples from 57 other species. DNA extracts of all target species had positive signals with no cross-reaction and the limit of detection was 0.00025 ng DNA. Ct values for raw and processed fruiting bodies as well as fruiting bodies (1% (w/w)) mixed with foodstuff or artificial gastric juice contents ranged from 17.16 to 26.60 for both examined species. This new assay proves specific to the target species, highly sensitive, and applicable to processed food samples and gastric juice contents, making it useful for rapid identification of *O. guepiniformis* and *L. edodes*.

Keywords: species identification, multiplex real-time PCR, *Omphalotus guepiniformis*

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Akiyama H, Minegishi Y[*1,4], Makiyama D, Mano J[*2], Sakata K, Nakamura K, Noguchi A, Takabatake R[*2], Futo S[*3], Kondo K, Kitta K[*2], Kato Y[*4], Teshima, R: Quantification and Identification of Genetically Modified Maize Events in Non-Identity Preserved Maize Samples in 2009 Using an Individual Kernel Detection System.

Food Hyg Saf Sci. 2012;53:157-65.

We investigated the GM maize grain content of non-identity preserved (non-IP) maize samples produced in 2009 from the USA using our individual kernel detection system, two multiplex qualitative PCR methods coupled to a microchip electrophoresis and partially real-time PCR array to clarify how many GM event maize grains they contained, and which GM event frequently appeared in 2009. The average percentage and standard deviation of GM maize grains on a kernel basis in five non-IP sample lots was 81.9% ± 2.8%, the average percentage of single GM event grains was 46.9%, and the average percentage of stacked GM event grains was 35.0%. MON88017 grains and NK603 grains were the most frequently observed as single GM event grains. The most frequent stacked GM event grains were MON88017 x MON810 grains. This study shows that our method can provide the beneficial information for GM maize events mixing in imported maize samples on a kernel basis.

Keywords: genetically modified maize, event, multiplex qualitative PCR

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Ito A[*1,2], Taguchi T[*1], Mogi T[*1], Wake H[*1], Tanaami, T[*1], Akiyama H, Teshima R, Sasaki N[*2], Yamada A[*2], Ozeki, Y[*2]: Comparison of Signal enhancement techniques using DNA microarrays for screening GM crops.

Jpn J Food Chem Safety. 2012;19:141-7.

For the qualification and quantification of genetically modified (GM) crops without PCR, one possible alternative method is the detection of DNA fragments synthesized by random primers by DNA microarrays. Here, we used four signal amplification methods adopted in protocols for model target preparation of DNA microarrays and evaluated the detectable copy numbers of the targets. A 100-fold higher detectable copy number of the target was achieved using a fluorescently labeled dendrimer agent with a lower background level than using Cy3-labeled target as the control. This level was estimated to be sufficient for the detection of a single copy gene in GM maize genomic DNA. This model experiment suggests that DNA microarrays will be able to detect introduced genes of GM crops without PCR.

Keywords: DNA microarray, genetically modified organism (GMO), signal amplification

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吉松嘉代*，河野徳昭*，川原信夫*，穐山浩，手島玲子，西島正弘：薬用及び環境浄化用遺伝子組換え植物の開発・生産に関する最近の動向.
YAKUGAKU ZASSHI. 2012;132:629-74.

Developments in the use of genetically modified plants for human and livestock health and phytoremediation were surveyed by information retrieval from Entrez PubMed, Chemical Abstracts Service, Google, congress abstracts and proceedings of related scientific societies, scientific journals, and so on. Obtained information was classified into 8 categories according to the research objective and the usage of the transgenic plants as 1: nutraceuticals (functional foods), 2: oral vaccines, 3: edible curatives, 4: vaccine antigens, 5: therapeutic antibodies, 6: curatives, 7: diagnostic agents and reagents, 8: phytoremediation. Totally 405 information was collected from 2006 to 2010. The numbers of cases were 120 for nutraceuticals, 65 for oral vaccines, 25 for edible curatives, 36 for vaccine antigens, 36 for therapeutic antibodies, 76 for curatives, 15 for diagnostic agents and reagents, and 40 for phytoremediation (sum of each cases was 413 because some reports were related to several categories). Nutraceuticals, oral vaccines and curatives were predominant. The edible crop most frequently used was rice (51 cases), and tomato (28 cases), lettuce (22 cases), potato (18 cases), corn (15 cases) followed.
Keywords: GM plant, molecular farming, nutraceutical

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Watanabe S[*1], Taguchi H[*1], Temmei Y[*1], Hirao T[*1], Akiyama H, Sakai S, Adachi R, Urisu A[*2], Teshima R: Specific detection of potentially allergenic peach and apple in foods using polymerase chain reaction.
J Agric Food Chem. 2012;60:2108-15.

Two PCR methods were developed for specific detection of the trnS-trnG intergenic spacer region of *Prunus persica* (peach) and the internal transcribed spacer region of *Malus domestica* (apple). The peach PCR amplified a target-size product from the DNA of 6 *P. persica* cultivars including 2 nectarine and 1 flat peach cultivar, but not from those of 36 nontarget species including 6 *Prunus* and 5 other *Rosaceae* species. The apple PCR amplified a target-size product from the DNA of 5 *M. domestica* cultivars, but not from those of 41 nontarget species including 7 *Maloideae* and 9 other *Rosaceae* species. Both methods detected the target DNA from strawberry jam and cookies spiked with peach and apple at a level equivalent to about 10 μg of total soluble proteins of peach or apple per gram of incurred food. The specificity and sensitivity were considered to be sufficient for the detection of trace amounts of peach or apple contamination in processed foods.
Keywords: food allergy, peach, apple

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Mano J[*1], Furui S[*1], Takashima K[*1], Koiwa T[*1,2], Futo S[*3], Minegishi Y[*4], Akiyama H, Teshima R, Kurashima T[*1], Takabatake R[*1], Kitta K[*1]: Development and validation of event-specific quantitative PCR method for genetically modified maize MIR604.
Food Hyg Saf Sci. 2012;53:166-71.

A GM maize event, MIR604, has been widely distributed and an analytical method to quantify its content is required to monitor the validity of food labeling. Here we report a novel real-time PCR-based quantitation method for MIR604 maize. We developed real-time PCR assays specific for MIR604 using event-specific primers designed by the trait developer, and for maize endogenous starch synthase IIb gene (SSIIb). Then, we determined the conversion factor, which is required to calculate the weight-based GM maize content from the copy number ratio of MIR604-specific DNA to the endogenous reference DNA. Finally, to validate the developed method, an interlaboratory collaborative trial according to the internationally harmonized guidelines was performed with blind samples containing MIR604 at the mixing levels of 0, 0.5, 1.0, 5.0 and 10.0%. The reproducibility (RSDr) of the developed method was evaluated to be less than 25%. The limit of quantitation of the method was estimated to be 0.5% based on the ISO 24276 guideline. These results suggested that the developed method would be suitable for practical quantitative analyses of MIR604 maize.
Keywords: MIR604, event-specific, genetically modified

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笠間菊子*，井上雪乃*，穐山浩，鈴木達也*，坂田こずえ，中村公亮，大島赴夫*，小島幸一*，近藤一成，手島玲子：プラスミドDNAを用いた中国産安全性未承認遺伝子組換えコメ検査に関する外部精度管理調査．

日本食品化学学会誌 2012;19:215-22.

中国産安全性未審査GMコメ検査を対象とした外部精度管理調査を実施した．通知法による検知を目的として，定性PCR用とリアルタイムPCR用の２種類の陽性コントロールプラスミドDNA溶液および非GMコメから抽出したDNA溶液を使用して調査試料を調製し，33の参加機関に送付した．通知法の定性PCRによる検出試験を対象とした試料は，定性PCR用のプラスミドDNA溶液を非GMコメDNA溶液中に容量比で１％および0.05％含むよう調製した．また，リアルタイムPCR法による検出試験を対象とした試料は，リアルタイムPCR用のプラスミドDNA溶液を非GMコメDNA溶液中に容量比で0.6％および0.12％含むよう調製した．外部精度管理調査の結果，参加33機関のうち31機関は調査試料をすべて予定通り判定した．しかし，２機関は陰性試料を陽性と判定し，プラスミドDNAを含めたその他の遺伝子の測定溶液への混入の可能性が疑われた．またリアルタイムPCRのマルチコンポーネント解析では，特定のリアルタイムPCR 装置のみでベースライン上昇が観察された．本精度管理調査の結果，プラスミドDNA溶液から調製した調査試料は中国産安全性未審査GMコメ検査の信頼性を確認する有効な手段となり得ることが示唆された．

Keywords：遺伝子組換えコメ，プラスミドDNA，外部精度管理調査

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Yoshimura M*, Akiyama H, Kondo K, Sakata K, Matsuoka H, Amakura Y*, Teshima R, Yoshida T*: Immunological Effects of Oenothein B, an Ellagitannin Dimer, on Dendritic Cells.

Int J Mol Sci. 2012;14:46-56.

Oenothein B is a unique macrocyclic ellagitannin dimer that has been found in various medicinal plants belonging to Onagraceae, Lythraceae, and Myrtaceae, with diverse biological activities. The immunological effects of tannins in terms of cytokine-release from macrophages and monocytes have been discussed, while the effects on other immunocompetent cells have been the subject of minimal investigation. We evaluated the immunomodulatory effects induced by tannin treatment in human dendritic cells (DCs), which play a critical role in the initial immune response, by measuring the changes in cytokine production, cell differentiation, and cell viability. Oenothein B showed significant down-regulation of the expression of cell surface molecules, CD1a and CD83, suggesting the inhibition of DC differentiation and/or maturation. The suppressive effect on DCs was associated with the induction of apoptosis without the activation of caspase-3/7, 8, and 9, and this was supported by the morphological features indicating significant nuclear condensation. Oenothein B also markedly suppressed the production of inflammatory cytokines, such as IL-1β and IL-6, in a dose-dependent manner. These data may, in part, be able to explain the traditional use of tannin-containing medicinal plants for the treatment of a variety of inflammatory diseases, including inflammatory bowel disease, celiac disease, and rheumatoid arthritis.

Keywords: dendritic cell, oenothein B, epigallocatechin gallate

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Takabatake R*1, Onishi M*2, Koiwa T*3, Futo S*2, Minegishi Y*4, Akiyama H, Teshima R, Kurashima T*1, Mano J*1, Furui S*1, Kitta K*1: Development and Interlaboratory Validation of Quantitative Polymerase Chain Reaction Method for Screening Analysis of Genetically Modified Soybeans.

Biol Pharm Bull. 2013;36:131-4.

A novel real-time polymerase chain reaction (PCR)-based quantitative screening method was developed for three genetically modified soybeans: RRS, A2704-12, and MON89788. The 35S promoter (P35S) of cauliflower mosaic virus is introduced into RRS and A2704-12 but not MON89788. We then designed a screening method comprised of the combination of the quantification of P35S and the event-specific quantification of MON89788. The conversion factor (Cf) required to convert the amount of a genetically modified organism (GMO) from a copy number ratio to a weight ratio was determined experimentally. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDr), respectively. The determined RSDr values for the method were less

than 25% for both targets. We consider that the developed method would be suitable for the simple detection and approximate quantification of GMO.
Keywords: Screening, Quantification, Genetically modified soybeans

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Sugimoto N*1, Yamaguchi M*1, Tanaka Y*1, Nakase Y*1, Nagase H*1, Akiyama H, Ohta K*1,2: The basophil activation test identified carminic acid as an allergen inducing anaphylaxis.
J Allergy Clin Immunol.: In Practice 2012;1:197-9

In the autumn of 2011, a 39-year-old Japanese woman was referred for evaluation of allergic reactions after ingestion of a commercial bottled supplement that contained vitamin

Bs and C and fruit flavors. The patient was strongly discouraged from consuming drinks and foods that contained cochineal dye (carminic acid). We tried the in vitro basophil activation test (BAT), which analyzes surface activation marker CD203c expression on basophils. in vitro BAT with the use of peripheral blood would be highly useful for analyzing cochineal dye-induced allergy. Flow cytometric assessment of surface markers, including CD203c, has enabled precise determination of the activation status of basophils. In regard to carminic acid-related allergy, our results suggest that basophils are highly sensitive to this allergen and that the cells might behave as a key initiator/effector in vivo.
Keywords: basophil activation test, carminic acid, allergy

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河﨑裕美，大西有希子，建部千絵，佐藤恭子，穐山浩，河村葉子：食品中のタール色素分析法の改良とマーケットバスケット試料への適用.
日本食品化学学会誌 2012;19:136-40.

１～６歳の小児を対象としたマーケットバスケット方式による一日摂取量調査のため，加工食品中の12種類の食用タール色素の従来の定量法を改良した．改良法では，イオンペア試薬としてTBA-Br溶液を採用することで，試料液の調製およびHPLC条件を簡略化した．また，クリーンアップにSep-Pak Plus tC18 Environmental Cartridgesを用いることで，精製操作を簡略化した．マーケットバスケット試料（1～8群）に適用したところ，７群の９種類の色素（食用赤色２号，同40号，同102号，同106号，黄色４号，同５号，緑色３号，青色１号，同２号）は妨害ピークのため定量できなかった．７群の上記の色素の回収率は，酵素処理後にポリアミドカラム処理を加えることにより向上した．２つの改良法により，加工食品中で不安定な食用青色２号の回収率は24～72%と低かったものの，その他のタール色素については良好な回収率が得られた（72～114%）．本法はタール色素の一日摂取量調査に適用可能である．
Keywords: tar dyes, HPLC, polyamide

Yokota A*, Kubota H, Komiya S, Sato K, Akiyama H, Koshiishi I*: Sensitive and Simple Determination of Bromate in Foods Disinfected with Hypochlorite Reagents Using High Performance Liquid Chromatography with Post-column Derivatization.
J Chromatogr A 2012;1262:219-22.

A novel analytical method for the quantification of bromate in fresh foods using high performance liquid chromatography (HPLC) with a post-column reaction has been developed. The fresh food sample solutions were pretreated with homogenization, centrifugal ultrafiltration and subsequent solid phase extraction using a strong anion-exchange resin. After separation on a strong anion-exchange chromatography column using a highly concentrated NaCl solution (0.3 M) as the eluent, the bromate was quantified by detection using a post-column reaction with a non-carcinogenic reagent (tetramethylbenzidine). The developed HPLC technique made it possible to quantify bromate in salt-rich fresh foods. The recoveries from fresh foods spiked with bromate at low levels (2 or 10 ng/g) satisfactorily ranged from 75.3 to 90.7%. The lowest quantification limit in fresh foods was estimated to be 0.6 ng/g as bromic acid. The method should be helpful for the quantification of bromate in fresh foods disinfected with hypochlorite solutions.
Keywords: carcinogenic, disinfection, tetramethylbenzidine

* Gunma University

Mikawa T, Kubota H, Ozeki Y*1, Yoshida M*2, Nakanishi T*2, Sato K, Akiyama H: Determination of sodium stearoyl lactylates in foods using HPLC after derivatization with 2-nitrophenyl hydrazine.
Jpn J Food Chem Safety 2012;19:178-84.

A high-performance liquid chromatographic method, following saponification and derivatization with 2-nitrophenyl hydrazine, was developed for determination of lactic acid derived from sodium stearoyl lactylates (SSL) in processed foods. Recoveries of SSL from ten kinds of processed foods spiked with SSL (2 g/kg) ranged from 79 to 102%, while the error associated with repeatability and intermediate reproducibility was less than 6.8% and 7.2%, respectively. This study showed that the proposed method can be applied for analysis of SSL in processed foods. The method is useful and reliable.

Keywords: sodium stearoyl lactylate, derivatization, 2-nitrophenylhydrazine

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Tokunaga H*, Osako T, Sato K: Determination of Ethylene Glycol and Diethylene Glycol as the Adulterant in Concentrated Glycerin, Glycerin and Propylene Glycol.
J Jpn Cosmetic Sci Soc. 2012;36:269-75.

The new modified method of EG and DEG for the impurity test in Concentrated Glycerin, Glycerin and Propylene Glycol of the Japanese Standards of Quasi-Drug Ingredients 2006 was established. This analytical method was the gas chromatographic method using the capillary column of 14 % cyanopropylmethylphenylsilicone and 86 % methylcilicone. The retention times of EG, propylene glycol, DEG and glycerin were 2.45, 2.78, 6.02 and 7.66 minutes, respectively. The working curve of EG was the good correlation between the concentrations of 2.5 to 80 μg/ml of EG and the peak areas and that of DEG was a good correlation between the concentrations of 5 to 80 μg/ml of DEG and the peak areas. When mixing EG and DEG with 50 mg/ml glycerin in methanol, the quantitation limits of EG and DEG in glycerin were 0.005% and 0.01%.

Keywords: diethylene glycol, glycerin, propylene glycol

* Pharmaceuticals and Medical Devices Agency

Kubota H, Sato K, Sasaki N*, Kawamura Y, Ozeki Y*, Akiyama H: Formation of volatile halogenated compounds in fresh-cut cabbage treated with sodium hypochlorite.
Jpn J Food Chem Safety 2012;19:94-103.

The factors affecting the formation of disinfection by-products in fresh-cut cabbage during sodium hypochlorite treatment investigated. Fresh cabbage was disinfected with a sodium hypochlorite solution (100 mg/L) for 10 min, with and without organic acids. Volatile organic compound residues in the fresh-cut cabbage were analyzed using HS-GC/MS. Chloroform was detected as the main by-product. Chloroform formation was dependent on contact time, pH, temperature and initial concentration of sodium hypochlorite solution. The use of sodium hypochlorite solution in combination with hydrochloric acid or some organic acids did not affect chloroform formation, except that citric acid reacted with hypochlorite to produce large amount of chloroform. When the citric acid was coupled with sodium hypochlorite solution, the chloroform level in the sample was dependent on the pre-mixing time of the solution, but was independent on the contact time of the mixed solution with the sample. Rinsing with water effectively reduced chloroform contaminants in the fresh-cut cabbage to the levels of chlorinated drinking water.

Keywords: trihalomethanes, chloroform, sodium hypochlorite

* Tokyo University of Agriculture and Technology

Tatebe C, Ohtsuki T, Otsuki N, Kubota H, Sato K, Akiyama H, Kawamura Y: Extraction Method and Determination of Sudan I Present in Sunset Yellow FCF by Isocratic High-Performance Liquid Chromatography.
Am J Anal Chem. 2012;3:570-5.

A method to extract and analyze Sudan I present in Sunset Yellow FCF (SYF) products was developed and validated. The method included the simple extraction of Sudan I from the SYF product using water, acetonitrile, and ethyl acetate and high-performance

liquid chromatography (HPLC) analysis with isocratic elution using acetonitrile:water (7:3) with a photodiode array detector at 485 nm. This method was found to remove most of the excess SYF colorant and other impurities before injection to the HPLC instrument, making it easy to maintain precision control in routine laboratory tests for Sudan I in the SYF colorant. The detection limit of Sudan I in SYF products was 0.2 μg/g. A survey conducted to determine Sudan I in 13 commercial SYF samples from Japanese manufacturers from 1970 to 2010 showed that the levels of Sudan I ranged from 0.3 to 1.9 μg/g in products manufactured from 1970 to 1996 and were below the limit of detection in products manufactured after 2005.

Keywords: Sudan I, Sunset Yellow FCF, HPLC

Ohtsuki T, Sato K, Sugimoto N, Akiyama H, Kawamura Y: Absolute quantitative analysis for sorbic acid in processed foods using proton nuclear magnetic resonance spectroscopy.
Anal Chim Acta. 2012;734:54-61.

An analytical method using solvent extraction and quantitative proton nuclear magnetic resonance (qH-NMR) spectroscopy was applied and validated for the absolute quantification of sorbic acid (SA) in processed foods. The proposed method showed good linearity. The recoveries for samples spiked at the maximum usage level specified for food in Japan and at 0.13 g kg^{-1} (beverage: 0.013 g kg^{-1}) were larger than 80%, whereas those for samples spiked at 0.063 g kg^{-1} (beverage: 0.0063 g kg^{-1}) were between 56.9 and 83.5%. The limit of quantification was 0.063 g kg^{-1} for foods (and 0.0063 g kg^{-1} for beverages containing *Lactobacillus* species). Analysis of the SA content of commercial processed foods revealed quantities equal to or greater than those measured using conventional steam-distillation extraction and high-performance liquid chromatography quantification. The proposed method was rapid, simple, accurate, and precise, and provided International System of Units traceability without the need for authentic analyte standards. It could therefore be used as an alternative to the quantification of SA in processed foods using conventional method.

Keywords: absolute quantification, quantitative proton nuclear magnetic resonance spectroscopy, sorbic acid

Ohtsuki T, Sato K, Sugimoto N, Akiyama H, Kawamura Y: Absolute quantification for benzoic acid in processed foods using quantitative proton nuclear magnetic resonance spectroscopy.
Talanta 2012;99:342-8.

The absolute quantification method of benzoic acid (BA) in processed foods using solvent extraction and quantitative proton nuclear magnetic resonance spectroscopy was developed and validated. BA levels were determined using proton signals (δ_H 7.53 and 7.98) referenced to 2-dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt (DSS-d_6) after simple solvent extraction from processed foods. All recoveries from several kinds of processed foods, spiked at their specified maximum Japanese usage levels (0.6–2.5 g kg^{-1}) and at 0.13 g kg^{-1} and 0.063 g kg^{-1}, were greater than 80%. The limit of quantification was confirmed as 0.063 g kg^{-1} in processed foods, which was sufficiently low for the purposes of monitoring BA. The accuracy of the proposed method is equivalent to the conventional method using steam-distillation extraction and high-performance liquid chromatography. The proposed method was both rapid and simple. Moreover, it provided International System of Units traceability without the need for authentic analyte standards. Therefore, the proposed method is a useful and practical tool for determining BA levels in processed foods.

Keywords: absolute quantification, quantitative proton nuclear magnetic resonance spectroscopy, benzoic acid

Tada A, Takahashi K, Ishizuki K, Sugimoto N, Suematsu T*1, Arifuku K*2, Tahara M, Akiyama T, Ito Y, Yamazaki T, Akiyama H, Kawamura Y: Absolute Quantitation of Stevioside and Rebaudioside A in Commercial Standards by Quantitative NMR.
Chem Pharm Bull. 2013;61:33-8.

The extract prepared from the leaves of *Stevia rebaudiana* Bertoni (Asteraceae) contains sweet steviol glycosides, mainly stevioside and rebaudioside A. Highly purified stevia extracts have become popular worldwide as a natural, low-calorie sweetener. They contain various types of steviol glycosides, and their main components are stevioside and rebaudioside A. The content of each steviol glycoside is quantified by comparing the ratios of the molecular weights and the chromatographic peak areas of the samples to those of stevioside or rebaudioside A standards of the Food and Agriculture Organization of the United Nations

(FAO)/ World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) and other specifications. However, various commercial standard reagents of stevioside and rebaudioside A are available. Their purities are different and their exact purities are not indicated. Therefore, the measured values of stevioside and rebaudioside A contained in a sample vary according to the standard used for the quantification. In this study, we utilized an accurate method, quantitative NMR (qNMR), for determining the contents of stevioside and rebaudioside A in standards, with traceability to the International System of Units (SI units). The purities of several commercial standards were determined to confirm their actual values.

Keywords: Stevioside, Quantitative NMR, Absolute quantitation

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*2 JEOL Ltd.

Ito Y, Ishizuki K, Sekiguchi W, Tada A, Akiyama T, Sato K, Yamazaki T, Akiyama H: Analysis of Residual Solvents in Annatto Extracts Using a Static Headspace Gas Chromatography Method.
Am J Anal Chem. 2012;3:638-45

An analytical method for the quantification of residual solvents in annatto extracts, natural food colorants, was established using a static headspace gas chromatography (HSGC) coupled with a flame ionization detector (FID). As a sample diluent in a headspace sampling, dimethylformamide (DMF) was selected owing to its high capacity for dissolving both bixin-based and norbixin-based annatto extracts. The quantification of residual solvents was performed using the external standard method. The linearity of the calibration curves was assured with relative coefficients (R2) that were greater than 0.999. The recoveries of all standard solvents spiked in the annatto extracts were in the range from 95.1% to 107.1% to verify the accuracy and the relative standard deviation (RSD%) values (n = 3) were in the range from 0.57% to 3.31%. The quantification limits (QL) were sufficiently lower than the limits specified by Joint FAO/WHO Expert Committee on Food Additives (JECFA). With the established HSGC method, six residual solvents (methanol, ethanol, 2-propanol, acetone, ethyl acetate, and hexane) in 23 commercial annatto-extract products that consist of seven bixin-based and 16 norbixin-based products were quantified. The levels of residual ethyl acetate and hexane in all products were lower than the specified limits of JECFA. However, three samples of bixin-based products showed higher levels of residual 2-propanol (approximately 313.9 - 427.7 ppm) than the specified limit. Other bixin products also showed higher concentrations of residual methanol (approximately 166.6 - 394.7 ppm) and residual acetone (approximately 75.2 - 179.8 ppm) than the limits of JECFA. In the case of norbixin-based products, nine samples showed higher levels of residual acetone (approximately 42.6 - 139.5 ppm) than the limits of JECFA. This is the first survey of residual solvents in annatto extracts using the validated HSGC method

Keywords: Annatto Extracts, Headspace Gas Chromatography, Residual Solvents

六鹿元雄，山口未来，平原嘉親，河村葉子：洗浄剤中のヒ素試験法および鉛試験法.
日本食品化学学会誌 2012;19:88-93.

洗浄剤中のヒ素および鉛試験法を確立した．ヒ素試験法は，試料（脂肪酸系洗浄剤：5 g，非脂肪酸系洗浄剤：1 g，食洗機用洗浄剤：0.3 g）に水を加えて150 mLとし試料溶液とした後，この液5 mLをODSミニカラムに通し，その溶離液を水で10 mLに定容して試験溶液とした．試験溶液およびヒ素標準溶液に塩酸1 mLおよびヨウ化カリウム溶液（1→5）1 mLを加え，水素化物発生装置-AASまたは水素化物発生装置-ICPで測定した．試料溶液当たりの定量限界は0.005 μg/mL（As_2O_3として）であった．鉛試験法は，試料（脂肪酸系洗浄剤：5 g，非脂肪酸系洗浄剤：1 g，食洗機用洗浄剤：0.3 g）に水120 mLを加えて溶解後，アルカリ性の場合には2 mol/L硝酸で中和した．水で150 mLに定容して試験溶液とし，AAS法またはICP法で測定した．試験溶液の定量限界は0.1 μg/mLであった．本法は現行の試験法と比べて非常に簡便である．これらの試験法を用いて製品10検体を調査した結果，ヒ素および鉛はいずれからも検出されなかった.

