Oshima N, Yamashita, T[*1], Hyuga S[*2], Hyuga M, Kamakura H, Yoshimura M[*3], Maruyama T, Hakamatsuka T, Amakura Y[*3], Hanawa T[*2], Goda Y: Efficiently prepared ephedrine alkaloids-free Ephedra Herb extract: a putative marker and antiproliferative effects.

*J Nat Med*. 2016;70:554-62

Ephedrine alkaloids (EAs) have been considered the main pharmacologically active substances in Ephedra Herb (麻黄, Mao; EH) since they were first identified by Prof. N. Nagai, and are known to induce palpitation, hypertension, insomnia, and dysuria as side effects. Therefore, the administration of drugs containing EH to patients with cardiovascular-related diseases is severely contraindicated. While our previous studies suggest that some of the effects of EH may not be due to EAs, considering their side effects would be expedient to develop a new EAs-free EH extract (EFE). Here,we established a preparationmethod forEFEand revealed its chemical composition, including the content of herbacetin, a flavonoid aglycon present in EH and a potential putative marker for EFE quality control. In addition, we showed the antiproliferative effects of EFE against the H1975 non-small cell lung cancer (NSCLC) cell line. EFE was prepared fromEHextract using the ion exchange resin SK-1B. LC/Orbitrap MS analysis revealed the removal of EAs, 6-methoxykynurenic acid, and 6-hydroxykynurenic acid from the original extract. Quantitative analysis of herbacetin using LC/MS in acid-hydrolyzed EFE showed that its content was 0.104 %. Although several alkaloidal constituents were removed from EH extract, the antiproliferative effect of EFE against H1975 cells was comparable to that of EH extract. These results indicate that EFE retained the anticancer effect of EH and demonstrated its potential for future development as a new herbal medicine with reduced side effects.

Keywords: Ephedra Herb, Ephedrine alkaloids-free Ephedra Herb extract, herbacetin

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神本敏弘[*1,2], 余村かおり[*1], 小幡竜弘[*1], 菊地祐一[*1], 平倉一弘[*1], 西村浩昭[*1], 五十嵐靖[*1], 濱口隆[*1], 諸田隆[*1], 袴塚高志, 合田幸広, 川原信夫[*3], 木内文之[*2]：定量用グリチルリチン酸に含まれる類縁物質に関する研究.

*医薬品医療機器レギュラトリーサイエンス* 2016;47:600-8

Glycyrrhizic acid reference standard (RS) for the Japanese Pharmacopoeia (JP) also contains an analogue of glycyrrhizic acid. Although the analogue is not separable from glycyrrhizic acid under the assay condition of "Glycyrrhiza" in the 16th edition of JP (JP16), an HPLC condition to separate the analogue from glycyrrhizic acid is adopted in the 17th edition of JP (JP17). Therefore, it is important to clarify the nature of the analogue. The structure of the analogue was presented at the 111st Annual Meeting of the Pharmaceutical Society of Japan, but details of the analogue have not yet been published. Thus, we isolated the analogue (compound X) and identified it as 3-[β-D-galactopyranuronosyl-(1→2)-β-D-glucopyranuronosyloxy]glycyrrhetic acid, named galacturoglycyrrhizic acid. We also examined the influence of the analogue on the assay of glycyrrhizic acid in "Glycyrrhiza" under the conditions adopted in JP16 and JP17. Mixtures of glycyrrhizic acid and compound X in various ratios showed a single peak under the condition of JP16 with standard deviation of peak areas of 1.31%, indicating that content of compound X do not affect the assay result. Contents of compound X in 20 lots of glycyrrhizic acid RS for JP distributed since 1996 were not more than 1.24%, and the contents in the lots distributed between 2004 and 2012 were less than 1%. These results indicate that the glycyrrhizic acid RS for JP distributed so far can be used in the assay of "Glycyrrhiza" in JP17.

Keywords: glycyrrhizic acid reference standard, Glycyrrhiza, galacturoglycyrrhizic acid

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Izutsu K, Kusano R*, Arai R*, Yoshida H, Ito M*, Shibata H, Sugano K*, Goda Y, Terada K*: Effect

of Co-solutes and Process Variables on Crystallinity and the Crystal form of Freeze-dried *Myo*-Inositol.
*Int J Pharm*. 2016;509:368-74

The purpose of this study was to elucidate how co-solutes affect the crystallization of small solute molecules during freeze-drying and subsequent storage. Crystallization profiles of *myo*-inositol and its mixture with dextran 40k in frozen solutions and dried solids were assessed by thermal analysis (DSC), powder-X-ray diffraction, and simultaneous DSC and PXRD analysis. Higher mass ratios of dextran maintained *myo*-inositol in the non-crystalline mixture state, in frozen solutions, during freeze-drying process, and exposure of dried solids to higher temperatures. Co-lyophilization with a lower mass ratio of dextran resulted in solids containing a variety of *myo*-inositol crystal forms and crystallinity depending on the composition and thermal history of the process. Heating of some inositol-rich amorphous solids showed crystallization of *myo*-inositol in the metastable form and its transition to stable form before melting. Heat-treatment of inositol-rich frozen solutions resulted in high crystallinity stable-form inositol solids, leaving dextran in the amorphous state. Sufficient direct molecular interactions (e.g., hydrogen bonding) should explain the stability of dextran-rich amorphous solids. Optimizing solute composition and processes should be a potent way to control crystal form and crystallinity of components in freeze-dried formulations.
Keywords: freeze-drying, crystallization, crystal form

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Izutsu K, Yoshida H, Shibata H, Goda Y: Amorphous-amorphous Phase Separation of Freeze-concentrated Protein and Amino Acid Excipients for Lyophilized Formulations.
*Chem Pharm Bull*. 2016;64:1674-80

The objective of this study was to elucidate the mixing state of proteins and amino acid excipients concentrated in the amorphous non-ice region of frozen solutions. Thermal analysis of frozen aqueous solutions was performed in heating scans before and after a heat treatment. Frozen aqueous solutions containing a protein (e.g., recombinant human albumin, gelatin) or a polysaccharide (dextran) and an amino acid excipient (e.g., L-arginine, L-arginine hydrochloride, L-arginine monophosphate, sodium L-glutamate) at varied mass ratios showed single or double $T_g'$ (glass transition temperature of maximally freeze-concentrated solutes). Some mixture frozen solutions rich in the polymers maintained the single $T_g'$ of the freeze-concentrated amorphous solute-mixture phase. In contrast, amino acid-rich mixture frozen solutions revealed two $T_g'$s that suggested transition of concentrated non-crystalline solute-mixture phase and excipient-dominant phase. Post-freeze heat treatment induced splitting of the $T_g'$ in some intermediate mass ratio mixture solutions. The mixing state of proteins and amino acids varied depending on their structure, salt types, mass ratio, composition of co-solutes (e.g., NaCl) and thermal history. Information on the varied mixing states should be valuable for the rational use of amino acid excipients in lyophilized protein pharmaceuticals.
Keywords: freeze-drying, excipient, phase separation

Shibata H, Yoshida H, Izutsu K, Goda Y: Use of bicarbonate buffer systems for dissolution characterization of enteric-coated proton pump inhibitor tablets.
*J Pharm Pharmacol*. 2016;68:467-74

OBJECTIVES: The aim of this study was to assess the effects of buffer systems (bicarbonate or phosphate at different concentrations) on the in vitro dissolution profiles of commercially available enteric-coated tablets.
METHODS: In vitro dissolution tests were conducted using an USP apparatus II on 12 enteric-coated omeprazole and rabeprazole tablets, including innovator and generic formulations in phosphate buffers, bicarbonate buffers and a media modified Hanks (mHanks) buffer.
KEY FINDINGS: Both omeprazole and rabeprazole tablets showed similar dissolution profiles among products in the compendial phosphate buffer system. However, there were large differences between products in dissolution lag time in mHanks buffer and bicarbonate buffers. All formulations showed longer dissolution lag times at lower concentrations of bicarbonate or phosphate buffers. The dissolution rank order of each formulation differed between mHanks buffer and bicarbonate buffers. A rabeprazole formulation coated with a methacrylic acid copolymer

showed the shortest lag time in the high concentration bicarbonate buffer, suggesting varied responses depending on the coating layer and buffer components. CONCLUSION: Use of multiple dissolution media during in vitro testing, including high concentration bicarbonate buffer, would contribute to the efficient design of enteric-coated drug formulations.

Keywords: bicarbonate buffer, dissolution, enteric-coated tablets

Yoshida H, Kuwana A, Shibata H, Izutsu K, Goda Y: Effects of Pump Pulsation on Hydrodynamic Properties and Dissolution Profiles in Flow-Through Dissolution Systems (USP 4).

*Pharm Res*. 2016;33:1327-36

Purpose: To clarify the effects of pump pulsation and flow-through cell (FTC) dissolution system settings on the hydrodynamic properties and dissolution profiles of model formulations.

Methods: Two FTC systems with different cell temperature control mechanisms were used. Particle image velocimetry (PIV) was used to analyze the hydrodynamic properties of test solutions in the flow-through dissolution test cell. Two pulsation pumps (semi-sine, full-sine) and a non-pulsatile pump were used to study the effects of varied flows on the dissolution profiles of United States Pharmacopeia standard tablets.

Results: PIV analysis showed periodic changes in the aligned upward fluid flow throughout the dissolution cell that was designed to reduce the temperature gradient during pump pulsation (0.5 s/pulse). The maximum instantaneous flow from the semi-sine pump was higher than that of the full-sine pump under all conditions. The flow from the semi-sine wave pump showed faster dissolution of salicylic acid and prednisone tablets than those from other pumps. The semi-sine wave pump flow showed similar dissolution profiles in the two FTC systems.

Conclusions: Variations in instantaneous fluid flow caused by pump pulsation that meets the requirements of pharmacopoeias are a factor that affects the dissolution profiles of tablets in FTC systems.

Keywords: dissolution testing, flow-through cell system, hydrodynamic

Ando D[*1], Kamada H[*1], Inoue M[*1], Taki S[*1], Furuya T[*1], Abe Y, Nagano K[*1], Tsutsumi Y[*2], Tsunoda S[*1]: Generation of a sensitive TNFR2-specific murine assays system.

*Pharmazie*. 2016;71:235-7

Tumor necrosis factor (TNF)/TNF receptors (TNFR1/TNFR2) are considered to be potential drug targets to treat refractory diseases, including autoimmune diseases and malignant tumors. However, their specific functions, especially in the case of TNFR2, are poorly understood. In this study, we constructed a mouse TNFR2 (mTNFR2)-mediated biological assay system that shows no effects of mouse TNFR1 (mTNFR1) in order to screen mTNFR2-selective stimulating agents. Mouse TNFR1-/-R2-/- preadipocytes were transfected with the gene encoding the mTNFR2/mouse Fas (mFas) chimeric receptor in which the extracellular and transmembrane domains of mTNFR2 were fused to the intracellular domain of mFas. Our results demonstrated that this cell line exhibits highly sensitive mTNFR2-mediated cytotoxic effects. We propose that this mTNFR2-mediated biological assay system would be a useful tool to screen for mTNFR2-selective stimulating agents.

Keywords: tumor necrosis factor-alpha, biological assay, TNFR2

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Ando D[*1], Inoue M[*1], Kamada H[*1], Taki S[*1], Furuya T[*1], Abe Y, Nagano K[*1], Tsutsumi Y[*2], Tsunoda S[*1]: Creation of mouse TNFR2-selective agonistic TNF mutants using a phage display technique.

*Biochem Biophys Rep*. 2016;7:309-15

Tumor necrosis factor-α (TNF), which is an immuno-modulatory cytokine, has been suggested to cause inflammatory responses as well as protection against tissue dysfunction by binding two types of TNF receptor (TNFR1/TNFR2). However, the physiological effects of TNFR2-specific activation remain unclear. We therefore aimed to generate a TNF mutant with full TNFR2-selective agonist activity as a functional analytical tool. In this study, we utilized a phage display technique to create mouse TNFR2 (mTNFR2)-selective TNF mutants that bind specifically to mTNFR2 and show full bioactivity

compared with wild-type TNF. A new phage library displaying TNF mutants was created, in which nine amino acid residues at the predicted receptor-binding site were randomized. From this library, an agonistic TNF mutant exhibiting high binding selectivity and bioactivity to mTNFR2 was isolated. We propose that this TNF mutant would be a powerful tool with which to elucidate the functional roles of mTNFR2.

Keywords: TNFR2, phage display, cytokine

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Sasaki T*1, Kambara O*1, Sakamoto T, Otsuka M*2, Nishizawa J*3: Single crystal growth and polarization absorption spectroscopy of theophylline anhydrous for terahertz vibrational mode assignment.
*Vibrational Spectroscopy*. 2016;85:91-6

In an attempt to provide a procedure for mode assignment in the terahertz (THz) frequency range, we fabricated an apparatus for single crystal growth via a temperature difference method to provide organic crystals that are sufficiently thin for wide- range THz transmission spectroscopy. Single-crystal theophylline anhydrous (TPAH) was successfully fabricated. THz polarization spectroscopy measurements were performed on TPAH crystal samples at 70 K. Assignment of the absorption peaks was carried out by comparing measurement results with those from density functional theory (DFT) calculations under periodic boundary conditions.

Keywords: terahertz spectroscopy, single crystal growth, DFT calculation

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Sakamoto T, Sasaki T*, Katori N, Goda Y: Analysis of Pseudo-polymorphism Conversion of Theophylline During Wet Granulation and Drying Processes and Effect of Binder on Dehydration and Amorphization.
*J Infrared Milli Terahz Waves*. 2016;37:1007-20

We conducted a time-course analysis of the pseudo-polymorphism conversion (i.e., the hydration and dehydration) of the xanthine-related compound theophylline during wet granulation and drying processes, using terahertz spectroscopy. We also investigated the amorphization mechanism of theophylline hydrate during a drying process in a vacuum using terahertz, mid-infrared (mid-IR), and near-infrared (near-IR) spectroscopy. After a high-shear granulation process using a mixture of theophylline, hydroxypropyl cellulose (HPC), and water, the terahertz spectrum (which was similar to that of an anhydride) was changed to a spectrum that was quite similar to that of a monohydrate. This result suggests that (1) an anhydride was converted to a monohydrate during the wet granulation process and (2) the spectrum was changed to the original waveform after the drying process with heat. This phenomenon indicates that the theophylline monohydrate was reconverted to an anhydride during the drying process. When wet granules were dried in a vacuum, the terahertz absorption lessened and finally disappeared with the passage of time, suggesting that the theophylline monohydrate in the granules was converted to an amorphous state. During the drying process with heat, the dehydration progressed temperature dependently regardless of the presence/absence of HPC. In addition, the reconversion from a monohydrate to an anhydride was completed concurrently with the completion of dehydration. The conversion rate of theophylline from a monohydrate to an amorphous form in granules (with HPC) was faster than that without HPC. This observation suggests that HPC promotes the amorphization of theophylline.