Keywords: detergent，arsenic，lead

Abe K*, Kumagai T*, Takahashi C*, Kezuka A*, Murakami Y*, Osawa Y*, Motoki H*, Matsuo T*, Horiuchi M*, Sode K*, Igimi S, Ikebukuro K*: Detection of pathogenic bacteria by using zinc finger protein fused with firefly luciferase.

Anal Chem. 2012;84:8028-32.

We constructed a novel bacterial genome detection system using zinc finger protein (ZF) fused with firefly luciferase (ZF-luciferase). Taking advantage of the direct recognition of double-stranded DNA (dsDNA) by ZF, we previously constructed bacterial genome detection systems that did not require dehybridization processes. To detect polymerase chain reaction (PCR) products rapidly and with a high sensitivity, we constructed two kinds of ZF-luciferase, Sp1-fused luciferase (Sp1-luciferase), and Zif268-fused luciferase (Zif268-luciferase). ZF-luciferase not only maintains luciferase activity but also shows dsDNA-binding ability and specificity. Furthermore, we succeeded in the detection of 10 copies of the genome of Legionella pneumophila and Escherichia coli O157. ZF-luciferase would be a useful tool for highly sensitive detection of pathogenic bacterial genome.

Keywords: zinc finger protein, luciferase, detection method

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Suzuki H: Susceptibility of Different Mice Strains to Okadaic Acid, A Diarrhetic Shellfish Poisoning Toxin.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2012;29:1307-10.

The mouse bioassay is widely used to detect diarrhetic shellfish poisoning (DSP) toxins. To the best of our knowledge, however, there have been no reports specifically on strain differences in susceptibility to DSP toxins. In this study, we investigated the susceptibility of different mice strains to okadaic acid (OA), one of the representative DSP toxins. A lethal dose of OA was injected intraperitoneally (i.p.) into mice. The mice were observed until 24 h after injection. Five inbred strains (A/J, BALB/c, C3H/He, C57BL/6, and DBA/2) and two non-inbred strains (ddY, and ICR) of mice were compared. All the mice were male, weighed 16-20 g, and were 4-5 weeks old. The lethality was 90-100% in the A/J, BALB/c, ddY, and ICR strains, 70-80% in the C3H/He and C57BL/6 strains, and 40% in DBA/2 strain. Survival analysis showed that the BALB/c, C57BL/6, ddY, and ICR strains died earlier and the A/J, C3H/He and DBA/2 strains survived longer. These results indicate that significant differences may exist in the susceptibility of mice strains to OA.

Keywords: mouse bioassay, okadaic acid, strain

Suzuki H: Age-Dependent Changes in Intraepithelial Lymphocytes (IEL) of the Small Intestine, Cecum, and Colon from Young Adult to Aged Mice.

Arch Gerontol Geriatr. 2012;55:261-70.

We previously reported the regional differences in the IELs present in the proximal (P), middle (M), and distal (D) parts of the small intestine, cecum (Ce), and colon (Co) of mice. In this study, we investigated the age-dependent changes in the regional differences of IELs from young adult to aged mice. In this experiment, 3-, 6-, 12-, 18-, and 24-month-old mice were examined. IELs were separately isolated from 5 parts of the intestines and analyzed by flow cytometry. Regional differences in the number and phenotype of IELs showed the same trends in all age groups. The number of IELs was highest in 6-month-old mice and then gradually decreased with age. As to IEL subsets, age-related changes were not seen except for a few subsets among the age groups. We conclude that age-related decreases in IELs in mouse small intestine may be one of the aging phenomena of the intestinal immune system. Such age-related decreases in IELs may be concerned with the increased liability to intestinal infections in the elderly.

Keywords: Intraepithelial lymphocytes (IEL), aging, mouse

Suzuki H: Differences in Susceptibility to Okadaic Acid, a Diarrhetic Shellfish Poisoning Toxin, between Male and Female Mice.

Toxins 2012;5:9-15.

The mouse bioassay (MBA) for diarrhetic shellfish poisoning (DSP) toxins has been widely used in many countries of the world. In the Japanese and EU methods, male mice are designated to be used for MBA. Female mice were described to be less susceptible than male mice. To the best of our knowledge, however, there have been no reports on the details of sex differences in susceptibility to DSP toxins. In this study, we investigated whether, and to what extent, female mice are less sensitive to DSP toxins. A lethal dose of okadaic acid (OA), one of the representative DSP toxins, was injected intraperitoneally into mice. The mice were observed until 24 hours after injection. Both male

and female mice of ICR and ddY strains, which are designated in the Japanese official method, were compared. All the mice were four weeks old and weighed 18-20 g. The experiments were repeated twice. The lethality was 70%-100%. Survival analysis showed no sex differences in susceptibility to OA, but ICR female mice showed significant resistance compared with other groups in one out of two trials. These results indicate that sex differences were not clear but, nonetheless, male mice showed more stable results.

Keywords: mouse bioassay, okadaic acid, sex

Asakura H, Brueggemann H[*1], Sheppard SK[*2], Ekawa T, Meyer TF[*3], Yamamoto S, Igimi S: Molecular evidence for the thriving of *Campylobacter jejuni* ST-4526 in Japan.

PLoS One 2012;7:e48394.

Campylobacter jejuni is a leading cause of human gastroenteritis worldwide. This study aimed at a better understanding of the genetic diversity of this pathogen disseminated in Japan. We performed multilocus sequence typing (MLST) of *Campylobacter jejuni* isolated from different sources (100 human, 61 poultry, and 51 cattle isolates) in Japan between 2005 and 2006. This approach identified 62 sequence types (STs) and 19 clonal complexes (CCs), including 11 novel STs. These 62 STs were phylogenetically divided into 6 clusters, partially exhibiting host association. We identified a novel ST (ST-4526) that has never been reported in other countries; a phylogenetic analysis showed that ST-4526 and related STs showed distant lineage from the founder ST, ST-21 within CC-21. Comparative genome analysis was performed to investigate which properties could be responsible for the successful dissemination of ST-4526 in Japan. Results revealed that three representative ST-4526 isolates contained a putative island comprising the region from Cj0737 to Cj0744, which differed between the ST-4526 isolates and the reference strain NCTC11168 (ST-43/CC-21). Amino acid sequence alignment analyses showed that two of three ST-4526 isolates expressed 693aa-filamentous hemagglutination domain protein (FHA), while most of other *C. jejuni* strains whose genome were sequenced exhibited its truncation. Correspondingly, host cell binding of FHA-positive *C. jejuni* was greater than that of FHA-truncated strains, and exogenous administration of rFHA protein reduced cell adhesion of FHA-positive bacteria. Biochemical assays showed that this putative protein exhibited a dose-dependent binding affinity to heparan sulfate, indicating its adhesin activity. Moreover, ST-4526 showed increased antibiotic-resistance and a reduced ability for DNA uptake. Taken together, our data suggested that these combined features contributed to the clonal thriving of ST-4526 in Japan.

Keywords: *Campylobacter jejuni*, MLST, Comparative genomics

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Asakura H, Ekawa T, Sugimoto N, Momose Y, Kawamoto K[*1], Makino S[*2], Igimi S, Yamamoto S: Membrane topology of *Salmonella* invasion protein SipB confers osmotolerance.

Biochem Biophys Res Commun. 2012;426:654-8.

Salmonella enterica serovar Typhimurium is a major cause of human gastrointestinal illness worldwide. This pathogen can persist in a wide range of environments, making it of great concern to public health. Here we report that the salmonella pathogenicity island (SPI)-1 effector protein SipB exhibits a membrane topology that confers bacterial osmotolerance. Disruption of the *sipB* gene or the *invG* gene (SPI-1 component) significantly reduced the osmotolerance of S. Typhimurium LT2. Biochemical assays showed that NaCl osmolarity increased the membrane topology of SipB, and a neutralising antibody against SipB reduced osmotolerance in the WT strain. The WT strain, but not the sipB mutant, exhibited elevated cyclopropane fatty acid C19:0 during conditions of osmotic stress, correlating with the observed levels of survival and membrane integrity. This result suggests a link between SipB and the altered fatty acid composition induced upon exposure to osmotic stress. Overall, our findings provide the first evidence that the *Salmonella* virulence translocon SipB affects membrane fluidity and alters bacterial osmotolerance.

Keywords: *Salmonella enterica*, SipB, osmotolerance

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Kusumoto A*, Asakura H, Kawamoto K*: General stress signma factor RpoS influences time required to enter the viable but non-culturable state in *Salmonella enterica*.

Microbiol Immunol. 2012;56:228-37.

We investigated the role of the alternative sigma factor RpoS in the viable but non-culturable（VBNC）induction in *Salmonella enterica*. Osmotic stress induced the VBNC state in S. Typhimurium LT2 and S. Oranienburg, but the S. Dublin exhibited slower entry into the VBNC state. The LT2 *rpoS* gene was initiated from an alternative initiation codon, TTG; therefore, LT2 had smaller amounts of RpoS than Dublin and Oranienburg. Oranienburg had a single amino acid substitution（D118N）in RpoS（$RpoS_{SO}$）. Disruption of *rpoS* caused rapid VBNC induction. VBNC induction was significantly delayed by Dublin-type RpoS, but only slightly by $RpoS_{SO}$. These indicate that RpoS delays VBNC induction and that the rapid induction of VBNC in LT2 and Oranienburg may be due to lower levels of RpoS and to the D118N amino acid substitution, respectively. Reduced RpoS intracellular level was observed during VBNC induction. During the VBNC induction, *Salmonella* might regulate RpoS which is important for maintenance of culturablity under stresses.

Keywords: *Salmonella enterica*, RpoS, osmotolerance

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Sasaki Y*1, Haruna M*1, Murakami M*1, Hayashida M*2, Ito K*1, Noda M, Yamada Y*1: Prevalence of Campylobacter spp., Salmonella spp., Listeria monocytogenes, and hepatitis E virus in swine livers collected at an abattoir.

Jpn J Infect Dis. 2013;66:161-4.

We investigated the prevalence of Campylobacter spp., Salmonella spp., Listeria monocytogenes, and hepatitis E virus（HEV）in swine liver. We collected swine livers from 110 pigs at an abattoir from September 2010 to March 2011. Pathogens were detected in the liver samples of 19（17.3%）pigs. Campylobacter spp. were isolated from the liver samples of 14（12.7%）pigs. In 10 of the 14 Campylobacter-positive pigs, bacteria were present in the internal regions of the liver. Salmonella spp. and L. monocytogenes were detected in the liver samples of 5（4.5%）pigs and 1（1%）pig, respectively. No HEV was detected in the swine liver samples tested. Regarding antimicrobial resistance in Campylobacter and Salmonella isolates, all isolates, except 1 Campylobacter jejuni isolate, were resistant to 1 or more antimicrobial agent. Campylobacter spp. resistant to erythromycin and/or enrofloxacin were isolated from the liver samples of 9（8%）pigs. These results suggest that the consuming swine liver without proper heat treatment may increase the risk of foodborne illnesses.

Keywords: swine liver, foodborne pathogens

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*2 Tokyo Kenbikyo-in Foundation

岡田由美子：食中毒の動向と対策.

化学療法の領域 2012;28:28-35.

過去10年間の感染性食中毒は，細菌性食中毒事件数の減少に伴い，食中毒事件総数はほぼ半減してきている．一方，患者総数は10年間ほぼ横ばい状態であり，ウイルス性食中毒患者数と同じ傾向を示している．個別の原因物質では，かつて国内の食中毒の中心であった腸炎ビブリオやサルモネラから，原因食品の中で増殖しないカンピロバクターやノロウイルスを原因とするものが中心となってきている．平成23年度から新しく厚生労働省食中毒統計に加わった原因物質も含め，感染性食中毒全般について，その動向や一般的留意点及び行政的対策について解説した．

Keywords: 感染性食中毒，動向

谷山茂人*1，高谷智裕*1，反町太樹*2，相良剛史*3，久保弘文*4，大城直雅，小野要*5，肖寧*2，橘勝康*1，荒川修*1：沖縄県沿岸に分布する腐肉食性および肉食性巻貝の毒性と毒成分.

食品衛生学雑誌 2013;54:49-55.

2009年１～６月に沖縄県沿岸で採集した小型巻貝８科15種計64個体のうち，５種にマウス毒性が認められた．このうち，キンシバイの毒力は総じて高く，筋肉で最高461 MU/gに達した．その他の4種（サツマビナ，ヘコミマクラ，イボヨフバイ，カゲロウヨフバイ）の毒力はおおむね10 MU/g前後であった．LC-MS分析により，有毒個体の毒の主体はいずれもTTXで，キンシバイではこれに加えて4,9-anhydroTTX，4-epiTTX，11-oxoTTXを含むことが示された．また，アワムシロの可食部

からもTTX（5.08 MU/g）が検出された．一方，残りの９種には，マウス毒性もTTXも全く認められなかった．

Keywords: 腐肉食性巻貝，キンシバイ，テトロドトキシン

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朝倉宏，岡田由美子，百瀬愛佳，山本茂貴，五十君靜信，春日文子：生食用食肉の規格基準策定に係る加熱条件の検討．
病原微生物検出情報 2012;33:132-3.

2011（平成23）年４月下旬～５月にかけて，富山県等で発生した，ユッケによる腸管出血性大腸菌（EHEC）集団食中毒の発生を契機として，厚生労働省では生食用食肉（牛肉）（内臓を除く）に対する規格基準の見直しについて検討が進められる運びとなった．コーデックス委員会の考え方を基にEHECの摂食時安全目標値が設定されたが，その達成に必要となる汚染低減のための応用的手法として温浴加熱の有効性を検証する過程および結論として，生食用牛肉の取り扱いにあっては，検体の熟成期間を考慮に入れつつ，表面より10mm内部までを対象とした衛生管理を行うことが，EHEC O157あるいはサルモネラの制御に有効かつ必要であることを示した．

Keywords: 腸管出血性大腸菌，温浴加熱

上田豊*1，花原悠太郎*1，阪本智宏*2，松村毅*3，北村勝，百瀬愛佳，朝倉宏，岡田由美子，五十君靜信，岩城正昭*4，加藤はる*4，柴山恵吾*4：鳥取県で発生した国内5年ぶりとなる食餌性ボツリヌス症．
病原微生物検出情報 2012;33:218-9.

2012年３月に鳥取県米子市で国内５年ぶりとなる食餌性ボツリヌス症が発生したので，その概要を報告した．

Keywords: 食餌性ボツリヌス症，あずきばっとう

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本村和嗣*1，横山勝*1，岡智一郎*1，片山和彦*1，野田衛，田中智之*2，佐藤裕徳*1：ノロウイルスのゲノム解析と流行発生のしくみ．
感染症学雑誌 2012;86:563-8.

我々は，ヒトノロウイルス感染症の流行予測とワクチン開発の基盤情報を得る目的で，2006年５月～2010年３月の間に全国19の道府県，20カ所の拠点衛生研究所で収集した感染者糞便中のGroup IIの中の遺伝子型4型（以下GII.4）全ゲノム配列を調べた（約7.5 kb，n=277）．糞便試料から核酸を抽出し，RT-PCRにより重複する２種のゲノム断片（5.3，2.5 kb）を増幅し，ダイレクトシークエンシング法で一感染者から１つのゲノム全長の配列情報を得た．ゲノム配列の進化系統，近縁関係は最尤法により解析した．流行株のアミノ酸の特徴を同定し，分子モデリング法を用いて，カプシド蛋白質で立体配置を視覚化した．解析期間内，2006b亜株が圧倒的に優勢なGII.4単系統群として存続した．一方，他にもGII.4単系統群が８種類発生したが，劣勢群として局地的流行に留まった．2006b亜株は，2006～2010秋冬期にかけて，全長にわたり，８カ所アミノ酸置換が生じていた．カプシド蛋白質に生じた変異は，立体構造上，ループに位置していた．この変異により，抗原性が変化すると推察された．高い変異率，感染力，増殖能が組み合わさり，ヒト社会では，日々膨大な数の変異ウイルスが発生していると推察される．抗原性が大きく変化したウイルスが出現すれば，ヒト社会の中で感染が広がり易いことが推定された．流行の変動に，ヒト集団のカプシド突端部への免疫が関与している可能性がある．

Keywords: norovirus, antigenic drift, genome recombination

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仁平稔*，高良武俊*，岡野祥*，喜屋武向子*，平良勝也*，久高潤*，崎枝央輝*，細田千花*，富永正哉*，野田衛：〈速報〉 ノロウイルスGII/4による集団食中毒事例－沖縄県．
病原微生物検出情報 2012;33:13-4.

2012年10月に沖縄県沖縄本島内の飲食店において，ノロウイルスGII/4の変異株（Sydney 2012）を原因とする集団食中毒事例が発生したので，その概要について報告した．

Keywords: Norovirus, GII/4 new variant, Sydney 2012

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稔[*9]，野田衛：〈速報〉 ノロウイルスGII/4の新しい変異株の遺伝子解析と全国における検出状況.
病原微生物検出情報 2012;33:14-5.

2012年10月に，新潟県長岡保健所管内の2つの福祉施設で胃腸炎の集団発生があった．今シーズン初の集団発生事例で，この2事例の患者から，遺伝子型GII/4のノロウイルスが検出された．COG2F/G2SKR増幅領域（N/S領域）の塩基配列に基づく系統樹解析の結果，本GII/4株は従来のGII/4変異株とは異なる，新しいGII/4変異株（GII/4 2012変異株，仮称）と思われた．そこで，本変異株のキメラウイルスの可能性および抗原性の変化を推定するために，RNAポリメラーゼ領域（Pol領域）およびP2ドメインを含むカプシド領域（P2d領域）の解析等を実施するとともに，全国の検出状況をとりまとめた．

Keywords: Norovirus, GII/4 new variant, Sydney 2012

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Hayashi H[*], Itahashi M[*], Taniai E[*], Yafune A[*], Sugita-Konishi Y, Mitsumori K[*], Shibutani M[*]: Induction of ovarian toxicity in a subchronic oral toxicity study of citrinin in female BALB/c mice.
J Toxicol Sci. 2012;37:1177-90.

The present study was performed to elucidate toxicity profile of citrinin (CTN) after repeated oral doses for 90 days, especially on the kidneys and female reproductive organs using female BALB/c mice. We first performed a 70-day repeated oral dose toxicity study of CTN by setting the doses at 1.25 and 7.5 ppm in the drinking water (Experiment 1). As a result, CTN did not produce anytoxicity in the kidneys, liver, and female genital organs/tracts, except for a slight increase of relative ovary weight. We, next, performed 90-day repeated oral dose toxicity study of CTN by increasing the dose levels at 15 and 30 ppm in the drinking water. The results suggested that CTN did not produce any toxicity in the kidneys, liver, and female genital organs/tracts, except for increase of both absolute and relative ovary weights accompanying increase of large follicles at ≥ 15 ppm. On the basis of these findings, the lowest-observable-adverse-effect level of CTN was 15 ppm (2.25 mg/kg body weight/day) in the drinking water for female BALB/c mice after 90-day oral treatment.

Keywords: Citrinin, Mycotoxin, Nephrotoxicity

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Aoyama K[*1], Akashi H[*2], Mochizuki N[*3], Ito Y[*4], Miyashita T[*5], Lee S[*6], Ogiso M[*7], Maeda M[*8], Kai S[*9], Tanaka H[*10], Noriduki H[*11], Hiraoka H[*1], Tanaka T[*12], Ishikuro E[*7], Itoh Y, Nagayama T[*13], Nakajima M[*14], Naito S[*15], Sugita-Konishi Y: Interlaboratory Study of LC-UV and LC-MS Methods for theSimultaneous Determination of Deoxynivalenol and Nivalenol in Wheat.
Food Hyg Saf Sci. 2012;53:152-6.

To evaluate LC methods with UV or MS detection for simultaneous analysis of deoxynivalenol (DON) and nivalenol (NIV) in wheat, an interlaboratory study was conducted in 11 laboratories.DON and NIV were purified using a multifunctional column, and their concentrations were determined using LC-UV or LC-MS(/MS). No internal standards were used. Three fortified wheat samples (0.1, 0.5 and 1 mg/kg), one naturally contaminated wheat sample, and one blank wheat sample were used. The recoveries ranged from 90% to 110% for DON and from 76% to 83% for NIV. For DON, the relative standard deviations for repeatability (RSDr) ranged from 1.1% to 7.6%. The relative standard deviations for reproducibility (RSDr) ranged from 7.2% to 25.2%. For NIV, the RSDr ranged from 2.0% to 10.7%, and the RSDr ranged from 7.0% to 31.4%. Regardless of sample and detector, the HorRat values for DON and NIV ranged from 0.4 to 1.4. Both LC-UV and LC-MS (/MS) methods were considered to be suitable for application as an official method.

Keywords: deoxynivalenol, nivalenol, interlaboratory study

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Lee K*1, French NP*2, Jones G*3, Hara-Kudo Y, Iyoda S*4, Kobayashi H*5, Sugita-Konishi Y, Tsubone H*1, Kumagai S*1: Variation in Stress Resistance Patterns among stx Genotypes and Genetic Lineages of Shiga Toxin-Producing *Escherichia coli* O157.
Appl Environ Microbiol. 2012;78:3361-8.

To evaluate the relationship between bacterial genotypes and stress resistance patterns, we exposed 57 strains of Shiga toxinproducing *Escherichia coli* (STEC) O157 to acid, freeze-thaw, heat, osmotic, oxidative, and starvation stresses. Inactivation rates were calculated in each assay and subjected to univariate and multivariate analyses, including principal component analysis (PCA) and cluster analysis. The stx genotype was determined for each strain as was the lineage-specific polymorphism assay (LSPA6) genotype. In univariate analyses, strains of the stx_1 stx_2 genotype showed greater resistance to heat than strains of the stx_1 stx_{2c} genotype; moreover, strains of the stx_1 stx_2 genotype showed greater resistance to starvation than strains of the stx_2 or stx_{2c} genotypes. LSPA6 lineage I (LI) strains showed greater resistance to heat and starvation than LSPA6 lineage II (LII) strains. PCA revealed a general trend that a strain with greater resistance to one type of stress tended to have greater resistance to other types of stresses. In cluster analysis, STEC O157 strains were grouped into stress-resistant, stress-sensitive, and intermediate clusters. In stx genotypes, all strains of the stx_1 stx_2 genotype were grouped with the stress-resistant cluster, whereas 72.7% (8/11) of strains of the stx_1 stx_{2c} genotype grouped with the stress-sensitive cluster. In LI strains, 77.8% (14/18) of the strains were grouped with the stress-resistant cluster, whereas 64.7% (11/17) of LII strains were grouped with the stress-sensitive cluster. These results indicate that the genotypes of STEC O157 that are frequently associated with human illness, i.e., LI or the stx_1 stx_2 genotype, have greater multiple stress resistance than do strains of other genotypes.

Keywords: *Escherichia coli* O157, stx genotype, lineage-specific polymorphism assay

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Kemmochi S*1,2, Hayashi H*1,2, Taniai E*1,2, Hasumi K*3, Sugita-Konishi Y, Kumagai S*4, Mitsumori K*1, Shibutani M*1: Protective Effect of Stachybotrys microspora Triprenyl Phenol-7 on the Deposition of IgA to the Glomerular Mesangium in Nivalenol-induced IgA Nephropathy Using BALB/c Mice.
J Toxicol Pathol. 2012;25:149-54.

Activators of tissue proteolysis including *Stachybotrys microspora* triprenyl phenol (SMTP)-7 are a new class of agents that are expected to be effective for amelioration of chronic tissue destructive diseases. The present study was performed to examine whether SMTP-7 is effective for the amelioration or protection of early-stage IgA nephropathy (IgAN) induced by nivalenol (NIV) in female BALB/c mice. In Experiment 1, mice were administered NIV at 24 ppm in diet for 8 weeks, and during the NIV treatment, they were intraperitoneally injected with SMTP-7 (10 mg/kg) three times a week. In Experiment 2, mice were injected similarly with SMTP-7 during the last 4 weeks of a 16-week NIV treatment. Immunofluorescence analysis revealed an inhibitory effect of SMTP-7 on the glomerular deposition of IgA in Experiment 1; however, it was ineffective in Experiment 2. On the other hand, SMTP-7 did not affect the serum concentration of IgA in both experiments. These results suggest that SMTP-7 has a potential to decrease the progression of IgAN induced

by NIV through inhibition of local accumulation of IgA in the glomerular mesangium, while it was ineffective for suppression of IgA production. On the other hand, SMTP-7 was found to be ineffective for already deposited IgA, suggesting that SMTP-7 may not be effective for ameliorating advanced IgAN.

Keywords: *Stachybotrys microspora* triprenyl phenol-7, IgA nephropathy, BALB/c mice, nivalenol

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Hara-Kudo Y, Saito S*1, Ohtsuka K*2, Yamasaki S*3, Yahiro S*4, Nishio T*5, Iwade Y*6, Otomo Y*7, Konuma H*8, Tanaka H*9, Nakagawa H*10, Sugiyama K*5, Sugita-Konishi Y, Kumagai S*11: Characteristics of a sharp decrease in *Vibrio parahaemolyticus* infections and seafood contamination in Japan.

Int J Food Microbiol. 2012;157:95-101.

Vibrio parahaemolyticus has been one of the most important foodborne pathogens in Japan since the 1960s, and a large epidemic was caused by the pandemic serotype O3:K6 from 1997 to 2001. *V. parahaemolyticus* infections, however, have sharply declined since that time. Data on serotypes isolated from 977 outbreaks were collected and analysed. Total and pathogenic, thermostable direct hemolysin (TDH) gene-positive *V. parahaemolyticus* were qualitatively and quantitatively detected in 842 seafood samples from wholesale markets in 2007-2009. Strains isolated from patients and seafood were analysed by serotyping, *tdh*-PCR, group-specific PCR for pandemic strains, and pulsed-field gel electrophoresis (PFGE). The sharp decrease in the infections from 1999 onwards was noted not only for O3:K6 infections but also for other serotypes. The change in the seafood contamination situation from 2001 to 2007－2009 was characterised by a decrease to three-fourths in the frequency of tdh-positive samples, although that decrease was small compared to the 18-fold decrease in the cases of *V. parahaemolyticus* outbreaks. PFGE detected the pandemic O3:K6 serotype in the same profile in seafood and patients from 1998 to the present. Because of no large decrease in seafood contamination by *V. parahaemolyticus* from the production to distribution stages and the presence of pandemic O3:K6 serotype in seafood to the present, it was suggested that the change of seafood contamination was unrelated to the sharp decrease in *V. parahaemolyticus* infections. *V. parahaemolyticus* infections might be prevented at the stages after the distribution stage.