Keywords: terahertz spectroscopy, granulation, gelatinization

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Yamamoto Y*1, Kumetani M*1, Onuki Y*2, Koide T, Suzuki T*3, Fukami T*4: Analysis of the stability of external-application dermatologic preparations: Consideration from rheological measurements.
*Chem Pharm Bull*. 2016;64:263-72

The present study examined the stability of mixtures of various combinations of moisturizers, water in oil (w/o)-type or oil in water (o/w)-type cream preparations containing heparinoids, and steroidal ointments or creams (o/w-type)

frequently used in children. Centrifugation at room temperature led to separation of mixtures of w/o-type moisturizers and steroidal ointments into three layers. Polarized microscopic observations, near-infrared (NIR) spectroscopy, and dye-based analyses revealed the presence of oily components in the upper and middle layers and water-soluble components in the lower layer. Separation into three layers upon centrifugation was also observed for mixtures of o/w-type moisturizers and steroidal ointments. In contrast, neither the o/w-type moisturizer and steroidal cream nor the w/o-type moisturizer and steroidal cream mixtures separated into layers upon centrifugation. Consideration of the characteristics of each preparation is necessary when mixing external-application dermatologic preparations. Centrifugation at 4°C did not result in layer separation of the w/o-type moisturizer and steroidal ointment mixture, suggesting that cold storage of such mixtures provides superior stability compared with room temperature storage. However, despite no obvious layer separation, the NIR spectra indicated that water movement was induced within the mixture. These results clearly indicate that methods such as NIR spectroscopy are useful for early determinations of the stability of mixed external-application dermatologic preparations.

Keywords: heparinoid, steroidal preparation, stability

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Koide T, Fukami T*1, Hisada H*1, Inoue M*1, Carriere J*2, Heyler R*2, Katori N, Okuda H, Goda Y: Identification of pseudopolymorphism of magnesium stearate by using Low-frequency Raman Spectroscopy.
*Org Process Res Dev*. 2016;20:1906-10

Magnesium stearate (Mg-St), which is currently available on the market, has a wide variety of properties, including pseudopolymorphism, relative content of stearic acid in fatty acid, and particle size. These properties of Mg-St influence manufacturing processes of pharmaceutical products, and therefore, it is necessary to control the quality of Mg-St from suppliers. The purpose of this study was to evaluate the low-frequency region of Raman spectroscopy for identification of pseudopolymorphism in Mg-St. Ten samples of Mg-St obtained from different suppliers were measured by powder X-ray diffraction (PXRD) and thermogravimetry and differential thermal analysis (TG-DTA) to identify the pseudopolymorphism of Mg-St. Then we investigated the relationship between their Raman spectra, including the low-frequency region, and pseudopolymorphism. The results were categorized as four types of Mg-St, namely, mono-, di-, and trihydrate and their mixture. The conventional region of the Raman spectrum (greater than 200 cm−1) was able to identify pseudopolymorphism to a certain degree, but it was not easy to completely distinguish pseudopolymorphism for the mixture of Mg-St. In contrast, the lowfrequency region of the Raman spectrum (less than 200 cm−1) was able to clearly distinguish them. These data suggest that Raman spectroscopy, especially in the low-frequency region, is an eff ective method for rapid identifi cation of pseudopolymorphism in Mg-St.

Keywords: Raman spectroscopy, magnesium stearate, low-frequency

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Inoue M*1, Hisada H*1, Koide T, Carriere J*2, Heyler R*2, Fukami T*1: In situ monitoring of crystalline transformation of carbamazepine using probe type low frequency Raman spectroscopy.
*Org Process Res Dev*. 2017;21:262-5

Crystallization is one of the most useful processes for the separation and purification of crystalline compounds. In crystallization processes, real-time monitoring is essential to obtain constant quality of crystalline compounds. This paper is the first to report in situ monitoring of crystalline transformations of active pharmaceutical ingredients by probe-type low-frequency Raman spectroscopy. In this study, carbamazepine was used as a model active pharmaceutical ingredient. We attempted to monitor the crystalline transformation of carbamazepine during heat treatment and the addition of solvent in a one-pot reaction. When carbamazepine form III was heated to 170 °C, the indicative spectrum of carbamazepine form I appeared over time. Subsequent addition of ethanol with heat treatment caused the carbamazepine

form I spectrum to disappear. After cooling to room temperature, the spectrum of carbamazepine form III reappeared. To optimize the solvent ratio, we monitored carbamazepine form III as it dispersed into a mixture of ethanol/water with different compositions (75/25, 62.5/37.5, 50/50, 37.5/62.5, and 25/75 (v/v)). The spectra of carbamazepine dihydrate were observed in all solvent compositions. When the mixture of ethanol/water was 62.5/37.5 (v/v), the conversion time to carbamazepine dihydrate was fastest. Therefore, probe-type lowfrequency Raman spectroscopy can be used for the in situ monitoring of crystalline transformation and may become a useful process analytical technology technique.

Keywords: low frequency, Raman spectroscopy, crystallization

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Kozaki M*, Kobayashi S*, Goda Y, Okuda H, Sakai-Kato K: Evaluating the Properties of Poly (lactic-*co*-glycolic acid) Nanoparticle Formulations Encapsulating a Hydrophobic Drug by Using the Quality by Design Approach.

*Chemical and Pharmaceutical Bulletin*. 2017;65:218-28

We applied the Quality by Design (QbD) approach to the development of poly (lactic-co-glycolic acid) (PLGA) nanoparticle formulations encapsulating triamcinolone acetonide, and the critical process parameters (CPPs) were identified to clarify the correlations between critical quality attributes and CPPs. Quality risk management was performed by using an Ishikawa diagram and experiments with a fractional factorial design (ANOVA). The CPPs for particle size were PLGA concentration and rotation speed, and the CPP for relative drug loading efficiency was the poor solvent to good solvent volume ratio. By assessing the mutually related factors in the form of ratios, many factors could be efficiently considered in the risk assessment. We found a two-factor interaction between rotation speed and rate of addition of good solvent by using a fractional factorial design with resolution V. The system was then extended by using a central composite design, and the results obtained were visualized by using the response surface method to construct a design space. Our research represents a case study of the application of the QbD approach to pharmaceutical development, including formulation screening, by taking actual production factors into consideration. Our findings support the feasibility of using a similar approach to nanoparticle formulations under development. We could establish an efficient method of analyzing the CPPs of PLGA nanoparticles by using a QbD approach.

Keywords: quality by design (QbD), poly (lactic-co-glycolic acid) (PLGA) , nanoparticle formulation

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Abe Y, Sakai-Kato K, Goda Y: Cell Type-Specific Response of Peripheral Blood CD14-Positive Monocytes to Liposome-Encapsulated Immunostimulatory siRNA.

*Biological and Pharmaceutical Bulletin*. 2016;39: 1859-67

RNA interference via small interfering RNA (siRNA) has many potential therapeutic applications, and liposomal-based systems are useful for improving the pharmacokinetics of siRNAs, including their intracellular release and distribution. However, for the successful translation of this technology into clinical applications, it is important to understand how liposomal encapsulation changes the cellular uptake and immunostimulatory adverse effects of siRNAs. Here we evaluated the cellular uptake and innate immune activation by an immunostimulatory siRNA encapsulated within a liposome carrier in commercially available human peripheral blood mononuclear cells (PBMCs). We found considerable lot-to-lot variation in cytokine production by the PBMCs. Flow cytometric analysis in conjunction with intracellular staining of tumor necrosis factor-α (TNF-α) revealed that after treating PBMCs with the liposomal siRNA, approximately 5% of the cells produced TNF-α and more than 90% of the TNF-α-producing cells were positive for CD14 expression. We also showed that peripheral blood CD14+ monocytes in the cytokine release assay had low inter-lot variabilities in TNF-α production, suggesting that the peripheral blood CD14+ monocyte-based cytokine release assay is a specific means of alleviating the lot-to-lot variability in the cytokine release profiles of commercially available PBMCs. Our results also show that the peripheral

blood CD14+ monocyte-based cytokine release assay can be used to identify the siRNA recognition receptors that mediate individual cytokine production.
Keywords: liposome, peripheral blood mononuclear cell, CD14 positive monocyte