Keywords: *Vibrio parahaemolyticus*, Epidemic, Seafood

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Hiroi M*1, Takahashi N*1, Harada T*1, Kawamori F*1, Iida N*1, Kanda T*1, Sugiyama K*1, Ohashi N*2, Hara-Kudo Y, Masuda M*1: Serotype, Shiga toxin (Stx) type, and antimicrobial resistance of Stx-producing *Escherichia coli* isolated from humans in Shizuoka prefecture, Japan (2003－2007).

Jpn J Infect Dis. 2012;65:198-202.

A total of 138 Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains isolated from humans between 2003 and 2007 in Shizuoka prefecture, Japan, were characterized with respect to serotype, Stx type, and antimicrobial resistance. The predominant O serogroups of STEC isolates were O157, O26, and O111. Antimicrobial susceptibility testing of STEC isolates showed that 31 of the 138 isolates (22.5%) were resistant to antibiotics. Compared to previous studies, we found that a higher rate of STEC O157 isolates were susceptible to all antimicrobial drugs examined in this study. However, antimicrobial susceptibility data from

this study showed that antimicrobial resistance patterns have increased by 6 compared to the survey performed by Masuda *et al.* between 1987 and 2002. This means that STEC isolates came to show more a variety of antimicrobial resistance patterns in comparison with the past. It is important to consider the population of isolates with decreased susceptibility to clinically relevant drugs such as CPFX and FOM. All 3 STEC isolates resistant to nalidixic acid showed the decreased susceptibility to ciprofloxacin (CPFX; MICs 0.25-0.5 μg/ml). In addition, a decreased susceptibility to fosfomycin (FOM) clearly emerged in *E. coli* O26 isolates. We found also the possibility that one STEC O26 strain is a chromosomal AmpC β-lactamase hyperproducer. These results suggest that antimicrobial agent therapy may be less successful for patients with non-O157 STEC infections than those with STEC O157 infections.

Keywords: STEC, Resistant to antibiotics

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Hiroi M*, Kawamori F*, Harada T*, Sano Y*, Miwa N*, Sugiyama K*, Hara-Kudo Y, Masuda T*: Antibiotic resistance in bacterial pathogens from retail raw meats and food-producing animals in Japan.
J Food Prot. 2012;75:1774-82.

To determine the prevalence and antimicrobial susceptibility profiles of *Campylobacter, Salmonella, Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) in food-producing animals and retail raw meats in Japan, raw meat samples as well as food-producing animal feces, cutaneous swabs and nasal swabs collected from 2004 to 2006 were analyzed. Isolation rates of *Campylobacter jejuni* and *Campylobacter coli*, *Salmonella* spp. and *S. aureus* were 34.6% (363/1050), 2.7% (28/1050) and 32.8% (238/725), respectively. MRSA was isolated from 3% (9/300) of meat samples. No VRE were isolated in this study. Three *C. jejuni* isolates from a patient with diarrhea in a hospital of Shizuoka prefecture and two chicken samples that exhibited resistance to ciprofloxacin (CPFX) had identical pulsed-field gel electrophoresis patterns, suggesting that *C. jejuni* that shows resistance to CPFX may be distributed in meat. Resistance to TC in *S. aureus* isolates from beef was lower than that seen in isolates from chicken and pork ($p < 0.01$). This study revealed that the prevalence of MRSA and VRE were low in food-producing animals and retail domestic meats in Japan although *Campylobacter* isolates resistant to fluoroquinolone and erythromycin were detected. The occurrence of antimicrobial-resistant pathogens should be monitored continuously to improve the management of the risks associated with antimicrobial drug resistance transferred from food-producing animals to humans.

Keywords: Antimicrobial resistance, Meat, *Campylobacter*

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Taniai E*1, 2, Yafune A*1-3, Hayashi H*1, 2, Itahashi M*1, 2, Hara-Kudo Y, Suzuki K*1, Mitsumori K*1, Shibutani M*1: Aberrant activation of ubiquitin D at G2 phase and apoptosis by carcinogens that evoke cell proliferation after 28-day administration in rats.
J Toxicol Sci. 2012;37:1093-111.

Following gene expression screening by microarrays in renal tubules with renal carcinogens, immunohistochemical analysis and TUNEL-assay were performed with carcinogens targeting different organs. All renal carcinogens tested (ferric nitrilotriacetic acid, ochratoxin A (OTA), monuron, tris (2-chloroethyl) phosphate, and potassium bromate) increased tubular cells immunoreactive for minichromosome maintenance 3 (Mcm3) or ubiquitin D (Ubd) or those showing apoptosis, compared with untreated controls or non-carcinogenic renal toxicants. Carcinogens targeting the liver (thioacetamide (TAA), fenbendazole, piperonyl butoxide (PBO) and methyleugenol), thyroid (sulfadimethoxine), urinary bladder (phenylethyl isothiocyanate), forestomach (butylated hydroxyanisol), glandular stomach (catechol), and colon (chenodeoxycholic acid and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) were examined for induction of Mcm3, Ubd, Topo IIα, Ki-67 and apoptosis using non-carcinogenic toxicants as negative controls. All cartinogens increased $Mcm3^+$, Ubd^+, Topo $IIα^+$, $Ki\text{-}67^+$ or $TUNEL^+$ cells, except for hepatocarcinogen PBO and both colon carcinogens, which did not increase cell proliferation. Ubd+ cells co-expressing Topo IIα was increased without changing phospho-Histone H3-co-expressing cell

population as examined with OTA and TAA. Results revealed cooperative responses of Topo IIα, Ubd and apoptosis by cartinogens inducing high proliferation activity, irrespective of target organs, examined here after a 28-day administration. Aberrant expression of Ubd at G_2 phase and increased apoptosis reflecting aberrant cell cycle regulation may be the common feature of these carcinogens.

Keywords: Apoptosis, Carcinogen, Cell proliferation

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Hiroi M[*1], Matsui S[*2], Kubo R[*3], Iida N[*1], Noda Y[*1], Kanda T[*1], Sugiyama K[*1], Hara-Kudo Y, Ohashi N[*4]: Factors for occurrence of extended-spectrum β-lactamase-producing *Escherichia coli* in broiler.
J Vet Med Sci. 2012;74:1635-7.

To clarify the factors for occurrence of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* on broiler, two flocks (1 day of age) feeding diet with or without antibiotics were kept to a broiler house sanitized with disinfectants. ESBL-producing *E. coli*, however, was detected at a concentration of over 10^6 CFU/g of feces from 9 days of age to 49 days of age in both broiler flocks. Therefore it was indicated that the antibiotics other than cephalosporins used in this study have no effect due to co-selection on the numbers of ESBL-producing *E. coli* in broiler feces in this term. When a flock was kept with diet with antibiotics for 49 days in a laboratory animal room, no ESBL-producing *E. coli* was detected in the flock. These results suggest that the occurrence of ESBL-producing *E. coli* may not be related to feed with antibiotics, and the contamination of ESBL-producing *E. coli* in broiler houses might be an important factor.

Keywords: Extended-spectrum β-lactamase-producing *Escherichia coli*, Broiler, Occurrence

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Matsutani S: Bacterial ArtA protein specifically binds to the internal region of IS*1 in vitro*.
Adv Biosci Biotechnol. 2012;3:869-75.

The internal region of bacterial translocatable IS*1* acts as a *cis*-element to stimulate transcription from the various promoters located upstream. The product of the *art*A gene is genetically shown to stimulate transcription with the *cis*-element. Here, a codon-optimized *art*A gene was synthesized and cloned to express the ArtA protein. ArtA was purified as the His-tagged protein. Nitrocellulose filter binding assay showed that ArtA specifically binds to the IS*1* internal region. Electrophoretic mobility shift assay also showed specific binding of ArtA to the IS*1* internal region. These results imply that ArtA directly binds to the IS*1* internal region and stimulates transcription.

Keywords: Transcription stimulation, Downstream element, DNA binding

鎌田洋一：ザルコシスティスが含まれる馬肉による食中毒.
日本食品微生物学会雑誌 2012;29:47-52.

生鮮冷蔵馬肉の生食によって発生する食中毒について，特徴，最新の事例紹介，原因究明の過程，その病因物質として*Sarcocystis fayeri*，虫体の検査法，毒性タンパク質，また危害の制御法と今後の検討課題について紹介した．今後は検査法が徹底され，食中毒診断を確実化でき，疫学情報が充実するだろう．本食中毒の予防には馬肉の冷凍処理が有効であることが明らかになっている．寄生環を遮断するという対処に加え，迅速スクリーニング法や冷凍以外の制御法の開発も行われてゆくだろう．

Keywords: 馬肉食中毒，住肉胞子虫，制御

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食品衛生研究 2012;62:23-6.

食後数時間で嘔吐・下痢を発症する原因不明の有症苦情事例における共通食に馬肉がある．熊本県では年間20件程度発生している．共通食の馬肉に対し寄生虫学的および毒性学的に検討し，原因が住肉胞子虫であることを明らかにした．馬肉の凍結処理が，住肉胞子虫の危害性を失活させることを示し，馬肉食中毒の制御方法を確立した．

Keywords: 馬肉食中毒，冷凍処理，危害性制御

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食品衛生学雑誌 2012;53:273-7.

セレウス嘔吐毒マーカータンパク質の検出システム（シングルパス・エメティックトキシンマーカー）の妥当性を検証した．わが国で発生した食中毒事例由来セレウス菌（84株），および市販食品分離株（21株），合計105株を1％ブドウ糖添加Casein-Glucose-Yeast Extract（CGY）培地で培養後，その培養液をキットのサンプル注入部に滴下し，検出ラインの有無を判定した．各菌株の嘔吐毒合成酵素遺伝子をPCR法を用いて検出し，陽性を示した株を嘔吐毒遺伝子保有株とした．食中毒検査で分離された嘔吐毒遺伝子保有菌58株は，本検出キットですべて陽性と判定された．一方，嘔吐毒遺伝子非保有株は，食中毒由来株では26株中２株，食品由来株21株中１株が陽性を示した．これら３菌株の毒素産生性を，HEp-2細胞を用いて検討したところ，毒素は検出されなかった．メーカーの報告によれば本イムノクロマトキットは，CGY培地による食品の培養液からも嘔吐毒産生セレウス菌が検出可能であるとのことから，食中毒検査時だけでなく，食材や調理食品中の嘔吐毒産生性セレウス菌のスクリーニングにも使用できると考えられた．

Keywords: セレウス菌，嘔吐毒素，イムノクロマト

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Watanabe M, Goto K*, Sugita-Konishi Y, Kamata Y, Hara-Kudo Y: Sensitive detection of whole-genome differentiation among closely-related species of the genus *Fusarium* using DNA-DNA hybridization and a microplate technique.

The Journal of Veterinary Medical Science. 2012;74:1333-6.

We developed a new system for detection of whole-genome differentiation using DNA-DNA hybridization, and tested its sensitivity with three closely-related ***Fusarium*** species. We compared DNA-DNA relatedness to nucleotide sequence homologies of five genetic regions between each of five strains of three *Fusarium* species. DNA-DNA relatedness by our system was 16.2-86.6%. Sequence homologies of 18S rDNA, rDNA cluster region from ITS1 to 28S rDNA, *β-tub*, *EF-1α* and *lys2* were 100.0, 99.0-100.0, 96.7-100.0, 95.1-99.4, and 94.7-100.0%, respectively. Our system could clearly detect differentiation between closely-related fungal species which have very similar morphological-characteristics, and exhibit little diagnoses in nucleotide sequences. Our results suggest that this system is a good tool for identification and phylogenetic analysis of closely-related fungal species.

Keywords: *Fusarium*, DNA-DNA hybridization, Whole-genome

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渡辺麻衣子，小沼ルミ*1，米澤隆弘*2，瓦田研介*1，小西良子，鎌田洋一：遺伝子塩基配列を指標とした食品由来*Fusarium*属分離株の同定．

日本食品微生物学雑誌 2012;29:221-9.

*Fusarium*属菌は，形態学的手法による同定が難しい場合が多く，近年，分子生物学的指標による同定手法が広く用いられつつある．しかし，近縁種を識別できないなどの問題点も多く，*Fusarium*属菌の同定に適した遺伝子指標を検討する必要がある．そこで，*Fusarium*属菌分離株を適切に同定できる遺伝子指標を特定することを目的として，塩基配列相同率を指標とした同定精度の比較検討を行った．菌株分譲機関由来株24菌種合計47菌株，およびspecies complexを形成する食品由来分離株８株を供試した．解析対象遺伝子として，18S rDNA，5.8S rDNA，ITS1，28S rDNA，*β-tub*および*lys2*を選択し，塩基配列を決定した．分譲機関から収集した47菌株の塩基配列のデータベースを作成し，これに対して，食品由来分離株の各遺伝子塩基配列の相同性検索を行った．この結果を参照し，遺伝子ごとに各分離株の菌種を特定した．塩基配列相同率による食品由来分離株の同定結果を遺伝子ごとに比較したところ，species complexを形成する菌種を同定するためには，従来広く用いられてきたリボゾーム関連遺伝子群の塩基配列を指標とする

ことはできないこと，全ての供試菌種を正しく同定できる遺伝子指標には*β-tub*が最も適するということが明らかとなった．しかし，菌種によっては塩基配列相同率の差が小さい場合もあり高いシークエンス精度が求められ，誤同定が起こることも考えられる．形態学的指標による同定も合わせて行う必要がある．

Keywords: *Fusarium*, Beta-tubulin, Nucleotide sequence homology

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Wu W*1, 2, Flannery BM*2, Watanabe M, Sugita-Konishi Y, Zhang H*1, Pestka JJ*2: Comparison of murine anorectic responses to the 8-ketotrichothecenes 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X and nivalenol.
Food and Chemical Toxicology 2012;50:2056-61.

While induction of food refusal by the trichothecene mycotoxin deoxynivalenol (DON) has been described in several animal models, much less is known about the anorectic effects of structurally related 8-ketotrichothecenes, 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FX) and nivalenol (NIV). Here, we compared the capacities of these congeners to induce anorexia in the mouse. As previously observed for DON, anorectic responses to 3-ADON and 15-ADON in the B6C3F1 female mouse following both intraperitoneal (IP) and oral exposure were transient, lasting only a few hours, with food intake recovering to control levels within 16 h. For both ADONs, the no observed adverse effect levels (NOAEL) and lowest observed adverse effect levels (LOAEL) were 0.5 and 1mg/kg bw following IP exposure, respectively, and 1 and 2.5mg/kg bw after oral exposure, respectively. In contrast, food refusal persisted from 48 to 96 h following IP and oral exposure to FX and NIV. For both IP and oral FX exposure, the NOAEL was 0.025 mg/kg bw and LOAEL was 0.25mg/kg bw, whereas the NOAELs and LOAELs for NIV were 0.01 and 0.1mg/kg bw, respectively, after IP exposure and 0.1 and 1mg/kg bw, respectively, following oral exposure. Both these data and a prior DON study suggest that anorectic responses to 8-ketotrichothecenes were always greater when administered IP as compared to oral exposure. Toxic potency data such as is described here will be applicable to future comparative risk assessments for this important group of trichothecene mycotoxins.

Keywords: Trichothecene, Anorexia

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Taniai E*1, 2, Hayashi H*1, 2, Yafune A*1, 2, Watanabe M, Akane H*1, Suzuki K*1, Mitsumori K*1, Shibutani M*1: Cellular distribution of cell cycle-related molecules in the renal tubules of rats treated with renal carcinogens for 28 days − Relationship between cell cycle aberration and carcinogenesis.
Archives of Toxicology 2012;86:1453-64.

To clarify the cell cycle-related changes during the early stages of renal carcinogenesis, we performed immunohistochemical analysis of tubular cells in male F344 rats treated with carcinogenic doses of representative renal carcinogens for 28 days. For this purpose, the karyomegaly-inducing carcinogens ochratoxin A (OTA), ferric nitrilotriacetic acid, and monuron, and the non-karyomegaly-inducing carcinogens tris (2-chloroethyl) phosphate and potassium bromate were examined. For comparison, a karyomegaly-inducing non-carcinogen, p-nitrobenzoic acid, and a non-carcinogenic non-karyomegaly-inducing renal toxicant, acetaminophen, were also examined. The outer stripe of the outer medulla (OSOM) and the cortex + OSOM were subjected to morphometric analysis of immunoreactive proximal tubular cells. Renal carcinogens, irrespective of their karyomegaly-inducing potential, increased proximal tubular cell proliferation accompanied by an increase in topoisomerase IIα-immunoreactive cells, suggesting a reflection of cell proliferation. Karyomegaly-inducing carcinogens increased nuclear Cdc2-, γH2AX-, and phosphorylated Chk2-immunoreactive cells in both areas, the former two acting in response to DNA damage and the latter one suggestive of sustained G_2. OTA could easily be distinguished from untreated controls and non-carcinogens by evaluation of molecules responding to DNA damage and G_2/M transition in the OSOM. Thus, all renal carcinogens examined facilitated proximal tubular proliferation by repeated short-term treatment. Among these, karyomegaly-inducing carcinogens may cause DNA damage and G2 arrest in the target tubular cells.

Keywords: Renal carcinogenesis, Karyomegaly, Cell

proliferationy

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Kobayashi N*, Watanabe M, Hara-Kudo Y: Distinctive identification of *Cladosporium sphaerospermum* and *Cladosporium halotolerans* based on physiological methods.
Journal of Systematics and Evolution 2012;50:235-43.

We aimed to detect physiological characteristics that clearly varied among the closely-related *Cladosporium sphaerospermum*-like species. We isolated the fungi identified as *C. sphaerospermum* s.l. based on traditional morphological criteria from various locations and substrata, and redefined this initial identification by the molecular phylogenetic methods. The isolates were identified as only *C. sphaerospermum* and *C. halotolerans*. We analyzed the substrate-utilization of 95 carbon sources using the Biolog system and made statistical comparisons of isolates by their abilities to grow at different osmolarities. The substrate-utilization patterns separated the isolates into two groups corresponding to the molecular data, and the osmotolerance was different between the species. We first showed that *C. sphaerospermum* and *C. halotolerans* were diverse not only at the molecular level but also at the ecological and the physiological levels, by analyzing substrate-utilization patterns and osmotolerance. Furthermore, we showed the potential utility of the Biolog system for discriminating among closely-related fugal species.
Keywords: *Cladosporium*, Identification, Substrate-utilization

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

Although the acute toxic effects of trichothecene mycotoxin deoxynivalenol (DON or vomitoxin), a known cause of human food poisoning, have been well characterized in several animal species, much less is known about closely related 8-ketotrichothecenes that similarly occur in cereal grains colonized by toxigenic fusaria. To address this, 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. With increasing doses, there were marked decreases in latency to emesis with corresponding increases in emesis duration and number of emetic events. The effective doses resulting in emetic events in 50% of the animals for 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: trichothecene, emesis, vomitoxin

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Iijima Y*, Nakanishi N*, Furusawa H, Ohnishi T, Sugita-Konishi Y: Inter-laboratory validation and applications of quantitative real-time PCR for the detection of *Kudoa septempunctata* in olive flounder (*Paralichthys olivaceus*).
Jpn J Infect Dis. 2012;65:436-8.

Kudoa septempunctata, a myxosporean parasite, was recently identified as the causative agent of food poisoning resulting from the consumption of raw olive flounder (*Paralichthys olivaceus*). A single blind inter-laboratory study, involving 5 laboratories, was conducted to validate a quantitative real-time PCR assay for the detection of the parasite. We obtained relatively constant values for log rDNA copies/g from these lab-

oratory analyses (SD = 0.35-0.86), suggesting the validity of the real-time PCR method for the detection of *K. septempunctata* in *P. olivaceus*. Detection of *K. septempunctata* in muscle tissue samples collected from both sides of the fish indicated that *K. septempunctata* infection spreads throughout the body of *P. olivaceus*. *K. septempunctata* infection in *P. olivaceus* is thought to occur during the early stage of fish growth because a *K. septempunctata* gene was detected in 1 of 300 *P. olivaceus* fry tested. Feeds seem not to be sources of infection. To prevent food poisoning due to *K. septempunctata*, the mechanism of infection and proliferation of *K. septempunctata* in *P. olivaceus* should be elucidated, and other hosts of the parasite should be identified. The sensitive real-time PCR method described here will be a useful tool for resolving these issues.

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

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Li YC*, Sato H*, Kamata Y, Ohnishi T, Sugita-Konishi Y: Three novel myxobolid species of genera *Henneguya* and *Myxobolus* (Myxosporea: Bivalvulida) from marine fish in Japan.

Parasitol Res. 2012;111:819-26.

Myxosporean* genera *Henneguya* and *Myxobolus (Bivalvulida: Myxobolidae) are closely related in morphology and molecular phylogeny, speciose with approximately 1,000 nominal species. The majority of them are recorded from freshwater fish worldwide, and few are known from marine fish. In this study, three myxobolid spp. are described from marine fish around Japan. Two novel *Henneguya* spp., *Henneguya ogawai* sp. n. and Henneguya yokoyamai sp. n., are described from two black sea breams (*Acanthopagrus schlegelii*) fished in the Inland Sea (Setonaikai), Japan. Plasmodia of the former species were localized in the esophageal or intestinal wall, and those of the latter species were in the wall of the gall bladder and peritoneum. Spore development in plasmodia of these two species was synchronous. The spore body of *H. ogawai* sp. n. was 11.0 (8.9-12.2)μm in length, 6.9 (6.3-7.5)μm in width, 5.9 (5.2-6.6)μm in thickness, with a bifurcated caudal process of equal length, 10.0 (8.4-12.7)μm long; total spore length, 21.1 (19.2-23.4)μm. It contained two polar capsule, 4.3 (3.8-5.2) × 1.9 (1.4-2.3)μm. The spore body of *H. yokoyamai* sp. n. was 11.0 (10.1-13.7)μm in length, 7.1 (6.6-7.5)μm in width, and 5.6 (4.5-6.4)μm in thickness, with a bifurcated caudal process of equal length, 14.1 (10.8-17.0)μm long; total spore length, 25.0 (21.9-29.2)μm. It contained two polar capsules, 3.7 (3.1-4.2) × 2.0 (1.8-2.4)μm. A novel *Myxobolus* sp., *Myxobolus machidai* sp. n., is described from a spotted knifejaw (*Oplegnathus punctatus*) fished in the Sea of Japan, off Shimonoseki, Yamaguchi Prefecture, Japan. Plasmodia were embedded in the esophageal wall. Its round spore was small in size, 9.0 (8.1-9.4)μm in length, 7.8 (7.5-8.3)μm in width, and 5.5 (5.1-6.0)μm in thickness. It contained two polar capsules, 3.5 (3.2-3.8) × 2.3 (2.2-2.5)μm. Spore development in a plasmodium was asynchronous. Nucleotide sequencing of the small subunit ribosomal RNA gene (SSU rDNA) of these two novel *Henneguya* spp. revealed a close phylogenetic relationship with the marine clade of *Henneguya* spp.; however, they were distinct in morphology and SSU rDNA sequence from any known species. *M. machidai* sp. n. was grouped with freshwater *Henneguya* spp. in a phylogenetic tree based on the SSU rDNA, distant from a known marine clade of *Myxobolus* spp. reported mainly from the Mediterranean Sea. This is the first record of *Henneguya-Myxobolus* spp. from natural marine water in Japan.

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

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Harada T*, Kawai T*, Jinnai M *, Ohnishi T, Sugita-Konishi, Y, Kumeda Y*: Detection of *Kudoa septempunctata* 18S ribosomal DNA in patient fecal samples from novel food-borne outbreaks caused by consumption of raw olive flounder (*Paralichthys olivaceus*).

J Clin Microbiol. 2012;50:2964-8.

***Kudoa septempunctata* is a newly identified myxo**sporean parasite of olive flounder (*Paralichthys olivaceus*) and a suspected causative agent of several food-borne gastroenteritis outbreaks in Japan. Here, we report the detection of *K. septempunctata* 18S ribosomal DNA in fecal samples of outbreak patients using an efficient method based on real-time PCR. We first performed a spiking experiment to assess whether our previously developed real-time PCR assay was applicable to detect *K. septempunctata* in feces. Simultaneously, we compared the relative extraction efficacy of *K.*

septempunctata DNA using three commercial kits. Finally, our detection method was validated by testing 45 clinical samples obtained from 13 food-borne outbreaks associated with the consumption of raw flounder and 41 fecal samples from diarrhea patients epidemiologically unrelated to the ingestion of raw fish. We found that the FastDNA Spin Kit for Soil (MP Biomedicals) was the most efficient method for extracting *K. septempunctata* DNA from fecal samples. Using this kit, the detection limit of our real-time PCR assay was $1.6 \times 10(1)$ spores per g of feces, and positive results were obtained for 21 fecal and 2 vomitus samples obtained from the food-borne outbreaks. To our knowledge, this is the first report to describe the detection of *K. septempunctata* DNA in patient fecal samples. We anticipate that our detection method will be useful for confirming food-borne diseases caused by *K. septempunctata* in laboratory investigations.

Keywords: Kudoa, Food-borne disease, Parasite

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Ohnishi T, Kikuchi Y, Furusawa H, Kamata Y, Sugita-Konishi Y: *Kudoa septempunctata* invasion increases the permeability of human intestinal epithelial monolayer.

Foodborne Pathog Dis. 2013;10:137-42.

Kudoa septempunctata is a myxosporean parasite of *Paralichthys olivaceus* (olive flounder) and causes a foodborne illness that affects more than 100 cases in Japan each year. We previously reported that the consumption of raw olive flounder meat containing a high concentration of *K. septempunctata* spores induces transient but severe diarrhea and emesis through an unknown mechanism. Here, we demonstrate that *K. septempunctata* sporoplasm plays an important role in mediating the toxicity of K. septempunctata. When *K. septempunctata* spores were inoculated in Caco-2 human intestinal cells, *K. septempunctata* sporoplasms were released from spores, and they invaded the cells. Electron microscopic observations revealed that the sporoplasm invasion severely damaged the Caco-2 cells. The inoculation of *K. septempunctata* spores eliminated the transepithelial electrical resistance (TER) across the cell monolayer. Inhibiting the invasion of the sporoplasms prevented the observed loss in cell layer integrity, as illustrated by the rapid elimination of the TER. These results suggest that the invasion by sporoplasms severely damaged individual intestinal cells, resulting in a loss of cell monolayer integrity.

Keywords: *Kudoa*, Foodborne disease, Parasite

Kawai T*1, Sekizuka T*2, Yahata Y*2, Kuroda M*2, Kumeda Y*1, Iijima Y*3, Kamata Y, Sugita-Konishi Y, Ohnishi T: Identification of *Kudoa septempunctata* as the causative agent of novel food poisoning outbreaks in Japan by consumption of Paralichthys olivaceus in raw fish.

Clin Infect Dis. 2012;54:1046-52.

BACKGROUND: Outbreaks of an unidentified food-borne illness associated with the consumption of raw fish have increased in Japan since 2003. Those affected with this illness develop diarrhea and emesis within 2-20 hours after a meal including raw fish. No known causative agents such as bacteria, viruses, bacterial toxins, or toxic chemicals have been detected in the foods that were ingested. Fortunately, this illness is self-limiting with good prognosis in all cases. METHODS: We conducted an epidemiological analysis of outbreaks that occurred during 2008 and 2010 and analysed a fish sample from one outbreak by metagenomic DNA sequencing, real-time polymerase chain reaction, and direct microscopic observations. The pathogenicity of a putative risk factor identified by these techniques was assessed using the suckling-mouse test and a house musk shrew emetic assay.

RESULTS: The epidemiological analysis of outbreaks in 24 municipalities involving >1300 subjects implicated an olive flounder (Paralichthys olivaceus) as the causative food source. The presence of *Kudoa septempunctata*, a recently-described myxosporean species in *P. olivaceus*, was prevalent in the causative foods. *K. septempunctata* induced watery stools and an elevated fluid accumulation ratio in suckling mice, as well as vomiting in house musk shrews.

CONCLUSIONS: These results identify *K. septempunctata* as the etiological agent of this novel food-borne illness outbreak associated with consumption of raw *P. olivaceus*. This is the first report, to our knowledge, demonstrating the human pathogenicity of *Kudoa* spores.

Keywords: *Kudoa*, Foodborne illness, Parasite

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Ohnishi T, Goto K*1, Kanda T*2, Kanazawa Y*3, Ozawa K*4, Sugiyama K*2, Watanabe M, Konuma H*5, Hara-Kudo Y: Microbial contamination associated with consumption and the growth in plastic bottled beverage.

J Environ Sci Health 2013;48:781-90.

Plastic bottles enable the storage of unfinished beverages, and most of microbial contamination has occurred in the unfinished beverage that was left. Therefore, we investigated microorganisms in various beverages contaminated by pouring and drinking directly by mouth from the bottle, and analyzed the growth of microorganisms in the beverages at room temperature. In the pouring test, microbial growth was detected in 60 of 320 samples, and 13 bacterial strains, 49 mold strains, and 8 yeast strains were isolated. Molds including *Cladosporium* spp., *Trametes* spp., *Bjerkandera* spp., and *Penicillium* spp. accounted for the majority of isolated microorganisms. In the drinking test, microbial growth was detected in 181 of 352 samples, and 225 bacterial strains, 27 mold strains and 77 yeast strains were isolated. Bacteria including *Streptococcus* spp. such as *S. salivarius* and *Staphylococcus* spp. such as *S. aureus* accounted for the majority of isolated microorganisms. Enterotoxin-producing *S. aureus* and *Bacillus cereus* were also isolated. The pH of the beverage influenced the growth of bacteria. The Brix values of the beverage did not correlate with the growth of microorganisms. These results revealed that various microorganisms including foodborne pathogens were able to grow in numerous types of beverages and that the storage of unfinished beverage in inappropriate condition, such as the storage at room temperature led microorganism to grow easily in beverage. Therefore, it is necessary to consume beverages as soon as possible after opening the bottle.

Keywords: Beverage, PET bottle, Contamination

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Yoshinari T, Ohnishi T, Kadota T*, Sugita-Konishi Y: Development of a purification method for simultaneous determination of deoxynivalenol and its acetylated and glycosylated derivatives in corn grits and corn flour by liquid chromatography-tandem mass spectrometry.

J Food Prot. 2012;75:1355-8.

We developed a purification method based on liquid chromatography-tandem mass spectrometry for the identification of DON, its acetylated derivatives (3ADON and 15ADON), and a glycosylated derivative (D3G) in corn-based products. The analytes were extracted from samples with acetonitrile-water (85:15, vol/vol) and then purified with multifunctional columns. Evaluation of five kinds of multifunctional columns revealed that DON and its acetylated derivatives were recovered well (96 to 120%) by all columns, but D3G was recovered adequately (93.5%) by only one column, InertSep VRA-3. Samples of corn grits and corn flour were analyzed using the purification method with InertSep VRA-3. DON, D3G, and 15-acetyl-deoxynivalenol were the major contaminants in the samples harvested in 2009, but only DON was detected in the samples harvested in 2010. These results suggest that the purification method using InertSep VRA-3 is effective for identification of DON and its derivatives in corn-based products.

Keywords: Deoxynivalenol, Liquid chromatography-tandem mass spectrometry, Corn grits

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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 deoxynivalenol and its acetylated derivatives, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in wheat.

Shokuhin Eiseigaku Zasshi 2013;54:75-82.

To validate an LC-MS/MS method for simultaneous determination of DON and its acetylated derivatives, 3ADON and 15ADON, in wheat using a multifunctional column, an inter-laboratory study was performed in 9 laboratories using one blank wheat sample, three spiked wheat samples (10, 50, 150 μg/kg) and one naturally contaminated wheat sample. The recoveries

ranged from 98.8 to 102.6% for DON, 89.3 to 98.7% for 3ADON, and from 84.9 to 90.0% for 15ADON. The relative standard deviations for repeatability (RSDr) and reproducibility (RSDR) of DON were in the ranges of 7.2-11.3% and 9.5-22.6%, respectively. For 3ADON, the RSDr ranged from 5.3 to 9.5% and the RSDR ranged from 16.1 to 18.0%, while for 15ADON, the RSDr ranged from 6.2 to 11.2% and the RSDR ranged from 17.0 to 27.2%. The HorRat values for the three analytes ranged from 0.4 to 1.2. These results validate this method for the simultaneous determination of DON and its acetylated derivatives, 3ADON and 15ADON.

Keywords: Acetyl deoxynivalenol, LC-MS/MS, Interlaboratory study

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Ohno A, Kawanishi T, Okuda H, Fukuhara K: A new approach to characterization of insulin derived from different species using ^{1}H-NMR coupled with multivariate analysis.

Chem Pharm Bull. 2012;60:320-4.

Most of the active components of polypeptides have a complex molecular structure, large molecular size. Such components may also be structurally heterogeneous. Therefore, development of a method that can confirm the consistency of polypeptides amino-acid sequences for product characterization is desirable. In general, it is extremely difficult to distinguish differences of a few amino acid residues in the 1H-NMR spectrum of polypeptides with molecular weights greater than several thousand. However, we have been able to distinguish between three insulin species differing in one to three amino acid residues using a combination of multivariate statistics and 1H-NMR spectra. These results demonstrate that this methodology could be useful for characterization of polypeptides.

Keywords: insulin, principal component analysis, 1H-NMR

Inami K*1, Iizuka Y*1, Furukawa M*1, Nakanishi I*2, Ohkubo K*3, Fukuhara K, Fukuzumi*3, Mochizuki M*1: Chlorine atom substitution influences radical scavanging activity of 6-chromanol.

Bioorg Med Chem. 2012;20:4049-55.

Synthetic 6-chromanol derivatives were prepared with several chlorine substitutions, which conferred both electron-withdrawing inductive effects and electron-donating resonance effects. A trichlorinated compound (2), a dichlorinated compound (3), and three monochlorinated compounds (4, 5, and 6) were synthesized; compounds 2, 3, and 6 were novel. The antioxidant activities of the compounds, evaluated in terms of their capacities to scavenge galvinoxyl radical, were associated with the number and positioning of chlorine atoms in the aromatic ring of 6-chromanol. The activity of compound 1 (2,2-dimethyl-6-chro- manol) was slightly higher than the activities of compounds 2 (2,2-dimethyl-5,7-dichloro-6-chromanol) or 3 (2,2-dimethyl-5,7,8-trichloro-6-chromanol), in which the chlorine atoms were ortho to the phenolic hydroxyl group of 6-chromanol. The scavenging activity of compound 3 was slightly higher than that of 2, which contained an additional chlorine substituted in the 8 position. The activities of polychlorinated compounds 2 and 3 were higher than the activities of any of the monochlorinated compounds (4-6). Compound 6, in which a chlorine was substituted in the 8 position, exhibited the lowest activity. Substi- tution of a chlorine atom meta to the hydroxyl group of 6-chromanol (compounds 2 and 6) decreased gal- vinoxyl radical scavenging activity, owing to the electron-withdrawing inductive effect of chlorine. Positioning the chloro group ortho to the hydroxyl group (compounds 4 and 5) retained antioxidant activ- ity because the intermediate radical was stabilized by the electron-donating resonance effect of chlorine in spite of the electron-withdrawing inductive effect of chlorine. Antioxidant activities of the synthesized compounds were evaluated for correlations with the O-H bond dissociation energies (BDEs) and the ion- ization potentials. The BDEs correlated with the second-order rate constants (k) in the reaction between galvinoxyl radical and the chlorinated 6-chromanol derivatives in acetonitrile. This indicated that the antioxidant mechanism of the synthesized compounds consisted of a one-step hydrogen atom transfer from the phenolic OH group rather than an electron transfer followed by a proton transfer. The synthe- sized compounds also exhibited hydroxyl radical scavenging

capacities in aqueous solution.

Keywords: antioxidant activities, radical scavanging activity, 6-chromanol derivatives

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Uchiyama S*1, Sakamoto H*2, Ohno A, Inaba Y*1, Nakagome H*3, Kunugita N*1: Reductive amination of glutaraldehyde 2,4-dinitrophenylhydrazone using 2-picoline borane and high-performance liquid chromatographic analysis.

Analyst 2012;137:4274-9.

A typical method for the measurement of glutaraldehyde（GLA）employs 2,4-dinitrophenylhydrazine（DNPH）to form GLA-DNPhydrazone derivatives. However, this method is subject to analytical errors because GLA-DNPhydrazone is a quaternary bis-derivative and forms three geometric isomers（*E-E*, *E-Z* and *Z-Z*）as a result of the two C=N double bonds. To overcome this issue, a method for transforming the C=N double bond into a C-N single bond, using reductive amination of DNPhydrazone derivatives, has been applied. The amination reaction of GLA-DNPhydrazones with 2-picoline borane is accelerated with catalytic amounts of acid and is completed within 10 minutes in the presence of 100 mmol L^{-1} phosphoric acid. Reduction of GLA-DNPhydrazone by 2-picoline borane is unique and results in the formation of N-(2,4-dinitrophenyl)-1-piperidinamine（DNPPA）. NMR and LC-APCI-MS data confirmed the product identification. DNPPA is very stable and did not change when stored for at least four weeks at room temperature. DNPPA has excellent solubility of 14.6 g L^{-1} at 20 °C in acetonitrile. The absorption maximum wavelength and the molar absorptivity of DNPPA were 351 nm and 4.2×10^4 L mol^{-1} cm^{-1} respectively. Complete separation between the reduced forms of C1-C10 aldehyde DNPhydrazones, including DNPPA, can be achieved by operating the reversed-phase high-performance liquid chromatograph at 351 nm in gradient mode using a C18 amide column. The reductive amination method for GLA overcomes analytical errors caused by *E-E*, *E-Z* and *Z-Z* geometrical isomers.

Keywords: 2,4-dinitrophenylhydrazine, GLA-DNPhydrazone derivatives, DNPPA

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Demizu Y, Nagoya S, Doi M*1, Sato Y, Tanaka M*2, Kurihara M: Twisted structure of a cyclic hexapeptide containing a combination of alternating L-Leu-D-Leu-Aib segments.

J Org Chem. 2012;77:9361-5.

We designed and synthesized a C_2-symmetric cyclic hexapeptide, cyclo（L-Leu-D-Leu-Aib）$_2$（2）, which contains L- and D-amino acids and achiral Aib residues. The conformation of 2 was analyzed in the crystalline state and in solution, which was a unique figure-eight-shaped conformation.

Keywords: amino acid, peptide, conformation

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Demizu Y, Yabuki Y, Doi M*1, Sato Y, Tanaka M*2, Kurihara M: Conformations of helical Aib peptides containing a pair of L- and D-amino acids.

J Pept Sci. 2012;18:466-75.

A pair of L-leucine（L-Leu）and D-leucine（D-Leu）was incorporated into a-aminoisobutyric acid（Aib）peptide segments. The dominant conformations of four hexapeptides, Boc-L-Leu-Aib-Aib-Aib-Aib-L-Leu-OMe（1a）, Boc-D-Leu-Aib-Aib-Aib-Aib-L-Leu-OMe（1b）, Boc-Aib-Aib-L-Leu-L-Leu-Aib-Aib-OMe（2a）, and Boc-Aib-Aib-D-Leu-L-Leu-Aib-Aib-OMe（2b）, were investigated by IR, ^{1}H NMR, CD spectra, and X-ray crystallographic analysis. All peptides 1a,b and 2a,b formed 3_{10}-helical structures in solution. X-ray crystallographic analysis revealed that right-handed（P）3_{10}-helices were present in 1a and 1b and a mixture of right-handed（P）and left-handed（M）3_{10}-helices was present in 2b in their crystalline states.

Keywords: amino acid, peptide, helical structure

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Anan K*1, Demizu Y, Oba M*2, Kurihara M, Doi M*3, Suemune H*1, Tanaka M*2: Helical structures of bicyclic a-amino acid homo-chiral oligomers with

the chiral centers at the side-chain fused-ring junctions.

Helv Chim Acta 2012;95:1694-713.

Chiral bicyclic α-amino acid (*R,R*)-$Ab_{5,6}$=c with stereogenic centers at the γ-position of fused-ring junctions, and its enantiomer (*S,S*)-$Ab_{5,6}$=c, were synthesized. The CD spectra of (*R,R*)-$Ab_{5,6}$=c oligomers indicated that the (*R,R*)-$Ab_{5,6}$=c hexapeptide formed a mixture of right-handed (*P*)- and left-handed (*M*)-3_{10}-helices, while, in the (*R,R*)-$Ab_{5,6}$=c nonapeptide, a right-handed (*P*)-3_{10}-helix slightly dominated over the (*M*)-helix. X-Ray crystallographic analyses of (*S,S*)-tripeptide and (*R,R*)-hexapeptide revealed that both the tripeptide and hexapeptide formed a mixture of (*P*)- and (*M*)-3_{10}-helices, respectively. These results indicated that the side-chain environments around the stereogenic centers are particularly important to control the helical-screw handedness of foldamers.

Keywords: amino acid, peptide, conformation

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Kato I*1, Oba M*1, Kurihara M, Takano Y*2, Tanaka M.*1: Synthesis of cyclic α,α-disubstituted amino acid bearing a pendent chiral center.

Pept Sci 2012 2013;129-30.

4-Aminopiperidine-4-carbocylic acid (Pip) derivatives, which are cyclic a,a-disubstituted a-amino acids bearing a d-nitrogen atom, were synthesized starting from dimethyl malonate. We also synthesized heteropeptides including N-substituted Pip derivatives, and studied the preferred secondary structure.

Keywords: α,α-disubstituted a-amino acids, peptide conformation, chiral center

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Imanishi A*1, Oba M*1, Demizu Y, Kurihara M, Doi M*2, Takazaki H*3, Suemune H*3, Tanaka M*1: Synthesis of chiral five-membered ring amino acids with an azido group, and their peptides.

Pept Sci 2012 2013;131-2.

Two diastereomeric five-membered ring a,a-disubstituted a-amino acids with an azido function, {1-amino-3-azidocyclopentanecarboxylic acid; (1*R*,3*R*)- and (1*S*,3*R*)-Ac_5c^{N3}}, were designed. Starting from L-malic acid, two diastereomeric cyclic amino acids with a hydroxyl group were synthesized. After separation of two diastereomers, each stereoisomer could be converted into a five-membered ring amino acid with an azido function.

Keywords: azido function, chiral center, α,α-disubstituted a-amino acids

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Sugiyama T*1, Imamura Y*2, Demizu Y, Kurihara M, Takano M*3, Kittaka A.*3: Synthesis of β-chiral peptide nucleic acids bearing lysine side chains.

Pept Sci 2012 2013;385-6.

Synthesis of a chiral PNA monomer with a lysine side chain at the β-position of the PNA backbone and its incorporation into PNA oligomers is described.

Keywords: peptide nucleic acid, preorganization, antigene

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Fujino T*1, Takeuchi A*1, Maruko-Ohtake A*1, Ohtake Y*2, Satoh J*1, Kobayashi T*2, Tanaka T*3, Ito H*1, Sakamaki R*1, Kashimura R*1, Ando K*1, Nishimaki-Mogami T, Ohkubo Y*2, Kitamura N*3, Sato R*4, Kikugawa K*1, Hayakawa M.*1: Critical role of farnesoid X receptor for hepatocellular carcinoma cell proliferation.

J Biochem. 2012;152:577-86.

Farnesoid X receptor (FXR), a pivotal factor maintaining bile acid homeostasis, has been recently shown to be a critical factor required for liver regeneration. The elucidation of the mechanism how FXR controls the proliferation of hepatocellular carcinoma cells is useful to establish the therapy for liver cancer. Here, we show that FXR plays a crucial role in the proliferation of human hepatocellular carcinoma cell line, HepG2, Huh7 and HLE. The treatment of HepG2 with FXR siRNA elevates the level of p16/INK4a expression resulting in the inhibition of cell proliferation. By

contrast, FXR activation reduces p16/INK4a expression and stimulates the cell proliferation. The ectopic expression of the active form of Ras that causes strong activation of extracellular signal-regulated kinase (ERK) leads to the decrease in FXR expression, suggesting that FXR expression is negatively regulated via Ras/ERK pathway. The elevation of p16/INK4a expression and the inhibition of cell proliferation by FXR knockdown are also observed in Huh7 and HLE. In this study, we have suggested a novel mechanism by which hepatocellular carcinoma cell proliferation is regulated: FXR stimulates cell proliferation by suppressing the p16/INK4a expression, whereas Ras/ERK pathway down-regulates the FXR expression, leading to the suppressed cell proliferation in hepatocellular carcinoma cell lines.

Keywords: FXR, hepatocytes, p16/INK4a

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Spann NJ*1, Garmire LX*1, McDonald JG*2, Myers DS*3, Milne SB*3, Shibata N, Reichart D*1, Fox JN*1, Shaked I*4, Heudobler D*1, Raetz CR*5, Wang EW*6, Kelly SL*6, Sullards MC*6, Murphy RC*7, Merrill AH Jr*6, Brown HA*3, Dennis EA*1, Li AC*1, Ley K*4, Tsimikas S*1, Fahy E*1, Subramaniam S*1, Quehenberger O*1, Russell DW*2, Glass CK*1: Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses.

Cell 2012;151:138-52.

Inflammation and macrophage foam cells are characteristic features of atherosclerotic lesions, but the mechanisms linking cholesterol accumulation to inflammation and LXR-dependent response pathways are poorly understood. To investigate this relationship, we utilized lipidomic and transcriptomic methods to evaluate the effect of diet and LDL receptor genotype on macrophage foam cell formation within the peritoneal cavities of mice. Foam cell formation was associated with significant changes in hundreds of lipid species and unexpected suppression, rather than activation, of inflammatory gene expression. We provide evidence that regulated accumulation of desmosterol underlies many of the homeostatic responses, including activation of LXR target genes, inhibition of SREBP target genes, selective reprogramming of fatty acid metabolism, and suppression of inflammatory-response genes, observed in macrophage foam cells. These observations suggest that macrophage activation in atherosclerotic lesions results from extrinsic, proinflammatory signals generated within the artery wall that suppress homeostatic and anti-inflammatory functions of desmosterol.

Keywords: macrophage foam cells, desmosterol, inflammatory responses

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Itoh Y*, Ishikawa M*, Kitaguchi R*, Okuhira K, Naito M, Hashimoto Y*: Double protein knockdown of cIAP1 and CRABP-II using a hybrid molecule consisting of ATRA and IAPs antagonist.

Bioorg Med Chem Lett. 2012;22:4453-7.

Protein knockdown can be achieved by the use of a small molecule that possesses affinity for both the target protein and ubiquitin ligase. We have designed such a degradation-inducing molecule targeting cIAP1 and CRABP-II, which are involved in proliferation of several cancer cell lines and in neuroblastoma growth, respectively. As a CRABP-II-recognizing moiety, all-trans retinoic acid (ATRA, 3), a physiological ligand of CRABP, was chosen. As a cIAP1-recognizing moiety, MV1 (5), which is a cIAP1/cIAP2/XIAP pan-ligand, was chosen. Although cIAP1 itself possesses ubiquitin ligase activity, we expected that its decomposition would be efficiently mediated by related molecules, including cIAP2 and XIAP, which also possess ubiquitin ligase activity. The designed degradation inducer 6, in which ATRA (3) and MV1 (5) moieties are connected via a linker, was synthesized and confirmed to induce efficient degradation of both cIAP1 and CRABP-II. It showed potently inhibited the proliferation of IMR32 cells.

Keywords: protein knockdown, cIAP1, CRABP-II

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Nakamura R, Ishiwatari A, Higuchi M, Uchida Y, Nakamura R, Kawakami H[*1], Urisu A[*2], Teshima R: Evaluation of the luciferase assay-based in vitro elicitation test for serum IgE.
Allergol Int. 2012;61:431-7.

BACKGROUND: An in vitro elicitation test employing human high-affinity IgE receptor-expressing rat mast cell lines appears to be a useful method for measuring mast cell activation using a patient's IgE and an allergen; however, such cell lines are sensitive to human complements in the serum. We have recently developed a new luciferase-reporting mast cell line (RS-ATL8) to detect IgE crosslinking-induced luciferase expression (EXiLE) with relatively low quantities of serum IgE.

METHODS: A total of 30 patients suspected of having egg white (EW) allergy were subjected to an oral food challenge (OFC) test; then, the performances of EW-specific serum IgE (CAP-FEIA), EW-induced degranulation, and EXiLE responses in RS-ATL8 cells were compared using receiver-operating characteristic (ROC) curve analysis. The patients' sera were diluted to 1: 100, which causes no cytotoxicity when sensitizing the RS-ATL8 cells for the degranulation and EXiLE tests.

RESULTS: The area under the ROC curves was highest in the EXiLE test (0.977), followed by CAP-FEIA (0.926) and degranulation (0.810). At an optimal cutoff range (1.648-1.876) calculated from the ROC curve of the EXiLE test, sensitivity and specificity were 0.944 and 0.917, respectively. A 95% positive predictive value was given at a cutoff level of 2.054 (fold increase in luciferase expression) by logistic regression analysis.

CONCLUSIONS: In contrast to in vivo tests, the EXiLE test appears to be a useful tool in diagnosing patients suspected of having IgE-dependent EW allergy without the risk of severe systemic reactions.
Keywords: allergen-specific IgE, allergy diagnosis, egg white allergy

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Nakamura R, Nakamura R, Adachi R, Itagaki Y[*1], Fukutomi Y[*2], Teshima R: Evaluation of allergenicity of acid-hydrolyzed wheat protein using an in vitro elicitation test.
Int Arch Allergy Immunol. 2013;160:259-64.

BACKGROUND: We performed an in vitro elicitation test to determine the ability of different types of wheat-allergic patients' IgE to induce humanized mast cell activation after the addition of various time-treated acid-hydrolyzed wheat proteins (HWPs).

METHODS: The reactivity of heat- and various time-treated acid-hydrolyzed glutens (acid-HGs) and commercial acid-HWP (HWP1), using serum IgE from wheat allergy accompanied by skin and rhinoconjunctival sensitization to HWP1 in the facial soap, pediatric subjects with food allergy to native wheat, adult wheat-dependent exercise-induced anaphylaxis subjects, and nonatopic healthy subjects, was elucidated by dot blot and a luciferase assay-based in vitro elicitation test (EXiLE test).

RESULTS: Serum from subjects sensitized with HWP1 reacted only to acid-HGs (acid-HGs treated for 0.5-3 or 6 h), but not native gluten, in the results of the dot blot. In contrast, sera from pediatric subjects sensitized with native wheat reacted to native gluten more strongly and showed only slight reactions to 0.5- to 1-hour-treated acid-HGs. The results of the in vitro elicitation test showed that acid hydrolyzation of the gluten attenuated antigen-induced luciferase expression in a time-dependent manner for sera from native-wheat-sensitized pediatric subjects. On the other hand, in the sera from HWP1-sensitized subjects, acid hydrolyzation of the gluten for 0.5 h dramatically increased luciferase expression.

CONCLUSIONS: Even after prolonged hydrolyzation, acid-HGs still retained the ability to activate mast cells in the case of HWP1-sensitized subjects.
Keywords: Food allergy, Wheat gluten, Acid hydrolysis

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Mano J[*1], Harada M[*1], Takabatake R[*1], Furui S[*1], Kitta K[*1], Nakamura K, Akiyama H, Teshima R, Noritake H[*2], Hatano S[*3], Futo S[*3], Minegishi Y[*4], Iizuka T[*5]: Comprehensive GMO detection using real-time PCR array: single-laboratory validation.

J AOAC Int. 2012;95:508-16.

We have developed a real-time PCR array method to comprehensively detect genetically modified (GM) organisms. In the method, genomic DNA extracted from an agricultural product is analyzed using various qualitative real-time PCR assays on a 96-well PCR plate, targeting for individual GM events, recombinant DNA (r-DNA) segments, taxon-specific DNAs, and donor organisms of the respective r-DNAs. In this article, we report the single-laboratory validation of both DNA extraction methods and component PCR assays constituting the real-time PCR array. We selected some DNA extraction methods for specified plant matrixes, i.e., maize flour, soybean flour, and ground canola seeds, then evaluated the DNA quantity, DNA fragmentation, and PCR inhibition of the resultant DNA extracts. For the component PCR assays, we evaluated the specificity and LOD. All DNA extraction methods and component PCR assays satisfied the criteria set on the basis of previous reports.

Keywords: Genetically modified organisms, Real-time PCR array

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Mano J*1, Masubuchi T*1, Hatano S*2, Futo S*2, Koiwa T*3, Minegishi Y*4, Noguchi A, Kondo K, Akiyama H, Teshima R, Kurashima T*1, Takabatake R*1, Kitta K*1: Development and validation of event-specific quantitative PCR method for genetically modified maize LY038.

Shokuhin Eiseigaku Zasshi 2013;54:25-30.

In this article, we report a novel real-time PCR-based analytical method for quantitation of the GM maize event LY038. We designed LY038-specific and maize endogenous reference DNA-specific PCR amplifications. After confirming the specificity and linearity of the LY038-specific PCR amplification, we determined the conversion factor required to calculate the weight-based content of GM organism (GMO) in a multilaboratory evaluation. Finally, in order to validate the developed method, an interlaboratory collaborative trial according to the internationally harmonized guidelines was performed with blind DNA samples containing LY038 at the mixing levels of 0, 0.5, 1.0, 5.0 and 10.0%. The precision of the method was evaluated as the RSD of reproducibility (RSD_R), and the values obtained were all less than 25%. The limit of quantitation of the method was judged to be 0.5% based on the definition of ISO 24276 guideline. The results from the collaborative trial suggested that the developed quantitative method would be suitable for practical testing of LY038 maize.