Takechi-Haraya Y, Sakai-Kato K, Abe Y, Kawanishi T, Okuda H, Goda Y: Atomic Force Microscopic Analysis of the Effect of Lipid Composition on Liposome Membrane Rigidity.
*Langmuir*. 2016;32:6074-82

Mechanical rigidity of the liposome membrane is often defined by the membrane bending modulus and is one of the determinants of liposome stability, but the quantitative experimental data are still limited to a few kinds of liposomes. Here, we used atomic force microscopy to investigate the membrane bending moduli of liposomes by immobilizing them on bovine serum albumin-coated glass in aqueous medium. The following lipids were used for liposome preparation: egg yolk phosphatidylcholine, dioleoylphosphatidylcholine, hydrogenated soybean phosphatidylcholine, dipalmitoylphosphatidylcholine, 1,2-dioleoyl-3-trimethylammonium-propane, cholesterol, and N-(carbonylmethoxypoly (ethylene glycol) 2000) -1,2-distearoyl-sn-glycero-3-phosphoethanolamine. By using liposomes of various compositions, we showed that the thermodynamic phase state of the membrane rather than the electric potential or liposome surface modification with poly (ethylene glycol) is the predominant determinant of the bending modulus, which decreased in the following order: solid ordered > liquid ordered > liquid disordered. By using the generalized polarization value of the Laurdan fluorescent probe, we investigated membrane rigidity in terms of membrane fluidity. Atomic force microscopic analysis was superior to the Laurdan method, especially in evaluating the membrane rigidity of liposomes containing hydrogenated soybean phosphatidylcholine and cholesterol. Positively charged liposomes with a large bending modulus were taken up by cells more efficiently than those with a small bending modulus. These findings offer a quantitative method of analyzing the membrane rigidity of nanosized liposomes with different lipid compositions and will contribute to the control of liposome stability and cellular uptake efficiency of liposomal formulations intended for clinical use.
Keywords: atomic force microscopy, liposomal membrane rigidity, cellular uptake

Takechi-Haraya Y, Sakai-Kato K, Abe Y, Kawanishi T, Okuda H, Goda Y: Observation of Liposomes of Differing Lipid Composition in Aqueous Medium by Means of Atomic Force Microscopy.
*Microscopy*. 2016;65:383-9

Liposomes present a challenge for atomic force microscopy (AFM) observation in aqueous medium because they easily collapse. Here, we demonstrate that bovine serum albumin coating of a glass substrate enables AFM observation of various liposomes in aqueous medium. With this AFM system, liposomes can be systematically observed and morphologically analyzed regardless of their surface charge, phase state, degree of lipid acyl chain unsaturation or PEG modification. This system thus has the potential to reveal the mechanical properties of liposomes of various lipid types and contents.
Keywords: atomic force microscopy, bovine serum albumin, liposome

Ito H[*1,2], Kaji H[*1], Togayachi A[*1], Azadi P[*3], Ishihara M[*3], Geyer R[*4], Galuska C[*4], Geyer H[*4], Kakehi K[*5], Kinoshita M[*5], Karlsson NG[*6], Jin C[*6], Kato K[*7], Yagi H[*7], Kondo S[*7], Kawasaki N[*8], Hashii N, Kolarich D[*9], Stavenhagen K[*9,10], Packer NH[*11], Thaysen-Andersen M[*11], Nakano M[*11,12], Taniguchi N[*13], Kurimoto A[*13], Wada Y[*14], Tajiri M[*14], Yang P[*15], Cao W[*15], Li H[*15], Rudd PM[*16], Narimatsu H[*1]: Comparison of analytical methods for profiling N- and O-linked glycans from cultured cell lines: HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study.
*Glycoconj J*. 2016;33:405-15

The Human Disease Glycomics/Proteome Initiative (HGPI) is an activity in the Human Proteome Organization (HUPO) supported by leading researchers from international institutes and aims at development of disease-related glycomics/glycoproteomics analysis techniques. Since 2004, the initiative has conducted three pilot studies. The first two were N- and O-glycan analyses of purified transferrin and immunoglobulin-G and assessed the most appropriate analytical approach employed at

the time. This paper describes the third study, which was conducted to compare different approaches for quantitation of N- and O-linked glycans attached to proteins in crude biological samples. The preliminary analysis on cell pellets resulted in wildly varied glycan profiles, which was probably the consequence of variations in the pre-processing sample preparation methodologies. However, the reproducibility of the data was not improved dramatically in the subsequent analysis on cell lysate fractions prepared in a specified method by one lab. The study demonstrated the difficulty of carrying out a complete analysis of the glycome in crude samples by any single technology and the importance of rigorous optimization of the course of analysis from preprocessing to data interpretation. It suggests that another collaborative study employing the latest technologies in this rapidly evolving field will help to realize the requirements of carrying out the large-scale analysis of glycoproteins in complex cell samples.

Keywords: glycoproteomics, Human disease glycomics/proteome initiative (HGPI), Human proteome organization (HUPO)

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原園景，川崎ナナ*，小林哲，石井明子：下垂体性性腺刺激ホルモン製剤の品質特性に関する研究.
*医薬品医療機器レギュラトリーサイエンス*　2016;47:388-92

Human menopausal gonadotropin (HMG), which is purified from urine of post-menopausal healthy women, has follicle-stimulating hormonal and luteinizing hormonal activities. Thus, HMG has been used to stimulate folliculogenesis for infertility therapy and assisted reproductive technology. HMG products from four manufacturers are commercially available in Japan. However, little information about the characteristics of these HMG products is available. In this study, protein contents, SDS-PAGE analysis, and quantification of FSH, LH and hCG by ELISA were performed, in order to characterize each product. These data might be useful for clinicians to choose the proper HMG product for each patient.

Keywords：下垂体性性腺刺激ホルモン製剤，FSH，LH

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小林哲，村山一茂*1，太田悠葵*2，川崎ナナ*2，豊島聰*1，石井明子：関節リウマチを主な適用疾患とするバイオ医薬品における有害事象プロファイルの比較.
*薬剤疫学*　2016;21:53-75

To identify the most frequently reported preferred terms (PTs) in the cases of rheumatoid arthritis (RA) patients treated with immunosuppressive biological drugs as suspected drugs, we analyzed the cases in the Japanese Adverse Drug Event Report (JADER) database. We found that pneumonia, interstitial lung disease, Pneumocystis jiroveci pneumonia (PCP), cellulitis, sepsis, and herpes zoster were the most frequently reported PTs. We obtained the reporting odds ratio (ROR) and the time to onset of these six PTs and compared them in the cases reported for each immunosuppressant as a suspected drug. We focused on RA treatment, including five tumor necrosis factor (TNF) antagonists (infliximab, etanercept, adalimumab, golimumab, and certolizumab pegol). For pneumonia, interstitial lung disease and sepsis, no specific correlation was observed for each immunosuppressant for RA. In the case of PCP, the highest ROR was observed in the patients treated with infliximab. The time to onset of PCP in the infliximab-treated patients (median, 0.19 yr) was significantly shorter than the onset time in the patients treated with tocilizumab, an interleukin-6 receptor blocker that is another type of drug for RA (0.32 yr, $p<0.01$, Mann-Whitney test). The onset time in the patients treated with golimumab (0.24 yr) was also significantly

shorter than the onset time for tocilizumab ($p<0.05$), but the ROR was not as high. These results suggested a correlation between PCP and infliximab. In the cases of cellulitis and herpes zoster, a similar correlation was observed with tocilizumab and certolizumab pegol, respectively. We should consider these results when patients have a respiratory disorder or skin/subcutaneous tissue disorder.