Keywords: Genetically modified organism, Event-specific, LY038

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Nakamura K, Akiyama H, Takahashi Y, Kobayashi T, Noguchi A, Ohmori K*1, Kasahara M*2, Kitta K*3, Nakazawa H*4, Kondo K, Teshima R: Application of a qualitative and quantitative real-time polymerase chain reaction method for detecting genetically modified papaya line 55-1 in papaya products.

Food Chem. 2013;136:895-901.

Genetically modified (GM) papaya (*Carica papaya* L.) line 55-1 (55-1), which is resistant to papaya ringspot virus infection, has been marketed internationally. Many countries have mandatory labeling regulations for GM foods, and there is a need for specific methods for detecting 55-1. Here, an event- and construct-specific real-time polymerase chain reaction (PCR) method was developed for detecting 55-1 in papaya products. Quantitative detection was possible for fresh papaya fruit up to dilutions of 0.001% and 0.01% (weight per weight [w/w]) for homozygous SunUp and heterozygous Rainbow cultivars, respectively, in non-GM papaya. The limit of detection and quantification was as low as 250 copies of the haploid genome according to a standard reference plasmid. The method was applicable to qualitative detection of 55-1 in eight types of processed products (canned papaya, pickled papaya, dried fruit, papaya-leaf tea, jam, puree, juice, and frozen dessert) containing papaya as a main

ingredient.

Keywords: Genetically modified papaya, Line 55-1, Detection method

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Adachi R, Nakamura R, Sakai S, Fukutomi Y*, Teshima R: Sensitization to acid-hydrolyzed wheat protein by transdermal administration to BALB/c mice, and comparison with gluten.

Allergy 2012;67:1392-9.

BACKGROUND: An increasing number of studies have shown that hydrolyzed wheat protein (HWP) can induce IgE-mediated hypersensitivity by skin contact and/or food ingestion. However, there has been no study of the sensitizing potential of HWP. In this study, the possibility of transdermal pathway for sensitization to acid-HWP (HWP1) was investigated using BALB/c mice, and compared with that of gluten.

METHODS: HWP1 or gluten (500 μg/mouse) was transdermally administered using patches. After three or four cycles of sensitization for 3 days/week, active systemic anaphylaxis (ASA) was induced by intraperitoneal injection of the antigen, and rectal temperatures, scores of anaphylactic responses, and plasma histamine levels were determined. Because HWP1 was included in facial soap in Japan, the effect of detergent on the sensitizing potential was also investigated.

RESULTS: Transdermal administration of HWP1 induced dose-dependent production of IgE and IgG1. After sensitization for 3 or 4 weeks, intraperitoneal injection of HWP1 caused ASA, leading to decreased rectal temperatures, increased anaphylaxis scores, and increased plasma histamine levels. In addition, splenocytes harvested after ASA produced IL-4, IL-5, and IL-10 by re-stimulation with HWP1. Transdermal exposure to gluten also induced IgE and IgG1 production, and intraperitoneal injection of gluten also induced ASA only in mice sensitized in the presence of sodium dodecyl sulfate.

CONCLUSIONS: Transdermal exposure to HWP1 is sufficient to activate key immune pathways necessary for sensitizing mice for immediate hypersensitivity reactions. This study shows that HWP has a sensitizing potential as well as gluten, whereas its allergenicity may be different from that of gluten.

Keywords: Acid-hydrolyzed wheat protein, Active systemic anaphylaxis, Food allergy

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登田美桜，畝山智香子，豊福肇*，森川馨：わが国における自然毒による食中毒事例の傾向（平成元年～22年）．

食品衛生学雑誌 2012;53:105-20.

厚生労働省監修の全国食中毒事件録（平成元年～平成22年版）の自然毒食中毒事例をもとに，わが国における中毒発生の傾向を検討した．平成元年以降の22年間を通じて自然毒食中毒の発生件数に経年的な減少傾向は見られず，発生を低減するために予防のための継続的な取り組みが必要であると考えられた．動物性及び植物性いずれの自然毒においても主な原因施設は「家庭」であり，食中毒の発生状況及び予防策，対応等について消費者向けの広い啓蒙・広報が重要である．また，食品の国際的な流通拡大や地球温暖化による海水温の上昇に伴い，これまで国内で食中毒が発生していない自然毒への対策も重要である．

Keywords：自然毒，食中毒，食品衛生

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Narumi K*1,2, Ashizawa K*3, Takashima R*1, Takasawa H*1, Katayama S*1, Tsuzuki Y*3, Tatemoto H*4, Morita T, Hayashi M*5, Hamada S*1: Development of a repeated-dose liver micronucleus assay using adult rats: An investigation of diethylnitrosamine and 2,4-diaminotoluene.

Mutat Res. 2012;747:234-9.

Repeated-dose liver micronucleus assay using adult rats were established by the investigation of diethylnitrosamine and 2,4-diaminotoluene. A new method to isolate hepatocytes without perfusion using only a part of the liver was also established which enables the integration of liver micronucleus assays into general toxicity studies.

Keywords: liver micronucleus assay, adult rat, repeated-dose toxicity study

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Takasawa H*1, Takashima R*1, Hattori A*1, Narumi K*1,2, Kawasako K*1, Morita T, Hayashi M*3, Hamada S*1: Development of a repeated-dose liver micronucleus assay using adult rats (II): Further investigation of 1,2-dimethylhydrazine and 2,6-diaminotoluene.
Mutat Res. 2013;751:12-8.

To further evaluate liver responses to hepatocarcinogens and noncarcinogens, the results of the liver micronucleus assay using adult rats and histopathology were compared between three hepatocarcinogens and a noncarcinogen. The results indicate that the liver micronucleus assay is effective for predicting hepatocarcinogenicity and may be integrated into repeateddose toxicity studies without disturbing routine examinations, such as histopathology.

Keywords: liver micronucleus assay, repeated-dose toxicity study, hepatocarcinogen

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Tohkin M, Kaniwa N, Saito Y, Sugiyama E, Kurose K, Nishikawa J, Hasegawa R, Aihara M*1, Matsunaga K*2, Abe M*2, Furuya H*3, Takahashi Y*4, Ikeda H*4, Muramatsu M*5, Ueta M*6, Sotozono C*6, Kinoshita S*6, Ikezawa Z*1: the Japan Pharmacogenomics Data Science Consortium: A whole-genome association study of major determinants for allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese patients.
Pharmacogenomics J. 2013;13:60-9.

Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are severe, cutaneous adverse drug reactions that are rare but life threatening. Genetic biomarkers for allopurinol-related SJS/TEN in Japanese were examined in a genome-wide association study in which Japanese patients (n=14) were compared with ethnically matched healthy controls (n=991). Associations between 890 321 single nucleotide polymorphisms and allopurinol-related SJS/TEN were analyzed by the Fisher's exact test (dominant genotype mode). A total of 21 polymorphisms on chromosome 6 were significantly associated with allopurinol-related SJS/TEN. The strongest association was found at rs2734583 in BAT1, rs3094011 in HCP5 and GA005234 in MICC ($P=2.44\times10^{-8}$; odds ratio=66.8; 95% confidence interval, 19.8-225.0). rs9263726 in PSORS1C1, also significantly associated with allopurinol-related SJS/TEN, is in absolute linkage disequilibrium with human leukocyte antigen-B*5801, which is in strong association with allopurinol-induced SJS/TEN. The ease of typing rs9263726 makes it a useful biomarker for allopurinol-related SJS/TEN in Japanese.

Keywords: *HLA-B*5801*, genetic biomarker, whole-genome association study

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Knights J*1, Chanda P*2, Sato Y*3, Kaniwa N, Saito Y, Ueno H*4, Zhang A*1, Ramanathan M*1: Vertical Integration of Pharmacogenetics in Population PK/PD Modeling: A Novel Information Theoretic Method.
CPT: Pharmacometr Systems Pharmacol. 2013;2:e25.

To critically evaluate an information-theoretic method for identifying gene-environmental interactions (GEI) associated with pharmacokinetic (PK), pharmacodynamic (PD), and clinical outcomes from genome-wide pharmacogenetic data. Our approach, which is built on the K-way interaction information (KWII) metric, was challenged with simulated data and clinical PK/PD data sets from the International Warfarin Pharmacogenetics Consortium (IWPC) and a gemcitabine clinical trial. The KWII efficiently identified both novel and known interactions for warfarin and gemcitabine. Interactions between herbal supplementation and VKORC1 genotype were associated with warfarin response. For gemcitabine-associated neutropenia, combination treatment with carboplatin and cytidine deaminase (CDA) 208G→A genotypes were identified as risk factors. Gemcitabine disposition was associated with drug metabolism-transporter interactions be-

tween deoxycytidine kinase (DCK) and the equilibrative nucleoside transporter (ENT). This novel approach is effective for detecting GEI involved in drug exposure and response and could enable integration of genome-wide pharmacogenetic data into the population PK/PD analysis paradigm.

Keywords: gene-environmental interactions, warfarin, gemcitabine

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Azuma Y, Hata K*1, Sai K, Udagawa R*1, Hirakawa A*2, Tohkin M, Ryushima Y*1, Makino Y*1, Yokote N*1, Morikawa N*3, Fujiwara Y*1, Saito Y, Yamamoto H*1: Association between Hand-Foot Syndrome and Efficacy of Capecitabine in Patients with Metastatic Breast Cancer.

Biol Pharm Bull. 2012;35:717-24.

Capecitabine, an oral prodrug of 5-fluorouracil (5-FU), is a promising treatment for colorectal, breast and gastric cancers, but often causes hand-foot syndrome (HFS), the most common dose-limiting toxicity. The current study was conducted to investigate the relationship between HFS and efficacy of capecitabine in 98 patients with metastatic breast cancer. Possible associations between HFS and efficacy endpoints, including time-to-treatment failure (TTF), tumor response in metastatic lesions and changes in tumor markers, were investigated retrospectively using electronic medical records. The TTF of group with HFS of grade 1 and ≥2 was significantly longer than that of group with no HFS, respectively (hazard ratio (HR), 0.39; 95% confidence interval (CI), 0.18-0.87 for group with grade 1; HR, 0.42, 95% CI, 0.19-0.90 for group with grade ≥2). Significantly higher disease control rates for the liver metastasis were observed in patients with HFS (grade 1 and greater) than in those without HFS (92.9 vs. 42.9%, p=0.009). Furthermore, prevention of increases in tumor marker levels (carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA15-3) and National Cancer Center-Stomach-439 (NCC-ST439)) was evident in patients with HFS. This study clearly showed a significant correlation between HFS and some efficacy markers of capecitabine therapy in patients with metastatic breast cancer, and suggests that early dose adjustment based on severity of HFS might improve efficacy. Studies are needed to explore predictive biomarkers for HFS/efficacy, so that capecitabine therapy can be further tailored to patient response.

Keywords: capecitabine, hand-foot syndrome, electronic medical record

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Saito M*1, Yoshida LS*2, Hayashi Y*1, Sai K, Takano-Omuro H*2, Yajima T*3, Sawada Y*4, Hasegawa R: Perception of physicians, pharmacists and pharmaceutical industries about information in package inserts in Japan.

Jpn J Drug Inform. 2012;14:2-13.

A perception survey of healthcare providers and pharmaceutical industries about the current package insert (PI) was conducted to evaluate whether its layout and issues such as the contents concerning drug-drug interactions are found appropriate. A questionnaire was sent via the Internet to physicians of various subspecialties, or via the postal service to pharmacy-employed pharmacists and pharmaceutical industries. It consisted of questions regarding the PI layout, the information contents on drug-drug interactions and other matters about PI revision. The survey showed that the PI is a major source of drug information for physicians (82.4%) and pharmacists (98.7%). The layout (order of appearance of headings and information about drug interactions in a tabular format) of the current PI is widely accepted by physicians, pharmacists, and pharmaceutical industries. There was, however, some degree of disagreement within these three groups in the perceptions about the presentation/contents of drug interactions is insufficient, and that information about adverse drug reactions and drug interactions is not enough updated in the PIs. Differences of perception were found between healthcare providers (i.e., PI users) and industries. Our survey revealed that the basic layout of the current PI should be preserved, but there are issues such as the contents and updating of information regarding drug interactions and adverse drug interactions that may require modi-

fications according to the healthcare providers' point of view.

Keywords: questionnaire survey, package insert, drug interaction

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Saito K, Moore R*, Negishi M*: Nuclear receptor CAR specifically activates the two-pore K+ channel Kcnk1 gene in male mouse livers, which attenuates phenobarbital-induced hepatic hyperplasia.

Toxicol Sci. 2013;132:151-61.

KCNK1, a member of the family of two-pore K(+) ion channels, is specifically induced in the livers of male mice after phenobarbital treatment. Here, we have determined the molecular mechanism of this male-specific activation of the Kcnk1 gene and characterized KCNK1 as a phenobarbital-inducible antihyperplasia factor. Upon activation by phenobarbital, nuclear receptor CAR binds the 97-bp response element (-2441/-2345) within the Kcnk1 promoter. This binding is observed in the livers of male mice, but not in the livers of female mice and requires the pituitary gland, because hypophysectomy abrogates it. Hyperplasia further progressed in the livers of Kcnk1 (-/-) male mice compared with those of Kcnk1 (+/+) males after phenobarbital treatment. Thus, KCNK1 suppresses phenobarbital-induced hyperplasia. These results indicate that phenobarbital treatment induces KCNK1 to elicit a male-specific and growth-suppressing signal. Thus, KCNK1 and Kcnk1 (-/-) mice provide an experimental tool for further investigation into the molecular mechanism of CAR-mediated promotion of the development of hepatocellular carcinoma in mice.

Keywords: CAR, K+ channel, hepatic hyperplasia

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Maekawa K, Nishikawa J, Kaniwa N, Sugiyama E, Koizumi T, Kurose K, Tohkin M*, Saito Y: Development of a rapid and inexpensive assay for detecting a surrogate genetic polymorphism of *HLA-B*58:01*: a partially predictive but useful biomarker for allopurinol-related Stevens-Johnson syndrome/toxic epidermal necrolysis in Japanese.

Drug Metab Pharmacokinet. 2012;27:447-50.

Allopurinol-induced Stevens-Johnson syndrome (SJS) /toxic epidermal necrolysis (TEN) is strongly associated with *HLA-B*58:01* in various populations including Japanese. We demonstrated that several single nucleotide polymorphisms (SNPs) around the HLA region on chromosome 6 were strongly linked with *HLA-B*58:01* in a previous study using Japanese allopurinol-related SJS/TEN patients. Their very strong linkage suggests that these SNPs could be used as surrogate biomarkers to find carriers of *HLA-B*58:01* to avoid these serious adverse effects. In the present study, to expedite the application of this pharmacogenomic information to the proper usage of allopurinol in a clinical situation, we developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for the genotyping of rs9263726 in the *psoriasis susceptibility 1 candidate 1* (*PSORS1C1*) gene, which is in absolute linkage disequilibrium (r^2 = 1, D' = 1) with *HLA-B*58:01*. The developed PCR-RFLP assay using FokI restriction enzyme was able to detect three different genotypes, GG, GA, and AA of rs9263726 robustly, and thus to find *HLA-B*58:01* carriers. This robust and inexpensive assay would be useful for pre-screening the subjects with *HLA-B*58:01*, a genetically high risk factor for allopurinol-induced SJS/TEN.

Keywords: allopurinol, PCR-RFLP, *HLA-B*58:01*

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Kurose K, Koizumi T, Nishikawa J, Maekawa K, Saito Y: Quality requirements for genomic DNA preparations and storage conditions for a high-density oligonucleotide microarray.

Biol Pharm Bull. 2012;35:1846-8.

High-density oligonucleotide microarrays are widely used in genome-wide association studies. The purpose of this study was to assess the influence of various factors during the preparation of DNA on genotype calling for the Affymetrix high-density oligonucleotide microarray 250K GeneChip. DNA was extracted from peripheral whole blood by solution-based and silica-membrane-based methods. Blood was stored at 4°C or 25°C for 4 or 24 h, followed by DNA extraction. To examine the effects of freeze-thaw cycles, blood and DNA

were also subjected to 5 and 10 or 20 of freeze-thaw cycles, respectively. The suitability of variously DNA preparations for the array was assessed by the call rate resulting from genotyping. All DNA samples showed mean call rates of more than 0.99, which passed the quality criteria for genotyping (greater than 0.95). The results indicated that the solution-based method and the silica-membrane-based DNA extraction method could provide DNA of sufficient quality for genotyping. In addition, DNA quality suitable for high-density oligonucleotide microarrays is not strongly dependent on the preparation conditions under standard procedures.

Keywords: DNA microarray, DNA preparation, storage condition

Kurose K, Hiratsuka K[*1], Ishiwata K[*1], Nishikawa J, Nonen S[*2], Azuma J[*2], Kato M[*3], Wakeno M[*3], Okugawa G[*3], Kinoshita T[*3], Kurosawa T[*1], Hasegawa R, Saito Y: Genome-wide association study of SSRI/SNRI-induced sexual dysfunction in a Japanese cohort with major depression.

Psychiatry Res. 2012;198:424-9.

Sexual dysfunction is a major side effect of selective serotonin reuptake inhibitors (SSRIs) and serotonin-noradrenaline reuptake inhibitors (SNRIs). We conducted a genome-wide association study to identify the genetic factors contributing to the risk of SSRI/SNRI-induced sexual dysfunction by testing 186 320 single nucleotide polymorphism (SNP) markers in a cohort of 201 Japanese major depression patients including 36 with sexual dysfunction induced by SSRI (paroxetine or fluvoxamine) or SNRI (milnacipran). The Cochran-Armitage trend test showed that 11 SNPs, tightly clustered in a distinct region on chromosome 14q21.3, were associated with SSRI/SNRI-induced sexual dysfunction at a genome-wide significance level after false discovery rate (FDR) correction, and the strongest SNP association was with rs1160351 ($P=3.04\times10(-7)$, risk ratio=2.92, 95% confidence interval (CI)=1.79-4.76). These SNPs mapped to the intronic region of the MDGA2 gene. A Manhattan plot showed that the strong association peak remained in MDGA2 after adjustment for sex and age in a multivariable logistic regression analysis although P values increased slightly and became non-significant. Replication studies with larger sample sizes are required to validate this exploratory study, but our findings may provide insights into the genetic basis of sexual dysfunction induced by SSRI/SNRI.

Keywords: GWAS, adverse drug reaction, antidepressant

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Duan H[*1], Yoshimura K[*1], Kobayashi N[*1], Sugiyama K[*1], Sawada J, Saito Y, Morisseau C[*2], Hammock BD[*2], Akatsuka T[*1]: Development of monoclonal antibodies to human microsomal epoxide hydrolase and analysis of "preneoplastic antigen"-like molecules.

Toxicol Appl Pharmacol. 2012;260:17-26.

Microsomal epoxide hydrolase (mEH) is a drug metabolizing enzyme which resides on the endoplasmic reticulum (ER) membrane and catalyzes the hydration of reactive epoxide intermediates that are formed by cytochrome P450s. mEH is also thought to have a role in bile acid transport on the plasma membrane of hepatocytes. It is speculated that efficient execution of such multiple functions is secured by its orientation and association with cytochrome P450 enzymes on the ER membrane and formation of a multiple transport system on the plasma membrane. In certain disease status, mEH loses its association with the membrane and can be detected as distinct antigens in the cytosol of preneoplastic foci of liver (preneoplastic antigen), in the serum in association with hepatitis C virus infection (AN antigen), or in some brain tumors. To analyze the antigenic structures of mEH in physiological and pathological conditions, we developed monoclonal antibodies against different portions of mEH. Five different kinds of antibodies were obtained: three, anti-N-terminal portions; one anti-C-terminal; and one, anti-conformational epitope. By combining these antibodies, we developed antigen detection methods which are specific to either the membrane-bound form or the linearized form of mEH. These methods detected mEH in the culture medium released from a hepatocellular carcinoma cell line and a glioblastoma cell line, which was found to be a multimolecular complex with a unique antigenic structure different from that of the membrane-bound form of mEH. These antibodies and antigen detection methods may be useful to study patho-

logical changes of mEH in various human diseases.
Keywords: hepatitis C virus, microsomal epoxide hydrolase, monoclonal antibody

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門脇京子*，石黒昭博*，高松昭司*，斎藤嘉朗，宇山佳明*：本邦の医薬品添付文書におけるゲノム薬理学関連情報およびその検査法の状況に関する調査・解析.
レギュラトリーサイエンス学会誌 2012;2:83-92.

【目的】本邦におけるゲノム薬理学（以下，「PGx」）関連情報の利用動向を把握するため，医療用医薬品の添付文書におけるPGx関連情報の収載状況を調査した．【方法】2002～2010年度に部会審議された医療用医薬品（441品目）について，PGx関連情報収載品目を特定した（56品目）．PGx関連情報の分類（ウイルス・細菌，代謝酵素，薬理学的標的，その他），検査必要性による分類（要解析，解析推奨，情報提供），バイオマーカーの用法による分類（有効性，安全性，ADME）および検査の薬事承認，保険収載状況の調査を行った．【結果・考察】医薬品添付文書におけるPGx関連情報を活用した情報提供は，年々品目数が増加し，2010年度には全441品目中12.7％にあたる56品目となっていた．PGx関連情報を評価対象に基づき4種に分類したところ，ウイルス・細菌が41％，代謝酵素が34％，薬理学的標的が14％，その他が11％であった．検査必要性による分類では，「要解析」はウイルス・細菌および薬理学的標的に比較的限られており，代謝酵素に関しては「情報提供」が主であった．一方で，PGx関連情報の診断に関しては，薬事法で承認されかつ保険収載されている検査方法は，約半数であった．また，用法による分類では，有効性に関する記載を含む品目が多く，安全性に関するPGx関連情報はごく少数にとどまっていた．今後は，医薬品の適正使用を推進するために，有効性だけではなく安全性に関するPGx関連情報をより多く添付文書に記載していくことが必要と考えられた．また，臨床現場においてPGx情報を広く活用していくためには，薬事法に基づき信頼性を確認し，かつ経済的な遺伝子検査方法をより多く提供していくことが課題であると考えられた．
Keywords: pharmacogenomics, drug package inserts, in vitro diagnostics

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Makino Y*1, Takahashi Y, Tanabe R*2, Tamamura Y*1, Watanabe T*1, Haraikawa M*2, Hamagaki M*1, Hata K*3, Kanno J, Yoneda T*3, Saga Y*4, Gosekl-Sone M*2, Kaneko K*5, Yamaguchi A*1, Iimura T*1: Spatiotemporal disorder in the axial skeleton development of the *Mesp2*-null mouse: A model of spondylocostal dysostosis and spondylothoracic dysostosis.
Bone 2013;53:248-58.

Spondylocostal dysostosis (SCDO) is a genetic disorder characterized by severe malformation of the axial skeleton. *Mesp2* encodes a basic helix-loop-helix transcription factor that is required for somite formation. Its human homologue, *Mesp2*, is a gene affected in patients with SCDO and a related vertebral disorder, spondylothoracic dysostosis (STDO). This work investigated how the loss of *Mesp2* affects axial skeleton development and causes the clinical features of SCDO and STDO. The current observations provide further insight into the pathogenesis of SCDO and STDO, and the physiological development of the axial skeleton.
Keywords: Spondylocostal dysostosis, Spondylothoracic dysostosis, Skeletal development

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Tsuboi I*, Harada T*, Hirabayashi Y, Kanno J, Inoue T, Aizawa S*: Age-related decline of mast cell regeneration in senescence-accelerated mice (SAMP1) after chemical myeloablation due to senescent stromal cell impairment.
Exp Biol Med (*Maywood*). 2012;237:1289-97

An age-related decline in immune functions is referred to as immunosenescence. Mast cells play an important role in the immune system. However, it has not yet been determined if aging may affect mast-cell development. In the present study, we examined the age-related change in mast-cell development after myeloablation with 5-fluorouracil (5-FU) in senescence accelerated mice (SAMP1), which exhibit senescence-mimicking stromal cell impairment after 30 weeks of age. We found that aged mice with stromal cell impairment (30-36 weeks old) showed a lower recovery of the number of femoral mast-cell progenitors (colony-forming unit [CFU]-mast) (64% of steady state), whereas young mice (8-12 weeks old) showed a higher

recovery (122% of steady state). Stromal cells influence mast-cell development by producing positive regulators such as stem cell factor (SCF) and negative regulators such as transforming growth factor-beta (TGF-beta). The ratio of the gene expression of SCF to that of TGF-beta (SCF/TGF-beta ratio) indicates the balance of positive and negative regulation of mast-cell development. SCF/TGF-beta ratio increased in both the young and aged mice after 5-FU treatment. However, the SCF/TGF-beta ratio rapidly decreased in aged mice, whereas it remained high in young mice. The number of femoral CFU-mast in the S-phase after 5-FU treatment reflects the activation of positive-dominant regulation for mast-cell development by stromal cells. Aged mice showed lower recovery of the number of femoral CFU-mast in the S-phase (47% of steady state), whereas young mice showed a higher recovery (205% of steady state). These results suggest that mast-cell development declines with aging due to stromal-cell functional impairment, which contributes to immunosenescence.

Keywords: aging, mast cells, 5-fluorouracil

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Abe S*, Kurata M*, Suzuki S*, Yamamoto K*, Aisaki K, Kanno J, Kitagawa M*: Minichromosome maintenance 2 bound with retroviral Gp70 is localized to cytoplasm and enhances DNA-damage-induced apoptosis.

PLoS One 2012;7:e40129.

The interaction of viral proteins with host-cellular proteins elicits the activation of cellular signal transduction pathways and possibly leads to viral pathogenesis as well as cellular biological events. Apoptotic signals induced by DNA-damage are remarkably up-regulated by Friend leukemia virus (FLV) exclusively in C3H hosts; however, the mechanisms underlying the apoptosis enhancement and host-specificity are unknown. Here, we show that C3H mouse-derived hematopoietic cells originally express higher levels of the minichromosome maintenance (MCM) 2 protein than BALB/c- or C57BL/6-deriverd cells, and undergo more frequent apoptosis following doxorubicin-induced DNA-damage in the presence of the FLV envelope protein gp70. Dual transfection with gp70/Mcm2 reproduced doxorubicin-induced apoptosis even in BALB/c-derived 3T3 cells. Immunoprecipitation assays using various deletion mutants of MCM2 revealed that gp70 bound to the nuclear localization signal (NLS) 1 (amino acids 18-24) of MCM2, interfered with the function of NLS2 (amino acids 132-152), and suppressed the normal nuclear-import of MCM2. Cytoplasmic MCM2 reduced the activity of protein phosphatase 2A (PP2A) leading to the subsequent hyperphosphorylation of DNA-dependent protein kinase (DNA-PK). Phosphorylated DNA-PK exhibited elevated kinase activity to phosphorylate P53, thereby up-regulating p53-dependent apoptosis. An apoptosis-enhancing domain was identified in the C-terminal portion (amino acids 703-904) of MCM2. Furthermore, simultaneous treatment with FLV and doxorubicin extended the survival of SCID mice bearing 8047 leukemia cells expressing high levels of MCM2. Thus, depending on its subcellular localization, MCM2 plays different roles. It participates in DNA replication in the nucleus as shown previously, and enhances apoptosis in the cytoplasm.

Keywords: Friend leukemia virus, DNA-damage, Apoptosis, MCM2

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Igarashi K, Kitajima S, Aisaki K, Tanemura K, Taquahashi Y, Moriyama N, Ikeno E, Matsuda N, Saga Y*[1,2], Blumberg B*[3], Kanno J: Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence.

J Toxicol Sci. 2012;37:373-80.

The human steroid and xenobiotic receptor (SXR), (also known as pregnane X receptor PXR, and NR1I2) is a low affinity sensor that responds to a variety of endobiotic, nutritional and xenobiotic ligands. SXR activates transcription of Cytochrome P450, family 3, subfamily A (CYP3A) and other important metabolic enzymes to up-regulate catabolic pathways mediating xenobiotic elimination. One key feature that demarcates SXR from other nuclear receptors is that the human and rodent orthologues exhibit different ligand preference for a subset of toxicologically important chemicals. This difference leads to a profound problem for rodent studies to predict toxicity in humans. The objective of this study is to generate a new humanized mouse line, which responds systemically to human-spe-

cific ligands in order to better predict systemic toxicity in humans. For this purpose, the ligand binding domain（LBD）of the human SXR was homologously knocked-in to the murine gene replacing the endogenous LBD. The LBD-humanized chimeric gene was expressed in all ten organs examined, including liver, small intestine, stomach, kidney and lung in a pattern similar to the endogenous gene expressed in the wild-type（WT）mouse. Quantitative reverse transcription-polymerase chain reaction（RT-PCR）analysis showed that the human-selective ligand, rifampicin induced Cyp3a11 and Carboxylesterase 6（Ces6）mRNA expression in liver and intestine, whereas the murine-selective ligand, pregnenolone-16-carbonitrile did not. This new humanized mouse line should provide a useful tool for assessing whole body toxicity, whether acute, chronic or developmental, induced by human selective ligands themselves and subsequently generated metabolites that can trigger further toxic responses mediated secondarily by other receptors distributed body-wide.

Keywords: human steroid and xenobiotic receptor（SXR）, knock-in, humanized mouse

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Okubo Y, Sugawara T*, Abe-Koduka N*, Kanno J, Kimura A*, Saga Y*: Lfng regulates the synchronized oscillation of the mouse segmentation clock via trans-repression of Notch signalling.

Nature Communications 2012;3:1141.

The synchronized oscillation of segmentation clock is required to generate a sharp somite boundary during somitogenesis. However, the molecular mechanism underlying this synchronization in the mouse embryos is not clarified yet. We used both experimental and theoretical approaches to address this key question. Here we show, using chimeric embryos composed of wild-type cells and Delta like 1（Dll1）-null cells, that Dll1-mediated Notch signalling is responsible for the synchronization mechanism. By analysing Lunatic fringe（Lfng）chimeric embryos and Notch signal reporter assays using a co-culture system, we further find that Lfng represses Notch activity in neighbouring cells by modulating Dll1 function. Finally, numerical simulations confirm that the repressive effect of Lfng against Notch activities in neighbouring cells can sufficiently explain the synchronization in vivo. Collectively, we provide a new model in which Lfng has a crucial role in intercellular coupling of the segmentation clock through a trans-repression mechanism.

Keywords: Notch signal, Somitogenesis, Lfng

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Fujimoto N*, Takagi A, Kanno J: Neonatal exposure to 2,3,7,8-tetrachlorodibenze-p-dioxin increases the mRNA expression of prostatic proteins in C57BL mice.

J Toxicol Sci. 2013;38:279-83.

The effects of neonatal exposure to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin（TCDD）on prostatic secretory protein expression were investigated. Male C57BL mice were treated with TCDD at 10, 100, or 1,000 ng/kg body weight at postnatal day（PND）6. At PND42, the ventral, dorsolateral, and anterior prostatic lobes were dissected and the mRNA expression of prostatic proteins including spermine-binding protein, serine protease inhibitor Kazal type 3, prostate secretory protein 94（PSP94）, immunoglobulin binding protein-like protein（IgGBPLP）, experimental autoimmune prostatitis antigen proteins, and peroxiredoxin-6（Prdx6）was measured by quantitative PCR. There was no significant difference in the weight of the prostatic lobes between the control and TCDD-treated groups. The expression of PSP94 and Prdx6 in the ventral prostate and IgGBPLP in the dorsolateral prostate at PND42 was significantly increased by neonatal TCDD treatment in a dose-dependent manner, while no changes were noted in other prostatic secretions. These data suggest that neonatal exposure to TCDD may have effects on the neonatal differentiation of the prostate and results in the hyper-expression of some prostatic proteins later in life.

Keywords: 2,3,7,8-Tetrachlorodibenzo-p-dioxin（TCDD）, Prostatic secretion, Neonatal effects

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Ohta R*, Takagi A, Ohmukai H*, Marumo H*, Ono A, Matsushima Y, Inoue T, Ono H, Kanno J: Ovariectomized mouse uterotrophic assay of 36 chemicals.

J Toxicol Sci. 2012;37:879-89.

The concern over endocrine disruptors prompted international establishment of a strategic framework for the identification of the estrogenic compounds. OECD has launched the Conceptual Framework tool box containing various screening and testing methods including the uterotrophic assay. The (anti)estrogenicity of 36 chemicals suspected to be estrogen-receptor interactive by in silico and/or in vitro screening in the Extended Scheme for Endocrine Disruptor Screening and Testing of the Ministry of Health, Labour and Welfare, Japan, were monitored by the uterotrophic assay using C57BL/6J ovariectomized adult female mice after a 7-day exposure by oral gavage (po) and subcutaneous injection (sc). Ethynyl estradiol was used as reference for agonist and antagonist detection. In addition, Bisphenol A (sc) and Genistein (po) were tested for the comparison to rat assays. Among the 36, 2- [Bis(4-hydroxy-phenyl)methyl] benzylalcohol, 2,2',4,4'-Tetrahydroxybenzophenone, 2,4-Dihydroxybenzophenone, 3,3',5-Triiodothyroacetic acid, New fuchsin and alpha-Naphtholbenzein, showed both estrogenic agonistic and antagonistic activities; first two showed U-shaped dose-response in antagonistic studies. N,N-Diphenyl-p-phenylenediamine, 2,2'-Dihydroxy-4,4'-dimethoxybenzophenone, n-Butyl 4-hydroxybenzoate, and Reserpine were agonistic by sc. Benzo [a] pyrene, Benz [a] anthracene, Dibenz [a,h] anthracene, 2-(2H-Benzotriazol-2-yl)-4,6-di(t-pentyl) phenol, Rosemarinic acid, meta-Thymol, 6-Gingerol, Colchicine, Malachite green base, Fenbuconazole, and Lead acetate were antagonistic. The rest, i.e. n-Heptyl 4-hydroxybenzoate, Tetrazolium violet, Pravastatin sodium salt, Physostigmine, salicylate (1:1), Nordihydroguaiaretic acid, o-Cresolphthalein, 1,3-Dinitrobenzene, C.I. Pigment orange, Tetrabromobis-phenol-A, 2-Hydroxy-4-methoxybenzophenone, Ethylparaben, Propyl p-hydroxybenzoate, Kaempferol, 2-(2-Benzotriazolyl) -p-cresol and Phenolphthalein were negative for both effects. Taking together with in silico/in vitro screening, the result suggested that the ovariectomized mouse uterotrophic bioassay has sufficient performance comparable to rat for the screening of (anti)estrogenicity of various chemicals.

Keywords: Mouse, Uterotrophic assay, Endocrine disruptors

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Takagi A, Hirose A, Futakuchi M*, Tsuda H*, Kanno J: Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice.

Cancer Sci. 2012;103:1440-4.

Among various types of multi-wall carbon nanotubes (MWCNT) are those containing fibrous particles longer than 5 μm with an aspect ratio of more than three (i.e. dimensions similar to mesotheliomagenic asbestos). A previous study showed that micrometer-sized MWCNT (μm-MWCNT) administered intraperitoneally at a dose of 3000 μg/mouse corresponding to 1×10^9 fibers per mouse induced mesotheliomas in p53 heterozygous mice. Here, we report a dose-response study; three groups of p53 heterozygous mice (n = 20) were given a single intraperitoneal injection of 300 μg/mouse of μm-MWCNT (corresponding to 1×10^8 fibers), 30 μg/mouse (1×10^7) or 3 μg/mouse (1×10^6), respectively, and observed for up to 1 year. The cumulative incidence of mesotheliomas was 19/20, 17/20 and 5/20, respectively. The severity of peritoneal adhesion and granuloma formation were dose-dependent and minimal in the lowest dose group. However, the time of tumor onset was apparently independent of the dose. All mice in the lowest dose group that survived until the terminal kill had microscopic atypical mesothelial hyperplasia considered as a precursor lesion of mesothelioma. Right beneath was a mononuclear cell accumulation consisting of CD45- or CD3-positive lymphocytes and CD45/CD3-negative F4/80 faintly positive macrophages; some of the macrophages contained singular MWCNT in their cytoplasm. The lesions were devoid of epithelioid cell granuloma and fibrosis. These findings were in favor of the widely proposed mode of action of fiber carcinogenesis, that is, frustrated phagocytosis where the mesotheliomagenic microenvironment on the peritoneal surface is neither qualitatively altered by the density of the fibers per area nor by the formation of granulomas against agglomerates.

Keywords: Multi-wall carbon nanotube, mesothelioma, p53 heterozygous mice

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Yanagihara K[*2], Fukamachi K[*1], Suzui M[*1], Kanno J, Hirose A, Ogata A[*3], Sakamoto Y[*3], Nakae D[*3], Omori T[*1], Tsuda H[*1]: Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats.

Cancer Sci. 2012;103:2045-50.

Multi-walled carbon nanotubes have a fibrous structure similar to asbestos and induce mesothelioma when injected into the peritoneal cavity. In the present study, we investigated whether carbon nanotubes administered into the lung through the trachea induce mesothelial lesions. Male F344 rats were treated with 0.5 mL of 500 μg/mL suspensions of multi-walled carbon nanotubes or crocidolite five times over a 9-day period by intrapulmonary spraying. Pleural cavity lavage fluid, lung and chest wall were then collected. Multi-walled carbon nanotubes and crocidolite were found mainly in alveolar macrophages and mediastinal lymph nodes. Importantly, the fibers were also found in the cell pellets of the pleural cavity lavage, mostly in macrophages. Both multi-walled carbon nanotube and crocidolite treatment induced hyperplastic proliferative lesions of the visceral mesothelium, with their proliferating cell nuclear antigen indices approximately 10-fold that of the vehicle control. The hyperplastic lesions were associated with inflammatory cell infiltration and inflammation-induced fibrotic lesions of the pleural tissues. The fibers were not found in the mesothelial proliferative lesions themselves. In the pleural cavity, abundant inflammatory cell infiltration, mainly composed of macrophages, was observed. Conditioned cell culture media of macrophages treated with multi-walled carbon nanotubes and crocidolite and the supernatants of pleural cavity lavage fluid from the dosed rats increased mesothelial cell proliferation in vitro, suggesting that mesothelial proliferative lesions were induced by inflammatory events in the lung and pleural cavity and likely mediated by macrophages. In conclusion, intrapulmonary administration of multi-walled carbon nanotubes, like asbestos, induced mesothelial proliferation potentially associated with mesothelioma development.

Keywords: Multi-walled carbon nanotube, Mesothelioma, rats, asbestos

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Kato K[*], Shirao T[*], Yamazaki H[*], Imamura K[*], Sekino Y: Regulation of AMPA receptor recruitment by the action binding protein drebrin in cultured hippocampal neurons.

J Neurosci Neuroengineer. 2012;1:153-60.

One of the key roles in synaptic strengthening is AMPA receptor (AMPA) trafficking to the postsynaptic density during synaptic plasticity. Morphological changes in dendritic spines related to actin remodeling have recently been shown to be closely associated with synaptic strengthening. During synaptic development, both of morphological changes and synaptic strengthening are observed. This suggests that the actin cytoskeleton and its binding proteins in spines play important roles in synaptic formation during development. In the present study, we investigated the role of drebrin, a spine resident actin binding protein, in synaptic transmission and strengthening, using RNA interference and perforated whole-cell patch-clamp techniques in developing hippocampal neurons cultured from embryonic rats. The amplitude and frequency of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) were significantly smaller in drebrin-knockdown (drebrin-KD) neurons than in control-GFP neurons. The Current-Voltage (I-V) relationship and mEPSC decay time constant of drebrin-KD and control-GFP neurons were comparable. These data suggest that postsynaptic change in the number of AMPARs caused by drebrin-KD is not accompanied by modulation of AMPAR. In addition, the initial phases of glutamate-induced LTP-like increment in mEPSC amplitude and frequency were attenuated in drebrin-KD neurons. Together it is indicated that drebrin is involved in the regulation of AMPAR trafficking in postsynapses.

Keywords: drebrin, AMPA receptor, neuron

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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;38:391-402.

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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Takaki J*, Fujimori K*, Miura M*, Suzuki T*, Sekino Y, Sato K: L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation.

J Neuroinflammation 2012;9:275.

Background:In the central nervous system (CNS), astrocytic L-glutamate (L-Glu) transporters maintain extracellular L-Glu below neurotoxic levels, but their function is impaired with neuroinflammation. Microglia become activated with inflammation; however, the correlation between activated microglia and the impairment of L-Glu transporters is unknown.

Methods: We used a mixed culture composed of astrocytes, microglia, and neurons. To quantify L-Glu transporter function, we measured the extracellular L-Glu that remained 30 min after an application of L-Glu to the medium (the starting concentration was 100 μM). We determined the optimal conditions of lipopolysaccharide (LPS) treatment to establish an inflammation model without cell death. We examined the predominant subtypes of L-Glu transporters and the changes in the expression levels of these transporters in this inflammation model. We then investigated the role of activated microglia in the changes in L-Glu transporter expression and the underlying mechanisms in this inflammation model.

Results: Because LPS (10 ng/ml, 72 h) caused a significant increase in the levels of L-Glu remaining but did not affect cell viability, we adopted this condition for our inflammation model without cell death. GLAST was the predominant L-Glu transporter subtype, and its expression decreased in this inflammation model. As a result of their release of L-Glu, activated microglia were shown to be essential for the significant decrease in L-Glu uptake. The serial application of L-Glu caused a significant decrease in L-Glu uptake and GLAST expression in the astrocyte culture. The hemichannel inhibitor carbenoxolone (CBX) inhibited L-Glu release from activated microglia and ameliorated the decrease in GLAST expression in the inflammation model. In addition, the elevation of the astrocytic intracellular L-Glu itself caused the downregulation of GLAST.

Conclusions: Our findings suggest that activated microglia trigger the elevation of extracellular L-Glu through their own release of L-Glu, and astrocyte L-Glu transporters are downregulated as a result of the elevation of astrocytic intracellular L-Glu levels, causing a further increase of extracellular L-Glu. Our data suggest the new hypothesis that activated microglia collude with astrocytes to cause the elevation of extracellular L-Glu in the early stages of neuroinflammation.

Keywords: L-glutamate transporter, microglia, astrocytes

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Takata F*[1,2], Dohgu S*[1], Yamauchi A*[1], Matsumoto J*[1], Machida T*[1], Fujishita K*[3], Shibata K*[3,4], Shinozaki Y*[3,4], Sato K, Kataoka Y*[1,2], Koizumi S*[3,4]: In vitro blood-brain barrier models using brain capillary endothelial cells isolated from infant and adult rats retain age-related barrier properties.

PLoS ONE 2012;8:e55166.

The blood-brain barrier (BBB) restricts the entry of circulating drugs and xenobiotics into the brain, and thus its permeability to substances is a critical factor that determines their central effects. The infant brain

is vulnerable to neurotoxic substances partly due to the immature BBB. The employment of in vitro BBB models to evaluate permeability of compounds provides higher throughput than that of in vivo animal experiments. However, existing in vitro BBB models have not been able to simulate the intrinsic neonatal BBB. To establish a neonatal BBB model that mimics age-related BBB properties, the neonatal and adult in vitro BBB models were constructed with brain endothelial cells isolated from 2- and 8-week-old rats, respectively. To evaluate BBB functions, transendothelial electrical resistance, permeability of sodium fluorescein and Evans blue-albumin, and transport of rhodamine123 were measured. Radiolabelled drugs were used for BBB permeability studies in the neonatal and adult BBB models (in vitro) and in age-matched rats (in vivo). The neonatal BBB model showed lower barrier and p-glycoprotein (P-gp) functions than the adult BBB model; these were well associated with lower expressions of the barrier-related proteins and P-gp, and a different distribution pattern of immunostained barrier-related proteins. Verapamil (a P-gp inhibitor) significantly increased the influx of rhodamine 123, supporting functional P-gp expression in the neonatal BBB model. Valproic acid, but not nicotine, showed higher BBB permeability in the neonatal BBB model, which was well in accordance with the in vivo BBB property. We established a neonatal BBB model in vitro. This could allow us to assess the age-dependent BBB permeability of drugs.

Keywords: blood brain barrier, in vitro, age-dependent

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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. 2012;33:47-58.

Abstract 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, MMP-9, P2 receptor

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Morizawa Y*1,2, Sato K, Takaki J*3, Kawasaki A*4, Shibata K*1, Suzuki T*3, Ohta S*2, Koizumi S*1: Cell-autonomous enhancement of glutamate-uptake by female astrocytes.

Cell Mol Neurobiol. 2012;32:953-6.

Since gonadal female hormones act on and protect neurons, it is well known that the female brain is less vulnerable to stroke or other brain insults than the male brain. Although glial functions have been shown to affect the vulnerability of the brain, little is known if such a sex difference exists in glia, much less the mechanism that might cause gender-dependent differences in glial functions. In this study, we show that in vitro astrocytes obtained from either female or male pups show a gonadal hormone-independent phenotype that could explain the genderdependent vulnerability

of the brain. Female spinal astrocytes cleared more glutamate by GLAST than male ones. In addition, motoneurons seeded on female spinal astrocytes were less vulnerable to glutamate than those seeded on male ones. It is suggested that female astrocytes uptake more glutamate and reveal a stronger neuroprotective effect against glutamate than male ones. It should be noted that such an effect was independent of gonadal female hormones, suggesting that astrocytes have cell-autonomous regulatory mechanisms by which they transform themselves into appropriate phenotypes.

Keywords: astrocytes, sex difference, GLAST

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Ihara Y*1, Kanda Y, Seo M*1, Watanabe Y*2, Akamizu T*3, Tanaka Y*1: Growth stimulating antibody, as another predisposing factor of Graves' disease (GD): analysis using monoclonal TSH receptor antibodies derived from patients with GD.
Endocr J. 2012;59:571-7.

TSH receptor antibody (TRAb) is clinically classified into thyroid stimulating antibody (TSAb) and thyroid-stimulation blocking antibody (TSBAb). In this study, we analyzed GSA of monoclonal TRAb established from patients with GD or idiopathic myxedema (IME). GSA was measured as the degree of FRTL-5 cell growth stimulated by each TRAb. The signaling pathways of the cell growth were pharmacologically analyzed. The cell growth stimulated by TSH was strongly suppressed by protein kinase A (PKA) inhibitor, but was not affected by extracellular signal regulated kinase kinase (MEK) inhibitor. Although TSAb from GD stimulated the cell growth, both inhibitors suppressed it. Surprisingly, the cell growth was also induced by TSBAb from GD and was only suppressed by MEK inhibitor. TSBAb from IME did not have GSA and attenuated the cell growth stimulated by TSH. We concluded that 1; in GD, not only TSAb but some TSBAb could stimulate thyrocyte growth. 2; TSBAb might be classified with respect to their effects on thyrocyte growth; i.e., thyrocyte growth stimulating antibody and thyrocyte growth-stimulation blocking antibody.

Keyword: Graves' disease, TSH receptor antibody

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Pitchakarn P*1, Suzuki S*1, Ogawa K, Pompimon W*2, Takahashi S*1, Asamoto M*1, Limtrakul P*3, Shirai T*1: Kuguacin J, a triterpenoid from *Momordica charantia* leaf, modulates the progression of androgen-independent human prostate cancer cell line, PC3.
Food Chem Toxicol. 2012;50:840-7.

In this study, we focused on the *in vitro* effects of Kuguacin J (KuJ), a purified component of bitter melon (*Momordica charantia*) leaf extract (BMLE), on the androgen-independent human prostate cancer cell line PC3 and the *in vivo* effect of dietary BMLE on prostate carcinogenesis using a PC3-xenograph model. KuJ exerted a strong growth-inhibitory effect on PC3 cells. Growth inhibition was mainly through G1-arrest: KuJ markedly decreased the levels of cyclins (D1 and E), cyclin-dependent kinases (Cdk2 and Cdk4) and proliferating cell nuclear antigen. Interestingly, KuJ also dramatically decreased the levels of survivin expressed by PC3 cells. In addition, KuJ exerted anti-invasive effects on PC3 cells, significantly inhibiting migration and invasion: KuJ inhibited secretion of the active forms of MMP-2, MMP-9 and uPA by PC3 cells. In addition, KuJ treatment significantly decreased the expression of membrane type 1-MMP (MT1-MMP) by PC3 cells. *In vivo*, 1% and 5% BMLE in the diet resulted in 63% and 57% inhibition of PC3 xenograft growth without adverse effect on host body weight. Our results suggest that KuJ is a promising new candidate chemopreventive and chemotherapeutic agent for prostate cancer.

Keywords: prostate cancer, bitter melon, Kuguacin J

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Tang M*, Takahashi S*, Naiki-Ito A*, Sato S*, Takahashi S*, Asamoto M*, Shirai T*: Silencing of connexin 43 suppresses invasion, migration and lung metastasis of rat hepatocellular carcinoma cells.
Cancer Sci. 2012;103:860-7.

To reduce cancer mortality, understanding of mechanisms of cancer metastasis is crucial. We have established 6 rat hepatocellular carcinoma (HCC) cell lines which exhibit differing metastatic potential to the lung after inoculation into the tail veins of nude mice. In the present experiment, we investigated the process of cell attachment to metastatic sites and possible regulating factors. One hour after inoculation, 2 of 2 HCC cell lines with high metastatic potential and 1 of 2 HCC cell lines with low metastatic potential exhibited many attached cells in the lung. One day after inoculation, lung metastatic foci were observed only with highly-metastatic cells with elevated connexin 43 (Cx43) expression as assessed by cDNA array analysis. Furthermore, 24 or 48 hrs after transfection of an siRNA targeting Cx43, *in vitro* invasion and migration were suppressed by 68% ($P<0.001$) and 36% ($P<0.05$) compared with control-siRNA transfected cells, despite no differences in cellular morphology, cell proliferation or apoptotic activity. Moreover, the number of metastatic nodules per lung area in nude mice was significantly ($P<0.01$) reduced. In conclusion, suppression of Cx43 expression in tumor cells reduced *in vitro* migration and invasion capacity and *in vivo* metastatic ability so that Cx43 has potential as a molecular target for prevention of cancer metastasis with Cx43 overexpressing tumors.

Keywords: connexin 43, metastasis, hepatocellular carcinoma

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Takahashi M, Matsuo S, Inoue K, Tamura K, Irie K, Kodama Y, Yoshida M: Development of an early induction model of medulloblastoma in Ptch1 heterozygous mice initiated with *N*-ethyl-*N*-nitrosourea.
Cancer Sci. 2012;103:2051-5.

Mice heterozygous for the ptch1 gene (ptch1 mice) are known as a valuable model of medulloblastoma, a common brain tumor in children. To increase the incidence and reduce the time required for tumor development, allowing for evaluation of modifier effects on medulloblastoma in a short time, we attempted to develop an early induction model of medulloblastoma in ptch1 mice initiated with *N*-ethyl-*N*-nitrosourea (ENU). Ptch1 mice and their wild-type littermates received a single intraperitoneal injection of ENU (10, 50 or 100 mg/kg) on postnatal day 1 (d1) or 4 (d4), and histopathological assessment of brains was conducted at 12 weeks of age. The width of the external granular layer (EGL), a possible origin of medulloblastoma, after injection of 100 mg ENU on d1 or d4 was measured in up to 21-day-old mice. Cerebellar size was apparently reduced at the 50 mg dose and higher regardless of genotype. Microscopically, early lesions of medulloblastomas occurred with a high incidence only in ptch1 mice receiving 10 mg on d1 or d4, but a significant increase was not observed in other groups. Persistent EGL cells and misalignment of Purkinje cells were increased dose-dependently. Although EGL was strikingly decreased after ENU injection, strong recovery was observed in mice of the d1-treated group. In summary, neonatal treatment with ENU is available for the induction of medulloblastoma in ptch1 mice, and 10 mg of ENU administered on d1 appeared to be an appropriate dose to induce medulloblastoma.

Keywords: medulloblastoma, Ptch1, *N*-ethyl-*N*-nitrosourea

Ota Y, Imai T, Hasumura M, Cho YM, Takami S, Oyamada T*1, Hirose M*2, Nishikawa A, Ogawa K: Prostaglandin synthases influence thyroid follicular cell proliferation but not carcinogenesis in rats initiated with *N*-bis(2-hydroxypropyl)nitrosamine.
Toxicol Sci. 2012;127:339-47.