Keywords: rheumatoid arthritis, reporting odds ratio, time to onset

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Kamada I*1, Saitou Y*1, Simizu T*1, Asakawa K*1, Masuoka K*1, Kobayashi T, Ishii-Watabe A, Toyoshima S*2: Evaluation of the clinical benefit of a G-CSF biosimilar.
*レギュラトリーサイエンス学会誌* 2017;7:3-15

Compared to conventional drugs, biomedicines are generally more clinically beneficial, but are relatively expensive and their use may contribute to increase in health care costs. In Japan, despite expectation of widespread use of biosimilars, the switch from original biomedicines to biosimilars has been slower than that from low-molecular-weight drugs to their generics. This could be because physicians and pharmacists are not fully aware that the efficacy and safety of an original biomedicine and its biosimilar are equivalent. We, therefore, examined the bioequivalency of original granulocyte-colony stimulating factor (G-CSF) biomedicines (G-CSF originators hereafter) and G-CSF biosimilars by comparing the efficacy and safety of the G-CSF originator, Gran® Syringe (Kyowa Hakko Kirin Co. Ltd., Japan), with a biosimilar, Filgrastim BS Injection Syringe"F" (Filgrastim BS hereafter, Fuji Pharma Co. Ltd., Japan), by using data collected over 3 years, starting in April 2012, at Mishuku Hospital. The economic effect of switching to Filgrastim BS was also examined. We compared both G-CSF preparations in terms of time-dependent change in leukocyte counts, incidence of febrile neutropenia, and abnormal laboratory values. Because the time to start the administration of G-CSF differed between the originator-treated and the Filgrastim BS-treated groups due to the revision of the Clinical Practice Guidelines for Cancer, and leukocyte count instead of neutrophil count was used as the parameter, their efficacy could not be simply compared. However, the results suggest that Filgrastim BS is equivalent to its originator in terms of efficacy and safety in this study. Further, the cost-saving effect of switching to Filgrastim BS accounted for 10% of the entire cost saved by switching to generics and biosimilars in 2014. Thus, we demonstrated that switching from G-CSF originators to Filgrastim BS was beneficial for maintaining clinical efficacy and safety and reducing costs for Mishuku Hospital.

Keywords: malignant lymphoma, G-CSF, biosimilar

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Hashimoto Y*1, Tada M, Iida M*1, Nagase S*1, Hata T*1, Watari A*1, Okada Y*1, Doi T*1, Fukasawa M*2, Yagi K*1, Kondoh M*1: Generation and characterization of a human-mouse chimeric antibody against the extracellular domain of claudin-1 for cancer therapy using a mouse model.
*Biochem Biophys Res Commun*. 2016;477:91-5

Claudin-1 (CLDN-1), an integral transmembrane protein, is an attractive target for drug absorption, prevention of infection, and cancer therapy. Previously, we generated mouse anti-CLDN-1 monoclonal antibodies (mAbs) and found that they enhanced epidermal absorption of a drug and prevented hepatitis C virus infection in human hepatocytes. Here, we investigated anti-tumor activity of a human-mouse chimeric IgG1, xi-3A2, from one of the anti-CLDN-1 mAbs, clone 3A2. Xi-3A2 accumulated in the tumor tissues in mice bearing with human CLDN-1-expressing tumor cells. Xi-3A2 activated Fc $\gamma$ receptor IIIa-expressing reporter cells in the presence of human CLDN-1-expressing cells, suggesting xi-3A2 has a potential to exhibit antibody-dependent cellular cytotoxicity against CLDN-1 expressing tumor cells. We also constructed a mutant xi-3A2 antibody with Gly, Ser, and Ile substituted with Ala, Asp, and Arg at positions 236, 239, and 332 of the Fc domain. This mutant antibody showed greater activation of Fc $\gamma$ receptor IIIa and in vivo anti-tumor activity in mice bearing human CLDN-1-expressing tumors than xi-3A2 did. These findings indicate that the G236A/S239D/I332E mutant of xi-3A2 might be a promising lead for

tumor therapy.

Keywords: claudin-1, monoclonal antibody, anti-tumor activity

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Hashimoto Y*1, Kawahigashi Y*1, Hata T*1, Li X*1, Watari A*1, Tada M, Ishii-Watabe A, Okada Y*1, Doi T*1, Fukasawa M*2, Kuniyasu H*3, Yagi K*1, Kondoh M*1: Efficacy and safety evaluation of claudin-4-targeted antitumor therapy using a human and mouse cross-reactive monoclonal antibody.

*Pharmacol Res Perspect*. 2016;4:e00266 eCollection 2016

Claudin-4 (CLDN-4), a tight-junction protein, is overexpressed in various malignant tumors, including gastric, colorectal, pancreatic, and breast cancers. However, CLDN-4 is also expressed in normal tissues, including the liver, pancreas, kidney, and small intestine. Whether CLDN-4 is an effective and safe target for cancer therapy has been unclear owing to the lack of a binder with both CLDN-4 specificity and cross-reactivity to human and murine cells. In this study, we successfully generated a rat anti-CLDN-4 monoclonal antibody (5D12) that was specific to, and cross-reactive with, human and mouse CLDN-4. 5D12 recognized the second extracellular domain of human CLDN-4 in a conformation-dependent manner. A human-rat chimeric IgG1 of 5D12 (xi-5D12) activated the Fc$\gamma$ IIIa receptor, indicating the activation of antibody-dependent cellular cytotoxicity in CLDN-4-expressing cells. Moreover, xi-5D12 significantly suppressed tumor growth in mice bearing human colorectal and gastric tumors without apparent adverse effects, such as weight loss or liver and kidney damage. These results suggest that CLDN-4 is a potent target for cancer therapy and that an anti-CLDN-4 antibody is a promising candidate anticancer agent.

Keywords：claudin-4, monoclonal antibody, antitumor therapy

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Nakamori S*1, Takahashi J*1, Hyuga S*2, Tanaka-Kagawa T, Jinno H, Hyuga M, Hakamatsuka T, Odaguchi H*2, Goda Y, Hanawa T*2, Kobayashi Y*1: Ephedra Herb extract activates/desensitizes transient receptor potential vanilloid 1 and reduces capsaicin-induced pain.

*J Nat Med*. 2017;71:105-13

Kampo medicines containing Ephedra Herb (EH) such as eppikajutsubuto and makyoyokukanto are used to treat myalgia, arthralgia, and rheumatism. The analgesic effects of these Kampo medicines are attributed to the anti-inflammatory action of EH. However, the molecular mechanism of the analgesic effect of EH remains to be clarified. In this study, the effects of EH extract (EHE) on transient receptor potential vanilloid 1 (TRPV1), a nonselective ligand-gated cation channel, which plays an essential role in nociception on sensory neurons, were investigated using mTRPV1/Flp-In293 cells (stable mouse TRPV1-expressing transfectants). Administration of EHE increased the intracellular Ca2+ concentration in these cells, which was inhibited by the TRPV1 antagonist, N-(4-tert-butylphenyl)-1,2-dihydro-4-(3-chloropyridine-2-yl) tetrahydropyrazine-1-carboxamide (BCTC), indicating that EHE activated TRPV1. Examination of EHE-induced nociceptive pain in vivo revealed that an intradermal (i.d.) injection of EHE into the hind paw of mice induced paw licking, a pain-related behavior, and that the extract increased paw licking times in a dose-dependent manner. The EHE-induced paw licking was also inhibited by BCTC. An i.d. injection of EHE 30 min before administration of capsaicin decreased capsaicin-induced paw licking times. Similarly, oral administration of the extract also suppressed capsaicin-induced paw licking, without affecting the physical performance of the mice. These results suggest that EHE suppresses capsaicin-induced paw licking by regulating TRPV1 activity. Thus, the antinociceptive effects of EHE seem to be produced by its direct action on sensory neurons through TRPV1.

Keywords: Ephedra Herb, pain, TRPV1

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Hotta MT*, Rodriguez-Caprio G*, Fierer DS*, Fernandez-Sesma A*, Simon V, Chen BK*: Enhanced FCGR2A and FCGR3A signaling by HIV viremic controller IgG.

*JCI Insight*. 2017;2:e88226

HIV-1 viremic controllers (VC) spontaneously control infection without antiretroviral treatment. Several studies indicate that IgG Abs from VCs induce enhanced responses from immune effector cells. Since signaling through Fc-$\gamma$ receptors (FCGRs) modulate these Ab-driven responses, here we examine if enhanced FCGR activation is a common feature of IgG from VCs. Using an infected cell-based system, we observed that VC IgG stimulated greater FCGR2A and FCGR3A activation as compared with noncontrollers, independent of the magnitude of HIV-specific Ab binding or virus neutralization activities. Multivariate regression analysis showed that enhanced FCGR signaling was a significant predictor of VC status as compared with chronically infected patients (CIP) on highly active antiretroviral therapy (HAART). Unsupervised hierarchical clustering of patient IgG functions primarily grouped VC IgG profiles by enhanced FCGR2A, FCGR3A, or dual signaling activity. Our findings demonstrate that enhanced FCGR signaling is a common and significant predictive feature of VC IgG, with VCs displaying a distinct spectrum of FCGR activation profiles. Thus, profiling FCGR activation may provide a useful method for screening and distinguishing protective anti-HIV IgG responses in HIV-infected patients and in monitoring HIV vaccination regimens.

Keywords: HIV, viremic controller, FCGR signaling

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Hyuga S*1, Hyuga M, Oshima N*2, Maruyama T, Kamakura H, Yamashita T*3, Yoshimura M*4, Amakura Y*4, Hakamatsuka T, Odaguchi H*1, Goda Y, Hanawa T*1: Ephedrine alkaloids-free Ephedra Herb extract: a safer alternative to ephedra with comparable analgesic, anticancer, and anti-influenza activities.

*J Nat Med*. 2016;70:571-83

It is generally accepted that the primary pharmacological activities and adverse effects of Ephedra Herb are caused by ephedrine alkaloids. Interestingly, our research shows that Ephedra Herb also has ephedrine alkaloid-independent pharmacological actions, such as c-MET inhibitory activity. This study describes the preparation of an ephedrine alkaloids-free Ephedra Herb extract (EFE) by ion-exchange column chromatography, as well as in vitro and in vivo evaluation of its pharmacological actions and toxicity. We confirmed that EFE suppressed hepatocyte growth factor (HGF)-induced cancer cell motility by preventing both HGF-induced phosphorylation of c-Met and its tyrosine kinase activity. We also investigated the analgesic effect of EFE. Although the analgesic effect of Ephedra Herb has traditionally been attributed to pseudoephedrine, oral administration of EFE reduced formalin-induced pain in a dose-dependent manner in mice. Furthermore, we confirmed the anti-influenza virus activity of EFE by showing inhibition of MDCK cell infection in a concentration-dependent manner. All assessments of toxicity, even after repeated oral administration, suggest that EFE would be a safer alternative to Ephedra Herb. The findings described here suggest that EFE has c-Met inhibitory action, analgesic effect, and anti-influenza activity, and that it is safer than Ephedra Herb extract itself. Therefore, EFE could be a useful pharmacological agent.

Keywords: Ephedra Herb, c-Met, influenza virus

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Takakura M, Tada M, Ishii-Watabe A: Development of cell-based assay for predictively evaluating the Fc $\gamma$ R-mediated human immune cell activation by therapeutic monoclonal antibodies.

*Biochem Biophys Res Commun*. 2017;485:189-94

Therapeutic monoclonal antibodies (mAbs) have important roles in treatments for various cancers and inflammatory diseases. Their highly target specificities provide controlled safety profiles. However, therapeutic mAbs commonly pose a risk of the induction of the release of cytokines, which may result in adverse events including infusion reaction and cytokine release syndrome. Several mechanisms are involved in the cytokine releases induced by therapeutic mAbs,

and the activation of immune effector cells via Fc γ receptors (Fc γ Rs) is one of the putative mechanisms for most IgG-subclass mAbs. The relationship between cytokine releases and mAbs' Fc functions is not fully understood. Here we developed a simple reporter cell-based assay for estimating the Fc γ R-mediated activation of human immune effector cells by mAbs. Our use of the cell-based assay to compare Fc-engineered mAbs with different Fc γ R-activation profiles revealed that the releases of inflammatory cytokines and chemokines from human peripheral blood mononuclear cells (hPBMCs) induced by the mAbs were elevated by treatment with Fc-engineered mAbs with higher Fc γ R-activation properties. Our results also suggested the involvement of monocytic effector cells in the activation of hPBMCs as sources of released cytokines and chemokines, which may lead to the immune cell-mediated adverse events. Our new reporter cell assay is a promising tool for evaluating and predicting the activation of human immune cells by novel Fc-engineered mAbs.

Keywords: monoclonal antibody, Fc γ receptor, immune cell activation

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*生薬学雑誌*　2016;70:51-6

Bamboo Leaf, the leaf of *Phyllostachys nigra* Munro var. *henonis* Stapf ex Rendle, *P. bambusoides* Siebold et Zuccarini, *Bambusa textilis* McClure or *B. emeiensis* L. C. Chia et H. L. Fung, is used as a crude drug in the Kampo medicine, Chikuyo-sekko-to. Lophatherum Herb, which is derived from the leaves of *Lophatherum gracile* Brongniart, is a crude drug similar to bamboo leaf. There has been a concern over the misuse of these crude drugs. Recently, because Chikuyo-sekko-to has been approved by the Ministry of Health, Labour and Welfare as an OTC Kampo formula, we investigated the standardization of bamboo leaf and decided to list it in the non-JP crude drug standards 2015. In the process of the standardization, we designed identification and purity tests for the crude drug by using TLC. In addition, each indicator spot of the tests was isolated from bamboo leaf and Lophatherum Herb using repeated column chromatography and HPLC. The chemical structures of these spots were elucidated as *p*-coumaric acid (**1**) and *trans*-aconitic acid (**2**) based on m.p. and spectroscopic data including 1D- and 2D-NMR and MS. The established TLC conditions were chromatographic support, silica gel; developing solvent, EtOAc/hexane/acetic acid (20/20/1) for the identification test, EtOAc/H2O/formic acid (10/1/1) for the purity test; developing length, 7 cm; visualization, 4-methoxybenzaldehyde-sulphuric acid reagent for the identification test, UV (254 nm) for the purity test; Rf values, 0.4 (*p*-coumaric acid; **1**) for the identification test, 0.6 (*trans*-aconitic acid; **2**) for the purity test.

Keywords: bamboo leaf, Lophatherum Herb, TLC

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Tokumoto H, Shimomura H, Hakamatsuka T, Ozeki Y*, Goda Y: Detection of *Nicotiana tabacum* leaf contamination in pharmaceutical products.