To clarify roles of prostaglandin synthases in rat thyroid follicular carcinogenesis, effects of an antithyroid agent, sulfadimethoxine (SDM), and two prostaglandin H synthase (COX) -inhibitors, indomethacin and nimesulide, on prostaglandin synthase expression, follicular cell proliferation and tumor induction in thyroids of rats with or without *N*-bis(2-hydroxypropyl) nitrosamine (DHPN) -initiation were examined. In Experiment 1, F344 male rats were allowed free access to drinking water containing SDM (0.1%), SDM+indomethacin (0.0025% in diet) or SDM+nimesulide (0.04% in diet) for 4 weeks. Both COX-inhibitors suppressed goitrogenic activity of SDM, but they did not significantly affect microsomal prostaglandin E syn-

thase-2 (mPGES-2) expression levels enhanced by SDM. In Experiment 2, all rats received a injection of DHPN (2800 mg/kg body weight), and starting 1 week later they were treated as in Experiment 1 for 4 or 10 weeks. Cell proliferation was suppressed or showed a tendency for suppression by the COX-inhibitors in the follicular preneoplastic/neoplastic lesions and surrounding parenchyma and this was obviously TSH-independent at least at week 4. However, neither of the COX-inhibitors altered the incidence or multiplicity of preneoplastic/neoplastic lesions. Immunohistochemistry revealed significant reduction and elevation of COX-2 and mPGES-2 expression, respectively, in the lesions, but these were also not changed by the COX-inhibitors. These results suggest that COX-2 and PGES, and in turn PGE(2) might play important roles in follicular cell proliferation but do not affect tumor induction in this rat thyroid carcinogenesis model. Further studies are needed to clarify the significance of the reduction of COX-2 expression in preneoplastic/neoplastic lesions.

Keywords: sulfadimethoxine, indomethacin, nimesulide

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Saegusa Y*1, Fujimoto H, Woo GH, Ohishi T*2, Wang L*2, Mitsumori K*2, Nishikawa A, Shibutani M*2: Transient aberration of neuronal development in the hippocampal dentate gyrus after developmental exposure to brominated flame retardants in rats.
Arch Toxicol. 2012;86:1431-42.

We immunohistochemically investigated the impact and reversibility of three brominated flame retardants (BFRs) known to be weak thyroid hormone disruptors on neuronal development in the hippocampal formation and apoptosis in the dentate subgranular zone. Pregnant Sprague-Dawley rats were exposed to 10, 100, or 1,000 ppm decabromodiphenyl ether (DBDE) ; 100, 1,000 or 10,000 ppm tetrabromobisphenol A (TBBPA) or 1,2,5,6,9,10-hexabromocyclododecane (HBCD) in the diet from gestational day 10 through to day 20 after delivery (weaning). On postnatal day (PND) 20, interneurons in the dentate hilus-expressing reelin increased with all chemicals, suggestive of aberration of neuronal migration. However, this increase had disappeared by PND 77. NeuN-positive mature neurons increased in the hilus on PND 77 with all chemicals. In the subgranular zone on PND 20, an increase in apoptotic bodies suggestive of impaired neurogenesis was observed after exposure to TBBPA or HBCD. The effects on neuronal development were detected at doses of ≥100 ppm DBDE; ≥1,000 ppm TBBPA; and at least at 10,000 ppm HBCD. On PND 20, the highest dose of DBDE and HBCD revealed mild fluctuations in the serum concentrations of thyroid-related hormones suggestive of weak developmental hypothyroidism, while TBBPA did not. Thus, DBDE and TBBPA may exert direct effect on neuronal development in the brain, but hypothyroidism may be operated for DBDE and HBCD at high doses. An excess of mature neurons in the hilus at later stages may be the signature of the developmental effects of BFRs. However, the effect itself was reversible.

Keywords: brominated flame retardants, hippocampal dentate gyrus, neurogenesis

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Suzuki Y, Umemura T, Hibi D, Inoue T, Jin M, Ishii Y, Sakai H*, Nohmi T, Yanai T*, Nishikawa A, Ogawa K: Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats.
Arch Toxicol. 2012;86:1593-601.

Estragole (ES) is a natural organic compound used frequently as a flavoring food additive. Although it has been reported to be tumorigenic and induce DNA adducts in the mouse liver, there have been no reports regarding ES hepatocarcinogenicity in rats. In the current study, we therefore examined potent carcinogenicity, DNA adduct formation and *in vivo* genotoxicity of ES in the livers of wild and reporter gene-carrying F344 rats. Males were administered 600 mg/kg bw ES by gavage and sequentially sacrificed at weeks 4, 8 and 16 for GST-P and PCNA immunohistochemistry and measurement of ES-specific DNA adducts by LC-MS/MS in the livers. GST-P-positive foci increased with time in ES-treated rats from week 4, PCNA-labeling indices being similarly elevated at both weeks 4 and 8. ES-specific DNA adducts such as ES-3'-N (2) -dG, 3'-8-dG and 3'-N (6) -dA were consistently detected, particularly at week 4. In a second study, male F344 *gpt* delta rats were administered 0, 22, 66, 200 or

600 mg/kg bw ES for 4 weeks. *Gpt* mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw in good correlation with PCNA-labeling indices. Mutation spectra analysis showed A:T to G:C transitions to be predominantly increased in line with the formation of ES-3'-N（6）-dA or 3'-8-dG. These results indicate that ES could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses.

Keywords: estragole, hepatocarcinogenesis, genotoxicity

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Fujii M[*1], Toyoda T, Nakanishi H[*1], Yatabe Y[*2], Sato A[*3], Matsudaira Y[*1], Ito H[*1], Murakami H[*1], Kondo Y[*1], Kondo E[*1], Hida T[*2], Tsujimura T[*2], Osada H[*1], Sekido Y[*1]: TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth.

J Exp Med. 2012;209:479-94.

Malignant mesothelioma（MM）is an incurable malignancy that is caused by exposure to asbestos and is accompanied by severe fibrosis. Because MM is usually diagnosed at an advanced stage and clinical identification of early lesions is difficult, its molecular pathogenesis has not been completely elucidated. Nearly 75% of MM cases have inactivating mutations in the NF2（neurofibromatosis type 2; Merlin）gene or in downstream signaling molecules of the Hippo signaling cascade, which negatively regulates the transcription factor Yes-associated protein（YAP）. In this study, we demonstrate a functional interaction between the Hippo and TGF-β pathways in regulating connective tissue growth factor（CTGF）. Expression of CTGF in MM cells was induced by the formation of a YAP-TEAD4-Smad3-p300 complex on the CTGF promoter. Knocking down CTGF expression in MM cells prolonged the survival of xenografted mice, and a significant association was seen between CTGF expression and extracellular matrix deposition in MM xenografts and in patient tissue specimens. We further suggest that CTGF may influence the malignancy of mesothelioma because of the different histological expression patterns observed in human MM tissues. These data suggest that CTGF is an important modulator of MM growth and pathology and represents a novel therapeutic target for this disease.

Keywords: malignant mesothelioma, TGF-β, connective tissue growth factor

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Fujimoto N[*1], Inoue K, Yoshida M, Nishikawa A, Ozawa S[*2], Gamou T[*2], Nemoto K[*3], Degawa M[*3]: Estrogen and androgen receptor status in hepatocellular hypertrophy induced by phenobarbital, clofibrate, and piperonyl butoxide in F344 rats.

J Toxicol Sci. 2012;37:281-6.

The present study examined hepatic estrogen receptor（ER）and androgen receptor（AR）levels as well as estrogen-signaling status in a model of rat hepatic hypertrophy induced by phenobarbital（PB）, chlofibrate（CF）, or piperonyl butoxide（PBO）. Male F344 rats were fed with PB at 2,500 ppm, CF at 2,500 ppm, and PBO at 20,000 ppm for 3 days, 4 weeks, and 13 weeks. CF and PBO induced diffuse hypertrophy, while centrilobular hypertrophy was observed with PB administration. The levels of mRNA for ERα, AR and leukemia inhibitory factor receptor（LIFR）which was found to be estrogen responsive in the present study, were determined by quantitative RT-PCR. In the CF and PBO groups, ERα mRNA expression was reduced, and consequently, the expression of a responsive gene, LIFR, was also decreased, while PB had no effect on ER mRNA levels. AR mRNA expression decreased in all the treated groups, but reduction was persistent only in PB group. Recently, LIFR was identified as a tumor suppressor gene in human HCC. Thus, LIFR may be one of the key mediators of hepatic carcinogenesis induced by CF and PBO, but PB appears to act via different mechanisms.

Keywords: hepatic hypertrophy, estrogen receptor, LIFR

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Hojo Y*, Shiraki A*, Tsuchiya T*, Shimamoto K*, Ishii Y, Suzuki K*, Shibutani M*, Mitsumori K*: Liver tumor promoting effect of etofenprox in rats and

its possible mechanism of action.

J Toxicol Sci. 2012;37:297-306.

To investigate the liver tumor-promoting effects of etofenprox (ETF), a pyrethroid-like insecticide, 6 week-old male F344 rats were given an intraperitoneal injection of N-diethylnitrosamine (DEN). After 2 weeks from the DEN treatment, 12 rats per group received a powdered diet containing 0, 0.25, 0.50, or 1.0% ETF for 8 weeks. At the time of 2nd week of ETF administration, all animals were subjected to two-thirds partial hepatectomy (PH). One rat per group except for the 0.25% ETF group died due to surgical operation of PH. The number and area of glutathione S-transferase placental form (GST-P) positive foci significantly increased in the livers of DEN-initiated rats given 0.50% and 1.0% ETF compared with the DEN-alone group. Quantitative real-time RT-PCR analysis revealed that the mRNA expression of phase I enzymes Cyp2b1/2, phase II enzymes such as Akr7a3, Gsta5, Ugt1a6, Nqo1 significantly increased in the DEN+ETF groups. The immunohistochemistry showed the translocation of CAR from the cytoplasm to the nuclei of hepatocytes in the ETF-treated groups. Reactive oxygen species (ROS) production increased in microsomes isolated from the livers of ETF-treated rats, and thiobarbituric acid-reactive substances (TBARS) levels and 8- hydroxy-2-deoxyguanosine (8-OHdG) content significantly increased in all of the ETF-treated groups and DEN+1.0% ETF group, respectively. The results of the present study indicate that ETF has a liver tumor-promoting activity in rats, and suggest that ETF activates the constitutive active/androstane receptor (CAR) and enhances microsomal ROS production, resulting in the upregulation of Nrf2 gene batteries; such an oxidative stress subsequently induces liver tumor-promoting effects by increased cellular proliferation.

Keywords: etofenprox, CYP2B inducer, liver

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Hayashi H*, Shimamoto K*, Taniai E*, Ishii Y, Morita R*, Suzuki K*, Shibutani M*, Mitsumori K*: Liver tumor promoting effect of omeprazole in rats and its possible mechanism of action.

J Toxicol Sci. 2012;37:491-501.

Omeprazole (OPZ), a proton pump inhibitor, is a cytochrome P450 (CYP) 1A1/2 inducer. Some CYP1A inducers are known to have liver tumor promoting effects in rats and the ability to enhance oxidative stress. In this study, we performed a two-stage liver carcinogenesis bioassay in rats to examine the tumor promoting effect of OPZ (Experiment 1) and to clarify a possible mechanism of action (Experiment 2). In Experiment 1, male F344 rats were subjected to a two-third partial hepatectomy, and treated with 0, 138 or 276 mg/kg OPZ by oral gavage once a day for six weeks after an intraperitoneal injection of N-diethylnitrosamine (DEN). Liver weights significantly increased in the DEN+OPZ groups, and the number and area of glutathione S-transferase placental form (GST-P) positive foci significantly increased in the DEN+276 mg/kg OPZ group. In Experiment 2, the same experiment as Experiment 1 was performed, but the dosage of OPZ was 0 or 276 mg/kg. The number and area of GST-P positive foci as well as liver weights significantly increased in the DEN+276 mg/kg OPZ group. The number of proliferative cell nuclear antigen (PCNA)-positive cells also significantly increased in the same group. Real-time RT-PCR showed that the expression of AhR battery genes including Cyp1a1, Cyp1a2, Ugt1a6 and Nqo1, and Nrf2 battery genes including Gpx2, Yc2, Akr7a3, Aldh1a1 Me1 and Ggt1 were significantly upregulated in this group. However, the production of microsomal reactive oxygen species (ROS) and formation of thiobarbituric acid-reactive substances (TBARS) decreased, and 8-hydroxydeoxyguanosine (8-OHdG) content remained unchanged in this group. These results indicate that OPZ, CYP1A inducer, is a liver tumor promoter in rats, but oxidative stress is not involved in the liver tumor promoting effect of OPZ.

Keywords: omeprazole, CYP1A1/2 inducer, tumor promotion

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Jin M, Kijima A, Suzuki Y, Hibi D, Ishii Y, Nohmi T, Nishikawa A, Ogawa K, Umemura T: *In vivo* genotoxicity of 1-methylnaphthalene from comprehensive toxicity studies with B6C3F1 *gpt* delta mice.

J Toxicol Sci. 2012;37:711-21.

1-Methylnaphthalene (1-MN), a constituent of the polycyclic aromatic hydrocarbons (PAHs), is a lung

carcinogen in mice. However, conventional genotoxicity tests such as the Ames test and sister chromatid exchange (SCE) test have yielded equivocal results. In the present study, the *in vivo* genotoxicity of 1-methylnaphthalene (1-MN) together with its toxicological profile was investigated in a 13-week repeated dose toxicity study of 1-MN using B6C3F1 *gpt* delta mice. In the serum biochemistry, significant increases in AST and ALP were observed in males of the 0.15% 1-MN group. From histopathological examination, the incidence of single cell necrosis in the liver was significantly increased in males of the 0.15% 1-MN group; however, no changes were observed in the lungs, the target organ of 1-MN. In an *in vivo* mutation assay, no changes in mutant frequencies of *gpt* and *red/gam* (Spi^-) in lung DNA of 1-MN treated mice were observed at 13 weeks. In addition, there were no significant differences in the proliferating cell nuclear antigen (PCNA)-positive ratios in bronchiolar epithelial cells among the groups for either sex. These results suggest that 1-MN at a carcinogenic dose not induce overt toxicity for any organs and has no *in vivo* genotoxicity in the lungs.

Keywords: *gpt* delta mice, mutagenicity, 1-methylnaphthalene

Cho YM, Imai T*, Takami S, Ogawa K, Nishikawa A: Female heterozygous (+/*fa*) Zucker rats as a novel leptin-related mammary carcinogenesis model.
J Toxicol Sci. 2012;37:1025-34.

The homozygous mutant fatty Zucker rat (*fa/fa*) is the prominent model for the research of obesity, one of the most well-known risk factor of postmenopausal mammary cancer. But the usage as a mammary gland carcinogenesis model is considered to be restricted due to the hypoplasia of mammary gland. In the present study, to find the validity of heterozygous mutant (+/*fa*) lean Zucker rats as a new leptin-related mammary carcinogenesis model, we examined whether the number of terminal end buds of mammary gland, the serum biochemistry, leptin concentration in serum and adipose tissue are changed in 7-week-old female +/+, +/*fa* and *fa/fa* rats, and whether these changes and leptin, TNF-α and VEGF mRNA expression in adipose tissue of +/+ and +/*fa* rats are influenced by 10% corn oil diet for 5 weeks. We confirmed that mild hyperleptinemia was more pronounced in 7-week-old +/*fa* as compared with wild type (+/+) and hypoplasia of mammary glands characterized by fewer numbers of terminal end buds in *fa/fa* was not observed in +/*fa*. With 10% corn oil diet, leptin mRNA expression in adipose tissue showed increasing tendency both in +/*fa* and +/+. Comparing with +/+, adipose tissue in +/*fa* treated with 10% corn oil diet was found to be significantly increased in the concentration of leptin protein and tended to be elevated expression of TNF-α mRNA. These results suggest that +/*fa* with 10% corn oil diet may be a useful model for investigation of the participation of leptin and TNF-α in mammary gland carcinogenesis.

Keywords: leptin, tumor models, mammary cancer

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Matsushita K, Ishii Y, Kijima A, Jin M, Takasu S, Kuroda K, Kodama Y, Ogawa K, Umemura T: Reporter gene mutation in the livers of *gpt* delta mice treated with 5-(hydroxymethyl)-2-furfural, a contaminant of various foods.
J Toxicol Sci. 2012;37:1077-82.

A major product formed during the Maillard reaction is 5-(hydroxymethyl)-2-furfural (HMF), which is present in various foods and beverages such as honey and fruit juice. HMF was shown to be a hepatocarcinogen in female mice using long-term bioassays. Although HMF is not a mutagen in conventional *in vitro* mutation assays, 5-sulfoxymethylfurfural (SMF), a reactive metabolite of HMF produced following sulfotransferase conjugation, does show mutagenicity. Thus, HMF-induced hepatocarcinogenesis likely involves genotoxic mechanisms. To clarify the mechanisms underlying HMF-induced hepatocarcinogenesis, female $B6C3F_1$ *gpt* delta mice were given HMF at carcinogenic doses (188 or 375 mg/kg b.w.) by gavage for 5 days per week for 4 weeks. This treatment produced no significant differences in mutant frequencies (MFs) of *gpt* and *red/gam* (Spi^-) genes among the groups. These results suggest that genotoxicity does not contribute to HMF-induced hepatocarcinogenesis. Parameters related to cell proliferation, such as proliferation cell nuclear antigen-labeling index and Cyclin D1 and E1 mRNA expression, exhibited no significant changes in the livers of HMF-treated groups. In view of the lack of carcinogenicity in rats, HMF may be consid-

ered to be a weak carcinogen. These results help us to understand the underlying mechanisms of action of HMF carcinogenesis.

Keywords: 5-(hydroxymethyl)-2-furfural, *gpt* delta mice, *in vivo* mutagenicity

Ishii Y, Inoue K, Takasu S, Jin M, Matsushita K, Kuroda K, Fukuhara K, Nishikawa A, Umemura T: Determination of lucidin-specific DNA adducts by liquid chromatography with tandem mass spectrometry in the livers and kidneys of rats given lucidin-3-*O*-primeveroside.

Chem Res Toxicol. 2012;25:1112-8.

Lucidin-3-*O*-primeveroside (LuP) is a component of madder color (MC), a compound which is carcinogenic in the kidney and liver of rats. Since LuP is metabolized to generate genotoxic compounds such as lucidin (Luc) and rubiadin, it is likely that these play key roles in MC carcinogenesis. In fact, after incubation of Luc with calf thymus DNA, Luc-N^2-dG and N^6-dA adducts were reportedly formed, possibly via the sulfotransferase metabolic pathway. However, the precise extent of formation *in vivo* remains uncertain. In the present study, to quantitatively determine Luc-specific DNA adducts in *in vivo* samples, we developed an online sample purification method using column-switching and an isotope dilution LC-ESI-MS/MS technique. The limits of quantification were 0.2 and 0.04 fmole on column for Luc-N^2-dG and N^6-dA adducts, respectively. Using the new analytical method, we attempted to measure adduct levels in the kidneys and livers of rats treated with 0.06, 0.3, and 1.5% LuP in the diet for one week. Luc-N^2-dG and N^6-dA adducts in these organs were detected at ranges from 7.97 to 51.67 /10^9 dG and from1.83 to 37.10 /10^9 dA, respectively. Dose-dependent increases of each adduct were observed in both organs. These quantitative data obtained with our newly developed analytical method might help to improve our understanding of MC carcinogenesis.

Keywords: lucidin-3-*O*-primeveroside, DNA adduct, madder color

Fujimoto H, Woo GH, Inoue K, Igarashi K, Kanno J, Hirose M[*1], Nishikawa A, Shibutani M[*2]: Increased cellular distribution of vimentin and Ret in the cingulum induced by developmental hypothyroidism in rat offspring maternally exposed to anti-thyroid agents.

Reprod Toxicol. 2012;34:93-100.

To elucidate target molecules of white matter development responding to hypothyroidism, global gene expression profiling of cerebral white matter from male rat offspring was performed after maternal exposure to anti-thyroid agents, 6-propyl-2-thiouracil and methimazole, on postnatal day 20. Genes involved in central nervous system development commonly up- or down-regulated among groups treated with anti-thyroid agents. Immunohistochemical distributions of vimentin, Ret proto-oncogene (Ret), deleted in colorectal cancer protein (DCC), and Claudin11 (Cld11) were examined based on the gene expression profile. Immunoreactive cells for vimentin and Ret in the cingulum, and the immunoreactive intensity of Cld11 and DCC in whole white matter were increased by treatment with anti-thyroid agents. Immunoreactive cells for vimentin and Ret were immature astrocytes and oligodendrocytes, respectively. Thus, immunoreactive cells for vimentin and Ret may be quantitatively measurable targets of developmental hypothyroidism in white matter.

Keywords: developmental hypothyroidism, cerebral white matter, vimentin

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Ochi A[*1], Ochiai K[*1], Kobara A[*1], Nakamura S[*1], Hatai H[*2], Handharyani E[*3], Tiemann I[*4], Tanaka III IB[*5], Toyoda T, Abe A[*1], Seok SH[*6], Sunden Y[*1], Torralba NC[*7], Park JH[*6], Hafez HM[*8], Umemura T[*1]: Epidemiological study of fowl glioma-inducing virus in chickens in Asia and Germany.

Avian Pathol. 2012;41:299-309.

Fowl glioma-inducing virus (FGV), which belongs to avian leukosis virus subgroup A (ALV-A), induces fowl glioma. This disease is characterized by multiple nodular gliomatous growths of astrocytes and has been previously reported in Europe, South Africa, Australia, the United States and Japan. FGV and FGV variants have spread to ornamental Japanese fowl, including Japanese bantams (*Gallus gallus domesticus*), in Japan. However, it is unclear how and where FGV emerged and whether FGV is related to the past fowl glioma in European countries. In this study, the prevalence of FGV in European, Asian and Japanese native chickens

were examined. FGV could not be isolated from any chickens in Germany and Asian countries other than Japan. Eighty (26%) out of 307 chickens reared in Japan were positive by FGV-screening nested PCR and 11 FGV variants with an FGV specific sequence in their 3' untranslated region (3'UTR) were isolated. In addition, 4 other ALVs lacking the FGV specific sequence were isolated from Japanese bantams with fowl glioma and/or cerebellar hypoplasia. These isolates were considered to be distinct recombinant viruses between FGV variants and endogenous/exogenous avian retroviruses. These results suggest that the variants as well as distinct recombinant ALVs are prevalent among Japanese native chickens in Japan and that FGV may have emerged by recombination among avian retroviruses in the chickens of this country.

Keywords: fowl glioma-inducing virus, avian leukosis virus, epidemiology

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Taketa Y, Yoshida M, Inoue K, Takahashi M, Sakamoto Y, Watanabe G*1, Taya K*1, Yamate J*2, Nishikawa A: The newly formed corpora lutea of normal cycling rats exhibit drastic changes in steroidogenic and luteolytic gene expressions.
Exp Toxicol Pathol. 2012;64:775-82.

In normal estrous cycling rats, corpora lutea (CL) regress over several cycles; however, the period during which they secrete progesterone (P4) is strictly limited. In the present study, we clarified the function of CL in normal cycling rats. We especially focused on expression levels of four steroidogenic and two luteolytic genes in the two different populations of the CL (new and old CL) at each estrous stage. The ovaries of female rats at each estrous cycle were collected, and new and old CL were separated with laser microdissection and analyzed for mRNA expression. In the new CL, the expressions of scavenger receptor class B type I (SR-BI), steroidogenic acute regulatory protein (StAR), and P450 cholesterol side-chain cleavage (P450scc) mRNA reached their highest levels at metestrus, and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA gradually increased from estrus to diestrus. Meanwhile, 20α-hydroxysteroid dehydrogenase (20α-HSD) and prostaglandin F2 alpha receptor (PGF2α-R) mRNA levels were remarkably low from estrus to metestrus and gradually increased thereafter. These gene levels in new CL corresponded to serum P4 levels during the estrous cycle. In the old CL, all steroidogenic and luteolytic gene levels were consistently high throughout the estrous cycle. These results provide clear evidence that new CL at metestrus have strong steroidogenic activity and through inhibition of luteolysis, maintain P4 production in normal cycling rats. The elevation of 20α-HSD and PGF2α-R levels in new CL at diestrus may be a trigger of functional luteolysis.

Keywords: corpora lutea, estrous cycle, laser microdissection

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Takami S, Imai T, Cho YM, Ogawa K, Hirose M, Nishikawa A: Juvenile rats do not exhibit elevated sensitivity to acrylamide toxicity after oral administration for 12 weeks.
J Appl Toxicol. 2012;32:959-67.

Acrylamide (AA), a neurotoxic, testicular toxic, genotoxic and carcinogenic chemical, has been reported to be formed in processed food, and sensitivity to AA intoxication in childhood is a concern. In the present study, to clarify the general toxicological profile of AA in juvenile rats, subchronic toxicity was evaluated in F344 rats administered AA in the drinking water at 0 (control), 10, 20 and 40 ppm, presented to the dams (three per group) immediately after the birth of their litters, through lactation (3 weeks), and directly to the offspring in their drinking water after weaning for a further 9 weeks (12 weeks total). Treatment with AA caused a decrease in body weights in 20 and 40 ppm F (1) females, compared with the controls. Average AA intake throughout the treatment period for the 10, 20 and 40 ppm groups after weaning was equivalent to 1.0, 2.1 and 4.4 mg kg(-1) body weight per day, respectively, in males and 1.2, 2.5 and 4.9 mg kg(-1) body

weight per day, respectively, in females. No toxicologically significant organ weight changes were observed. AA-induced histopathological changes were limited to focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelium in the ducts of epididymides, noted only in 40 ppm males. Taken together with previous reports, juvenile rats are not necessarily more susceptible to AA-induced toxicity as compared with young adults.

Keywords: acrylamide, juvenile rats, histopathology

Suzuki Y, Umemura T, Ishii Y, Hibi D, Inoue T, Jin M, Sakai H*, Kodama Y, Nohmi T, Yanai T*, Nishikawa A, Ogawa K: Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice.
Mutat Res. 2012;749:23-8.

Estragole (ES), a natural organic compound, is frequently used as a flavoring in food even though it is a hepatocarcinogen in mice. Although formation of ES-specific DNA adducts following conversion from ES to the nucleophilic metabolite by sulfotransferase 1A1 (SULT1A1) has been reported, the modes of action underlying ES-induced hepatocarcinogenesis remain uncertain because conventional genotoxicity tests for ES yield negative results. In the present study, taking notice of the fact that there is a sex difference in SULT1A1 activity in the mouse liver, we assessed the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency (MF) of reporter genes in female *gpt* delta mice treated with ES at doses of 0 (corn oil), 37.5, 75, 150 or 300mg/kg body weight (bw) by gavage for 13 weeks. Results were compared with those obtained in males. Since one female was found dead at week one, the highest dose was reduced to 250mg/kg bw in females from week two. As reported previously in C57BL/6 mice, the mRNA levels of Sult1a1 in female *gpt* delta mice were significantly higher than those in the males. The levels of ES-specific DNA adducts in the females were higher than those in the males at all doses except the highest dose. In addition, MFs of the *gpt* gene were significantly increased from doses of 75mg/kg bw of females, but the increment was observed only at the highest dose in males. There were no changes in the micronucleus test among the groups. Thus, the overall data suggest that specific DNA modifications by the SULT1A1-mediated carbocation formation and the resultant genotoxicity are key events in the early stage of ES-induced hepatocarcinogenesis of mice.