*Biol Pharm Bull.* 2016;39:263-72

*Nicotiana tabacum* (Solanaceae) is the only species whose leaves can be legally marketed as tobacco according to the Japanese Tobacco Business Act. Nicotine, a major alkaloid produced by *N. tabacum* leaves, is regulated in pharmaceuticals by the Japanese Pharmaceutical Affairs Law. However, the use of *N. tabacum* stems as an excipient in pharmaceuticals is permitted, because these contained only a small amount of nicotine. Recently, several reports showed that a substantial amount of nicotine was detected in an OTC pharmaceutical product, in which *N. tabacum* stems were used as an excipient. Therefore, products containing *N. tabacum* stems could be contaminated with the leaf material. In the present study, we established a method to detect contamination of *N. tabacum* stem materials with its leaves, using microscopy to obtain standard reference microphotographs for identification. Cultivated *N. tabacum* stems and leaves, commercial cigarette leaves,

and *N. tabacum* tissue imported as excipient material were used for preparing the microphotographs. The characteristic *N. tabacum* leaf structures found in the powdered fragments included: epidermal cells with sinuous anticlinal cell walls, hairs, mesophyll parenchyma with crystalized calcium oxalate (calciphytoliths), and branching vascular bundles derived from reticulate net-veins. A comparison of the microscopic characteristics of an OTC powder with those from the standard reference microphotographs was an effective method for *N. tabacum* stem and leaf identification. Thus, we evaluated the powdered pharmaceutical product containing *N. tabacum* stem tissue and *Hydrangea serrata* (Hydrangeaceae) leaf tissue as excipients, and confirmed the presence of *N. tabacum* leaf material.

Keywords: microscopic morphology, *Nicotiana tabacum*, nicotine

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佐藤（増本）直子，粂田幸恵，内山奈穂子，袴塚高志：薄層クロマトグラフィー及び高速液体クロマトグラフィーを用いた単味生薬エキス製剤の品質確保に資する評価法－平成27年薬生審査1225第6号通知「生薬のエキス製剤の製造販売承認申請に係るガイダンスについて」の適用例について－.

*医薬品医療機器レギュラトリーサイエンス* 2017;48:186-94

平成27年12月，長年医薬品として用いられてきた単味生薬をエキス製剤として承認する道筋として，薬生審査発1225第6号通知が発出された．本通知においてエキス製剤の品質を担保する上で特に重要な項目である定量法では，各生薬エキスにつき原則2つの指標成分について定量することが規定されている．しかし日本薬局方収載生薬において定量成分は1つであり，複数の指標成分定量は前例がない．本研究では，通知収載生薬エキスのうち分析法設定に特に注意を要したモクツウエキスとソウハクヒエキスについて，それぞれ指標成分候補を2つ以上選定し，TLCを用いた確認試験及びHPLCを用いた定量法の分析条件を検討した．

Keywords：単味生薬エキス製剤，TLC，HPLC

Masada S, Takahashi Y*, Goda Y, Hakamatsuka T: Qualitative and Quantitative Evaluation of Drug and Health Food Products Containing Red Vine Leaf Extracts on the Japanese Market.

*Chem Pharm Bull.* 2016;64:1275-80

We analyzed OTC drug and health food products containing RVLEs with different lot numbers by LC/MS. Subsequent multivariate analyses clearly indicated that the quality of the health food products was highly variable compared to that of the drug products. Surprisingly, the component contents in the health foods were different even within a same lot in a same brand. The quantitative analyses of flavonols and stilbene derivatives in the drugs and health foods indicated that the concentration of each substance was kept constant in the drugs but not in the health foods. These results strongly indicated that the quality of RVLEs as a whole was not properly controlled in the manufacturing process of health foods. Since RVLE is an active ingredient with pharmaceutical evidences and is used for drugs, the proper regulation for ensuring the consistent quality of RVLEs from product to product would be recommended even in the health foods.

Keywords: direct OTC drug, health food product, red vine leaf extract

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Masada S, Uchiyama N, Goda Y, Hakamatsuka T: An analysis of anthocyanins in “Foods with Functional Claims” containing bilberry.

*日本食品化学学会誌* 2017;24:32-8

We evaluated the quality and quantity of 5 Foods with Functional Claims (FFCs) whose functional substances are bilberry anthocyanins. Twenty compounds (15 anthocyanins and 5 anthocyanidins) in the FFCs were separated by the HPLC method according to the European Pharmacopoeia instead of the journal featuring dietary supplements. Cyanidin-3-rutinoside was detected in 3 FFCs containing black currant extracts as well as bilberry extracts, and the anthocyanins in these FFCs were considered to be derived from both plants. Since the bilberry extracts are the active ingredient with pharmaceutical evidence and its health benefits are displayed on the product’s packaging, the proper regulation for ensuring a consistent quality of bilberry-containing FFCs would be recommended.

Keywords: bilberry, anthocyanin, foods with functional

claims

Tanaka R, Shibata H*, Sugimoto N, Akiyama H, Nagatsu A*: Application of quantitative $^1$H-NMR method for the determination of paeonol in Moutan cortex, Hachimijiogan and Keishibukuryogan.
*J Nat Med*. 2016;70:797-802

Quantitative $^1$H-NMR ($^1$H-qNMR) was applied to the determination of paeonol concentration in Moutan cortex, Hachimijiogan, and Keishibukuryogan. Paeonol is a major component of Moutan cortex, and its purity was calculated from the ratio of the intensity of the paeonol H-3' signal at $\delta$ 6.41 ppm in methanol-$d_4$ or 6.40 ppm in methanol-$d_4$ + TFA-$d$ to that of a hexamethyldisilane (HMD) signal at 0 ppm. The concentration of HMD was corrected with SI traceability by using potassium hydrogen phthalate of certified reference material grade. As a result, the paeonol content in two lots of Moutan cortex as determined by $^1$H-qNMR was found to be 1.59 % and 1.62 %, respectively, while the paeonol content in Hachimijiogan and Keishibukuryogan was 0.15 % and 0.22 %, respectively. The present study demonstrated that the $^1$H-NMR method is useful for the quantitative analysis of crude drugs and Kampo formulas.
Keywords: paeonol, quantitative $^1$H-NMR, Moutan cortex

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Tanaka R, Inagaki R*, Sugimoto N, Akiyama H, Nagatsu A*: Application of quantitative $^1$H-NMR ($^1$H-qNMR) method for the determination of geniposidic acid and acteoside in Plantaginis semen.
*J Nat Med*. 2017;71:315-20

A quantitative $^1$H-NMR method ($^1$H-qNMR) was used to determine the concentration of acteoside and geniposidic acid in Plantaginis semen. The purity of geniposidic acid and acteoside was determined by the ratio of the intensity of the H-3 signal at $\delta$ 7.51 ppm or the H-7" signal at $\delta$ 7.58 ppm in methanol-$d_4$ to that of a hexamethyldisilane (HMD) signal at 0.04 ppm, respectively. The concentration of HMD was corrected with International System of Units (SI) traceability by using potassium hydrogen phthalate (PHP) of certified reference material (CRM) grade. As a result, the geniposidic acid content in two lots of Plantaginis semen as determined by $^1$H-qNMR was found to be 0.84 % and 1.00 %, respectively. And the acteoside content in two lots of Plantaginis semen was 0.80 % and 0.93 %, respectively. We demonstrated that this method is useful for the quantitative analysis of crude drugs.
Keywords: geniposidic acid, acteoside, quantitative $^1$H-NMR

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Tohyama S*, Fujita J*, Hishiki T*, Matsuura T*, Hattori F*, Ohno R*, Kanazawa H*, Seki T*, Nakajima K*, Kishino Y*, Okada M*, Hirano A*, Kuroda T, Yasuda S, Sato Y, Yuasa S*, Sano M*, Suematsu M*, Fukuda K*: Glutamine oxidation is indispensable for survival of human pluripotent stem cells.
*Cell Metabolism*. 2016;23:663-74

Human pluripotent stem cells (hPSCs) are uniquely dependent on aerobic glycolysis to generate ATP. However, the importance of oxidative phosphorylation (OXPHOS) has not been elucidated. Detailed amino acid profiling has revealed that glutamine is indispensable for the survival of hPSCs. Under glucose- and glutamine-depleted conditions, hPSCs quickly died due to the loss of ATP. Metabolome analyses showed that hPSCs oxidized pyruvate poorly and that glutamine was the main energy source for OXPHOS. hPSCs were unable to utilize pyruvate-derived citrate due to negligible expression of aconitase 2 (ACO2) and isocitrate dehydrogenase 2/3 (IDH2/3) and high expression of ATP-citrate lyase. Cardiomyocytes with mature mitochondria were not able to survive without glucose and glutamine, although they were able to use lactate to synthesize pyruvate and glutamate. This distinguishing feature of hPSC metabolism allows preparation of clinical-grade cell sources free of undifferentiated hPSCs, which prevents tumor formation during stem cell therapy.
Keywords: hiPSCs, metabolome analyses, cardiomyocyte

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Kono K, Hiruma H, Kobayashi S*, Sato Y, Tanaka M*, Sawada R, Niimi S: *In vitro* endothelialization test of biomaterials using immortalized endothelial cells.