Keywords: estragole, *gpt* delta mice, DNA adduct

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Yoshida M, Katsuda S*1, Maekawa A*2: Involvements of estrogen receptor, proliferating cell nuclear antigen and p53 in endometrial adenocarcinoma development in Donryu rats.
J Toxicol Pathol. 2012;25:241-7.

Involvements of estrogen receptor (ER)α, proliferating cell nuclear antigen (PCNA) and p53 in the uterine carcinogenesis process in Donryu rats, a high yield strain of the uterine cancer were investigated immunohistochemically. ERα was expressed in atypical endometrial hyperplasia, accepted as a precancerous lesion of the uterine tumors, as well as well- and in moderately-differentiated endometrial adenocarcinomas, and the intensities of expression were similar to those in the luminal epithelial cells of the atrophic uterus at 15 months of age. The expression, however, was negative in the tumor cells of poorly differentiated type. Good growth of implanted grafts of the poorly-differentiated adenocarcinomas in both sexes with or without gonadectomy supported the estrogen independency of tumor progression to malignancy. PCNA labeling indices were increased with tumor development from atypical hyperplasia to adenocarcinoma. The tumor cells in poorly-differentiated adenocarcinomas were positive for p53 positive but negative for p21 expression, suggesting accumulation of mutated p53. These results indicate that the consistent ERα expression is involved in initiation and promotion steps of uterine carcinogenesis, but not progression. In addition, PCNA is related to tumor development and the expression of mutated p53 might be a late event during endometrial carcinogenesis.

Keywords: endometrial adenocarcinoma, Donryu rats, estrogen receptor

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Nohmi T, Yasui A[*2], Tanaka K[*1]: Mutations in UVSSA cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair.
Nature Genetics. 2012;44:593-7.

UV-sensitive syndrome（UV^{S}S）is an autosomal recessive disorder characterized by photosensitivity and deficiency in transcription-coupled repair（TCR）, a subpathway of nucleotide-excision repair that rapidly removes transcription-blocking DNA damage. Cockayne syndrome is a related disorder with defective TCR and consists of two complementation groups, Cockayne syndrome（CS）-A and CS-B, which are caused by mutations in ERCC8（CSA）and ERCC6（CSB）, respectively. UV^{S}S comprises three groups, UV^{S}S/CS-A, UV^{S}S/CS-B and UV^{S}S-A, caused by mutations in ERCC8, ERCC6 and an unidentified gene, respectively. Here, we report the cloning of the gene mutated in UV^{S}S-A by microcell-mediated chromosome transfer. The predicted human gene UVSSA（formerly known as KIAA1530）corrects defective TCR in UV^{S}S-A cells. We identify three nonsense and frameshift UVSSA mutations in individuals with UV^{S}S-A, indicating that UVSSA is the causative gene for this syndrome. The UVSSA protein forms a complex with USP7, stabilizes ERCC6 and restores the hypophosphorylated form of RNA polymerase II after UV irradiation.

Keywords: UV-sensitive syndrome, transcription-coupled repair, Cockayne syndrome

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Bailey AD[*1], Gray LT[*1], Pavelitz T[*1], Newman JC[*2], Horibata K, Tanaka K[*3], Weiner AM[*1]: The conserved Cockayne syndrome B-piggyBac fusion protein（CSB-PGBD3）affects DNA repair and induces both interferon-like and innate antiviral responses in CSB-null cells.
DNA Repair. 2012;11:488-501.

Cockayne syndrome is a segmental progeria most often caused by mutations in the CSB gene encoding a SWI/SNF-like ATPase required for transcription-coupled DNA repair（TCR）. Over 43Mya before marmosets diverged from humans, a piggyBac3（PGBD3）transposable element integrated into intron 5 of the CSB gene. As a result, primate CSB genes now generate both CSB protein and a conserved CSB-PGBD3 fusion protein in which the first 5 exons of CSB are alternatively spliced to the PGBD3 transposase. Using a host cell reactivation assay, we show that the fusion protein inhibits TCR of oxidative damage but facilitates TCR of UV damage. We also show by microarray analysis that expression of the fusion protein alone in CSB-null UV-sensitive syndrome（UVSS）cells induces an interferon-like response that resembles both the innate antiviral response and the prolonged interferon response normally maintained by unphosphorylated STAT1（U-STAT1）; moreover, as might be expected based on conservation of the fusion protein, this potentially cytotoxic interferon-like response is largely reversed by coexpression of functional CSB protein. Interestingly, expression of CSB and the CSB-PGBD3 fusion protein together, but neither alone, upregulates the insulin growth factor binding protein IGFBP5 and downregulates IGFBP7, suggesting that the fusion protein may also confer a metabolic advantage, perhaps in the presence of DNA damage. Finally, we show that the fusion protein binds in vitro to members of a dispersed family of 900 internally deleted piggyBac elements known as MER85s, providing a potential mechanism by which the fusion protein could exert widespread effects on gene expression. Our data suggest that the CSB-PGBD3 fusion protein is important in both health and disease, and could play a role in Cockayne syndrome.

Keywords: SWI/SNF-like ATPase, piggyBac elements, Cockayne syndrome

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Sugiyama K, Kinoshita M[*1], Kamata Y, Minai Y[*1], Tani F[*2], Sugita-Konishi Y: Thioredoxin-1 contributes to protection against DON-induced oxidative damage in HepG2 cells.
Mycotoxin Res. 2012;28:163-8.

Leucocytes are susceptible to the toxic effects of deoxynivalenol（DON）, which is a trichothecene mycotoxin produced by a number of fungi including Fusarium species. One mechanism of action is mediated by

reactive oxygen species (ROS). The liver is an important target for toxicity caused by foreign compounds including mycotoxins. On the other hand, little is known about the influence of the redox state on hepatocytes treated with DON. The present study investigated the effect of DON on the cytosolic redox state and antioxidative system in the human hepatoma cell line HepG2. The cell viability of human monocyte cell line THP-1 or leukemia cell line KU812 treated with 2.5 and 5 μmol/l DON were significantly reduced. However, HepG2 cells showed no toxic effects under the same conditions and did not exhibit an increased oxidative state. Further experiments showed that thioredoxin-1 (Trx-1) protein levels but not glutathione increased in the cells treated with 10 μmol/l DON. In addition, the enhancement of Trx-1 content was repressed by antioxidants. These results suggest that DON-induced accumulation of Trx-1 in HepG2 cells plays one of the key roles in protection against cytotoxicity caused by DON and that the mechanism may be mediated by the antioxidant properties of Trx-1.

Keywords: thioredoxin-1, deoxynivalenol, oxidative damage

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Honma M, Takahashi T*, Asada S*, Nakagawa Y*, Ikeda A*, Yamakage K*: In vitro clastogenicity and phototoxicity of fullerene(C(60)) nanomaterials in mammalian cells.
Mutat Res. 2012;749:97-100.

Carbon nanomaterials such as carbon nanotubes, graphene, and fullerenes (C(60)) are widely used in industry. Because of human health concerns, their toxic potential has been examined in vivo and in vitro. Here we used mammalian cells to examine the in vitro clastogenicity as well as the phototoxicity of C(60). While C(60) induced no structural chromosome aberrations in CHL/IU cells at up to 5mg/ml (the maximum concentration tested), it significantly induced polyploidy at 2.5 and 5mg/ml with and without metabolic activation. In BALB 3T3 cells, C(60) showed no phototoxic potential but the anatase form of titanium oxide did. Since insoluble nanomaterials cause polyploidy by blocking cytokinesis rather than by damaging DNA, we concluded that the polyploidy induced by C(60) in CHL/IU cells was probably due to non-DNA interacting mechanisms.

Keywords: fullerene, nanomaterials, in vitro genotoxicity

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Yamada M, Shimizu M*1, Katafuchi A, Grúz P, Fujii S*2, Usui Y*1, Fuchs RP*2, Nohmi T: *Escherichia coli* DNA polymerase III is responsible for the high level of spontaneous mutations in *mutT* strains.
Mol Microbiol. 2012;86:1364-75.

Reactive oxygen species induce oxidative damage in DNA precursors, i.e. dNTPs, leading to point mutations upon incorporation. *Escherichia coli mutT* strains, deficient in the activity hydrolysing 8-oxo-dGTP, display more than a 100-fold higher spontaneous mutation frequency over the wild-type strain. Here, we report that DNA pol III incorporates 8-oxo-dGTP ≈ 20 times more efficiently opposite template A compared with template C. Single, double or triple deletions of pol I, pol II, pol IV or pol V had modest effects on the *mutT* mutator phenotype. Only the deletion of all four polymerases led to a 70% reduction of the mutator phenotype. While pol III may account for nearly all 8-oxo-dGTP incorporation opposite template A, it only extends ≈ 30% of them, the remaining 70% being extended by the combined action of pol I, pol II, pol IV or pol V. The unique property of pol III to preferentially incorporate 8-oxo-dGTP opposite template A during replication might explain the high spontaneous mutation frequency in *E. coli mutT* compared with the mammalian counterparts lacking the 8-oxo-dGTP hydrolysing activities.

Keywords: *mutT*, DNA polymerases, 8-oxo-dGTP

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Sassa A*1, Kamoshita N, Matsuda T*2, Ishii Y, Kuraoka I*3, Nohmi T, Ohta T*1, Honma M, Yasui M: Miscoding properties of 8-chloro-2'-deoxyguanosine, a hypochlorous acid-induced DNA adduct, catalysed by human DNA polymerases.
Mutagenesis 2013;28:81-8.

Many chronic inflammatory conditions are associated with an increased risk of cancer development. At

the site of inflammation, cellular DNA is damaged by hypochlorous acid (HOCl), a potent oxidant generated by myeloperoxidase. 8-Chloro-2'-deoxyguanosine (8-Cl-dG) is a major DNA adduct formed by HOCl and has been detected from the liver DNA and urine of rats administered lipopolysaccharide in an inflammation model. Thus, the 8-Cl-dG lesion may be associated with the carcinogenesis of inflamed tissues. In this study, we explored the miscoding properties of the 8-Cl-dG adduct generated by human DNA polymerases (pols). Site-specifically modified oligodeoxynucleotide containing a single 8-Cl-dG was prepared and used as a template in primer extension reactions catalysed by human pol α, κ or η. Primer extension reactions catalysed by pol α and κ in the presence of all four dNTPs were slightly retarded at the 8-Cl-dG site, while pol η readily bypassed the lesion. The fully extended products were analysed to quantify the miscoding frequency and specificity of 8-Cl-dG using two-phased polyacrylamide gel electrophoresis (PAGE). During the primer extension reaction in the presence of four dNTPs, pol κ promoted one-base deletion (6.4%), accompanied by the misincorporation of 2'-deoxyguanosine monophosphate (5.5%), dAMP (3.7%), and dTMP (3.5%) opposite the lesion. Pol α and η, on the other hand, exclusively incorporated dCMP opposite the lesion. The steady-state kinetic studies supported the results obtained from the two-phased PAGE assay. These results indicate that 8-Cl-dG is a mutagenic lesion; the miscoding frequency and specificity varies depending on the DNA polymerase used. Thus, HOCl-induced 8-Cl-dG adduct may be involved in inflammation-driven carcinogenesis.

Keywords: DNA adduct, inflammation

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Grúz P, Nohmi T*: Expression and activity of human DNA polymerase η in *Escherichia coli*.
Genes and Environment. 2013;35;10-20.

DNA polymerase η (hPolη) is a key protein in translesion DNA synthesis (TLS) in human cells. Its primary function is the error free replication through UV-induced TT cyclobutane dimers which present a barrier to DNA synthesis by other eukaryotic replicative polymerases. hPolη defects underlie the genetic disease xeroderma pigmentosum variant (XPV) characterized by higher susceptibility to UV-light induced skin cancers due to erroneous replication of the UV adducts. However, hPolη is also a very low fidelity enzyme when copying undamaged DNA or DNA with other adducts and is actively recruited during the somatic hypermutation of the immunoglobulin genes. Here, we demonstrate that hPolη restores partially the mutability and completely the survival of a UV non-mutable umuDC-deletion mutant of *Escherichia coli* after UVB irradiation. We chose UVB instead of UVC as a radiation source because UVB is a major cause of human skin cancer induced by sunlight. The umuDC genes encode endogenous TLS DNA polymerase V. The catalytic core lacking the C-terminal part of hPolη was even more biologically active than the full size protein and its activity was further enhanced by attaching the prokaryotic β-subunit binding motif to it. The mutagenicity and survival effects were enhanced upon the induction of hPolη expression and its catalytically inactive variant was unable to promote any mutagenesis. This suggests that hPolη directly participates in the replication of damaged DNA in the prokaryotic bacteria. To demonstrate that our system can be useful in studying different variants of hPolη in vivo we have constructed 4 amino acid substitution mutants with altered geometry of the catalytic site analyzed previously biochemically and confirmed their altered abilities to promote mutagenesis and survival after UVB irradiation. This study paves a way to generate a variety of useful derivatives of hPolη in prokaryotic systems.

Keywords: DNA polymerase η, translesion DNA synthesis, UVB

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Hasegawa R, Hirata-Koizumi M, Dourson ML*, Parker A*, Ono A, Hirose A: Safety assessment of boron by application of new uncertainty factors and their subdivision.
Regul Toxicol Pharmacol. 2013;65:108-14.

The available toxicity information for boron was re-evaluated and four appropriate toxicity studies were selected in order to derive a tolerable daily intake (TDI) using newly proposed uncertainty factors (UFs) presented in Hasegawa et al. (2010). No observed adverse effect levels (NOAELs) of 17.5 and 8.8

mgB/kg/day for the critical effect of testicular toxicity were found in 2-year rat and dog feeding studies. Also, the 95% lower confidence limit of the benchmark doses for 5% reduction of fetal body weight (BMDL (05)) was calculated as 44.9 and 10.3 mgB/kg/day in mouse and rat developmental toxicity studies, respectively. Measured values available for differences in boron clearance between rats and humans and variability in the glomerular filtration rate (GFR) in pregnant women were used to derive chemical specific UFs. For the remaining uncertainty, newly proposed default UFs, which were derived from the latest applicable information with a probabilistic approach, and their subdivided factors for toxicokinetic and toxicodynamic variability were applied. Finally, overall UFs were calculated as 68 for rat testicular toxicity, 40 for dog testicular toxicity, 247 for mouse developmental toxicity and 78 for rat developmental toxicity. It is concluded that 0.13 mgB/kg/day is the most appropriate TDI for boron, based on rat developmental toxicity.

Keywords: Derivation of boron TDI, Chemical risk assessment, Application of new uncertainty factor (UF)

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Takahashi M, Kato H, Doi Y*, Hagiwara A*, Hirata-Koizumi M, Ono A, Kubota R, Nishimura T, Hirose A: Sub-acute oral toxicity study with fullerene C60 in rats.

J Toxicol Sci. 2012;37:353-61.

To obtain initial information on the possible repeated-dose oral toxicity of fullerene C60, Crl:CD (SD) rats were administered fullerene C60 by gavage once daily at 0 (vehicle: corn oil), 1, 10, 100, or 1,000 mg/kg/day for 29 days, followed by a 14-day recovery period. No deaths occurred in any groups, and there were no changes from controls in detailed clinical observations, body weights, and food consumption in any treatment groups. Moreover, no treatment-related histopathological changes were found in any organs examined at the end of the administration period and at the end of the recovery period. Blackish feces and black contents of the stomach and large intestine were observed in males and females at 1,000 mg/kg/day in the treatment group. There were no changes from controls in the liver and spleen weights at the end of the administration period, but those weights in males in the 1,000 mg/kg/day group increased at the end of the recovery period. Using liquid chromatography-tandem mass spectrometry, fullerene C60 were not detected in the liver, spleen or kidney at the end of the administration period and also at the end of the recovery period. In conclusion, the present study revealed no toxicological effects of fullerene C60; however, the slight increases in liver and spleen weights after the 14-day recovery period may be because of the influence of fullerene C60 oral administration. In the future, it will be necessary to conduct a long-term examination because the effects of fullerene C60 cannot be ruled out.

Keywords: Fullerene C60, Rat, Repeated oral (gavage) toxicity

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Matsumoto M, Serizawa H*1, Sunaga M*2, Kato H, Takahashi M, Hirata-Koizumi M, Ono A, Kamata E, Hirose A: No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats.

J Toxcol Sci. 2012;37:463-74.

Three female Crl:CD(SD) rats/group were dosed with single wall carbon nanotube (SWCNT) or multi wall carbon nanotube (MWCNT) four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals). Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects, and thus the oral LD_{50} values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw, in rats respectively. Five or ten Crl:CD (SD) rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). Six or twelve Crl:CD (SD) rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 50 mg/kg bw/day groups). Based on no toxicological effects, the NOAELs of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day (the highest dose tested), respectively. It was suggested that SWCNT and MWCNT dosed by gavage reached the gastro-intestinal tract as agglomerates and were mostly excreted

via feces.

Keywords: Single wall carbon nanotube, Multi wall carbon nanotube

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Takahashi M, Yabe K*, Kato H, Kawamura T, Matsumoto M, Hirata-Koizumi M, Ono A, Hirose A: Reproductive and developmental toxicity screening test of 3-cyanopyridine in rats.
Reprod Toxicol. 2013;35:7-16.

Crl:CD(SD) rats were given 3-cyanopyridine by gavage at 0, 5, 30 or 180 mg/kg/day. Males were dosed for 42 days beginning 14 days before mating, and females for 40-53 days beginning 14 days before mating to day 3 of lactation, including throughout the mating and gestation periods. General toxicity, mainly liver damage, was observed in males at ≥30 mg/kg/day and in females at ≥5 mg/kg/day. Sertoli cell vacuolation was observed at 180 mg/kg/day, and spermatocyte damages were observed at ≥30 mg/kg/day. Effects on estrous cycles, corpora lutea and implantations, and unsuccessfully mated females, despite additional mating, were observed at 180 mg/kg/day. Delayed initiation of delivery, dystocia, and deaths or moribundities of pregnant females were observed at 180 mg/kg/day, and only two pregnant rats delivered live pups at that dose. The NOAEL for reproductive/developmental toxicity was concluded to be 30 mg/kg/day.

Keywords: 3-Cyanopyridine, Reproductive and developmental toxicity, Rat

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Minowa Y*1, Kondo C*2, Uehara T*1-3, Morikawa Y*1, Okuno Y*4, Nakatsu N*1, Ono A, Maruyama T*2, Kato I*2, Yamate J*3, Yamada H*1, Ohno Y, Urushidani T*5: Toxicogenomic multigene biomarker for predicting the future onset of proximal tubular injury in rats.
Toxicology 2012;297:47-56.

Drug-induced renal tubular injury is a major concern in the preclinical safety evaluation of drug candidates. Toxicogenomics is now a generally accepted tool for identifying chemicals with potential safety problems. The specific aim of the present study was to develop a model for use in predicting the future onset of drug-induced proximal tubular injury following repeated dosing with various nephrotoxicants. In total, 41 nephrotoxic and nonnephrotoxic compounds were used for the present analysis. Male Sprague-Dawley rats were dosed orally or intravenously once daily. Animals were exposed to three different doses (low, middle, and high) of each compound, and kidney tissue was collected at 3, 6, 9, and 24h after single dosing, and on days 4, 8, 15, and 29 after repeated dosing. Gene expression profiles were generated from kidney total RNA using Affymetrix DNA microarrays. Filter-type gene selection and linear classification algorithms were employed to discriminate future onset of proximal tubular injury. We identified genomic biomarkers for use in future onset prediction using the gene expression profiles determined on day 1, when most of the nephrotoxicants had yet to produce detectable histopathological changes. The model was evaluated using a five-fold cross validation, and achieved a sensitivity of 93% and selectivity of 90% with 19 probes. We also found that the prediction accuracy of the optimized model was substantially higher than that produced by any of the single genomic biomarkers or histopathology. The genes included in our model were primarily involved in DNA replication, cell cycle control, apoptosis, and responses to oxidative stress and chemical stimuli. In summary, our toxicogenomic model is particularly useful for predicting the future onset of proximal tubular injury.

Keywords: Toxicogenomics, Nephrotoxicity, Rat

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Noriyuki N*1, Igarashi Y*1, Ono A, Yamada H*1, Ohno Y, Urushidani T*2: Evaluation of DNA microarray results in the Toxicogenomics Project (TGP) consortium in Japan.
J Toxicol Sci. 2012;37:791-801.

An important technology used in toxicogenomic drug discovery research is the microarray, which en-

ables researchers to simultaneously analyze the expression of a large number of genes. To build a database and data analysis system for use in assessing the safety of drugs and drug candidates, in 2002 we conducted a 5-year collaborative study in the Toxicogenomics Project（TGP1）in Japan. Experimental data generated by such studies must be validated by different laboratories for robust and accurate analysis. For this purpose, we conducted intra- and inter-laboratory validation studies with participating companies in the second collaborative study in the Toxicogenomics Project（TGP2）. Gene expression in the liver of rats treated with acetaminophen（APAP）was independently examined by the participating companies using Affymetrix GeneChip microarrays. The intra- and inter-laboratory reproducibility of the data was evaluated using hierarchical clustering analysis. The toxicogenomics results were highly reproducible, indicating that the gene expression data generated in our TGP1 project is reliable and compatible with the data generated by the participating laboratories.

Keywords: validation, toxicogenomics, microarray

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Okubo S*1, Miyamoto M*1, Takami K*1, Kanki M*2, Ono A, Nakatsu N*3, Yamada H*3, Ohno Y, Urushidani T*4: Identification of novel liver-specific mRNAs in plasma for biomarkers of drug-induced liver injury and quantitative evaluation in rats treated with various hepatotoxic compounds.

Toxicol Sci. 2013;132:21-31.

Circulating liver-specific mRNAs such as albumin（Alb）and α-1-microglobulin/bikunin precursor（Ambp）have been reported to be potential biomarkers for drug-induced liver injury（DILI）. We identified novel circulating liver-specific mRNAs and quantified them, together with the two previously reported mRNAs, in plasma from rats treated with various hepatotoxicants to validate circulating liver-specific mRNAs as biomarkers for DILI. Among six genes selected from the database, high liver specificity of apolipoprotein h（Apoh）and group-specific component（Gc）mRNAs were confirmed by reverse transcription（RT）-PCR and the copy numbers of these mRNAs elevated in plasma from rats treated with thioacetamide. Liver-specific mRNAs（Alb, Ambp, Apoh, and Gc）were quantified by real-time RT-PCR in plasma from rats with single dosing of seven hepatotoxicants. There were noticeable interindividual and intercompound variabilities in the severity of liver injury. The levels of four mRNAs increased almost in parallel and correlated with changes in the alanine aminotransferase（ALT）values and the hepatocellular necrosis scores at 24h after dosing. It was noteworthy that the magnitude of the increases in mRNA levels was greater than that in the ALT value. Time course analysis within 24h after dosing revealed that the timing of the increase was different among mRNA species, and the plasma levels of Alb and Gc mRNAs increased substantially earlier than the ALT values, suggesting that patterns of changes in circulating liver-specific mRNAs indicate the progression of liver injury. These results strongly support the reliability and usefulness of the four circulating liver-specific mRNAs as biomarkers for DILI.

Keywords: drug-induced liver injury, biomarker, circulating liver-specific mRNAs

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Uehara T*1-3, Kondo C*1, Morikawa Y*1,2, Hanafusa H*1, Ueda S*2, Minowa Y*2, Nakatsu N*2, Ono A, Maruyama T*1, Kato I*1, Yamate J*3, Yamada H*2, Ohno Y, Urushidani T*2,4: Toxicogenomic biomarkers for renal papillary injury in rats.

Toxicology 2013;303:1-8.

Renal papillary injury is a common side effect observed during nonclinical and clinical investigations in drug development. The present study aimed to identify genomic biomarkers for early and sensitive detection of renal papillary injury in rats. We hypothesized that previously identified genomic biomarkers for tubular injury might be applicable for the sensitive detection of papillary injury in rats. We selected 18 genes as candidate biomarkers for papillary injury based on previously published studies and analyzed their expression profiles by RT-PCR in each kidney region, namely the cortex, cortico-medullary junction, and papilla in various nephrotoxicity models. Comparative

analysis of gene expression profiles revealed that some genes were commonly upregulated or downregulated in the renal papilla, reflecting papillary injuries induced by 2-bromoethylamine hydrobromide, phenylbutazone, or n-phenylanthranilic acid. By applying receiver operator characteristics analysis, six candidate biomarkers were identified and their usefulness was confirmed by using an independent data set. The three top-ranked genes, Timp1, Igf1, and Lamc2, exhibited the best prediction performance in an external data set with area under the curve（AUC）values of greater than 0.91. An optimized support vector machine model consisting of three genes achieved the highest AUC value of 0.99. In conclusion, even though definitive validation studies are required for the establishment of their usefulness and reliability, these identified genes may prove to be the most promising candidate genomic biomarkers of renal papillary injury in rats.

Keywords: Toxicogenomics, Rat, Papillary injury

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Yamada T*1, Tanaka Y*1, Hasegawa R, Sakuratani Y*1, Yamada J*1, Kamata E, Ono A, Hirose A, Yamazoe Y*2, Mekenyan O*3, Hayashi M*4: A category approach to predicting the repeated-dose hepatotoxicity of allyl esters.

Regul Toxicol Pharmacol. 2013;65:189-95.

We tested a category approach to predict the hepatotoxic effects of repeated doses of allyl esters using a new database for repeated-dose toxicity. Based on information on hepatotoxic mechanism of allyl acetate, the category was defined as allyl esters that are hydrolyzed to allyl alcohol. Allyl alcohol is readily oxidized to acrolein in the liver, causing hepatotoxicity. Seventeen marketed allyl esters were obtained and grouped into category by identifying or predicting allyl alcohol formation. Allyl esters with a saturated straight alkyl carboxylic acid moiety（allyl acetate, hexanoate and heptanoate as tested species, and allyl butyrate, pentanoate, octanoate, nonanoate and decanoate as untested species）are likely similar in rate of ester hydrolysis, thereby defining subcategory 1. NOAEL and LOAEL for the hepatotoxic effects were estimated at 0.12 and 0.25mmol/kg/d for the untested species, based on those of allyl acetate. The remaining nine allyl esters with other alkyl or aromatic carboxylic acid moieties were placed in subcategory 2: their hepatotoxicity levels were not predictable due to an unclear match between their degree of structural complexity and rate of hydrolysis. Our results demonstrate the usefulness of the category approach for predicting the hepatotoxicity of untested allyl esters with saturated straight alkyl chains.

Keywords: Category approach, Hepatotoxicity, Adverse outcome pathway

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