*PLOS ONE*. 2016;11(6):e0158289

Functionalizing biomaterials with peptides or polymers that enhance recruitment of endothelial cells (ECs) can reduce blood coagulation and thrombosis. To assess endothelialization of materials *in vitro*, primary ECs are generally used, although the characteristics of these cells vary among the donors and change with time in culture. Recently, primary cell lines immortalized by transduction of simian vacuolating virus 40 large T antigen or human telomerase reverse transcriptase have been developed. To determine whether immortalized ECs can substitute for primary ECs in material testing, we investigated endothelialization on biocompatible polymers using three lots of primary human umbilical vein endothelial cells (HUVEC) and immortalized microvascular ECs, TIME-GFP. Attachment to and growth on polymer surfaces were comparable between cell types, but results were more consistent with TIME-GFP. Our findings indicate that TIME-GFP is more suitable for *in vitro* endothelialization testing of biomaterials.

Keywords: endothelialization, biocompatible polymers, TIME-GFP

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Hasebe-Takada N, Kono K, Yasuda S, Sawada R, Matsuyama A*, Sato Y: Application of cell growth analysis to the quality assessment of human cell-processed therapeutic products as a testing method for immortalized cellular impurities.

*Regenerative Therapy*. 2016;5:49-54

In human cell-processed therapeutic products (hCTPs) for clinical application, tumorigenic cellular impurities in the manufacturing process are a major concern. Because cellular immortalization is one of the prerequisite steps in tumorigenesis, we tested whether cell growth analysis can be employed to check for immortalized (and potentially tumorigenic) cellular impurities in hCTPs. We monitored the growth of human bone marrow-derived mesenchymal stem cells (BMSCs) mixed with HeLa cells at a ratio of 1/106 or more and compared their growth rates with that of BMSCs alone. The cell growth analysis detected a significant increase in the growth rate of the BMSCs spiked with 0.0001% HeLa within 30 days at a probability of 47%. When human adipose-derived stem cells (ADSCs) were spiked with ASC52telo cells, a human telomerase reverse transcriptase (hTERT) -immortalized adipose-derived mesenchymal stem cell line, at a ratio of 0.001% or more, their growth rates were significantly increased within 15 passages, compared with that of ADSCs alone. These results indicate that cell growth analysis for the detection of immortalized cellular impurities in human somatic stem cells is simple and can be useful for the quality assessment of hCTPs in the manufacturing process.

Keywords: regenerative medicine, tumorigenicity, quality assessment

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Hayakawa T*[1], Harris I*[2], Joung J*[3], Kanai N*[4], Kawamata S*[5], Kellathur S*[6], Koga J*[7], Lin YC*[8], Maruyama Y*[9], McBlane J*[10], Nishimura T*[9], Renner M*[11], Ridgway A*[12], Salmikangas P*[13], Sakamoto N*[9], Sato D*[14], Sato Y, Toda Y*[15], Umezawa A*[16], Werner M*[17], Wicks S*[18]: Report of the International Regulatory Forum on Human Cell Therapy and Gene Therapy Products.

*Biologicals*. 2016;44(5):467-79

The development of human cell therapy and gene therapy products has progressed internationally. Efforts have been made to address regulatory challenges in the evaluation of quality, efficacy, and safety of the products. In this forum, updates on the specific challenges in quality, efficacy, and safety of products in the view of international development were shared through the exchange of information and opinions among experts from regulatory authorities, academic institutions, and industry practitioners. Sessions identified specific/critical points to consider for the evaluation of human cell therapy and gene therapy products that are different from conventional biological products; common approaches and practices among regulatory regions were also shared. Certain elements of current international guidelines might not be appropriate to be applied to these products. Further, international discussion on the concept of potency and in vivo tumorigenicity studies, among others, is needed. This forum concluded that the continued collective actions are expected to promote international convergence of regulatory approaches

of the products. The Pharmaceuticals and Medical Devices Agency and Japanese Society for Regenerative Medicine jointly convened the forum with support from the National Institutes of Biomedical Innovation, Health and Nutrition. Participants at the forum include 300 experts in and outside of Japan.

Keywords: cell therapy, gene therapy, regulation

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Kitajima N*1,2, Numaga-Tomita T*1,3, Watanabe M*4, Kuroda T, Nishimura A*1,3, Miyano K*5, Yasuda S, Kuwahara K*6, Sato Y, Ide T*7, Birnbaumer L*8,9, Sumimoto H*5, Mori Y*10, Nishida M*1,2,3: TRPC3 positively regulates reactive oxygen species driving maladaptive cardiac remodeling.

*Sci Rep.* 2016;6:37001

Reactive oxygen species (ROS) produced by NADPH oxidase 2 (Nox2) function as key mediators of mechanotransduction during both physiological adaptation to mechanical load and maladaptive remodeling of the heart. This is despite low levels of cardiac Nox2 expression. The mechanism underlying the transition from adaptation to maladaptation remains obscure, however. We demonstrate that transient receptor potential canonical 3 (TRPC3), a Ca2+-permeable channel, acts as a positive regulator of ROS (PRROS) in cardiomyocytes, and specifically regulates pressure overload-induced maladaptive cardiac remodeling in mice. TRPC3 physically interacts with Nox2 at specific C-terminal sites, thereby protecting Nox2 from proteasome-dependent degradation and amplifying Ca2+-dependent Nox2 activation through TRPC3-mediated background Ca2+ entry. Nox2 also stabilizes TRPC3 proteins to enhance TRPC3 channel activity. Expression of TRPC3 C-terminal polypeptide abolished TRPC3-regulated ROS production by disrupting TRPC3-Nox2 interaction, without affecting TRPC3-mediated Ca2+ influx. The novel TRPC3 function as a PRROS provides a mechanistic explanation for how diastolic Ca2+ influx specifically encodes signals to induce ROS-mediated maladaptive remodeling and offers new therapeutic possibilities.

Keywords: reactive oxygen species, maladaptive cardiac remodeling, cardiomyocyte

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Numaga-Tomita T*[1,2], Kitajima N*[1,3], Kuroda T, Nishimura A*[1,2], Miyano K*[4], Yasuda S, Kuwahara K*[5], Sato Y, Ide T*[6], Birnbaumer L*[7,8], Sumimoto H*[4], Mori Y*[9], Nishida M*[1,2,3,10]: TRPC3-GEF-H1 axis mediates pressure overload-induced cardiac fibrosis.
*Sci Rep*. 2016;6:39383

Structural cardiac remodeling, accompanying cytoskeletal reorganization of cardiac cells, is a major clinical outcome of diastolic heart failure. A highly local Ca2+ influx across the plasma membrane has been suggested to code signals to induce Rho GTPase-mediated fibrosis, but it is obscure how the heart specifically decodes the local Ca2+ influx as a cytoskeletal reorganizing signal under the conditions of the rhythmic Ca2+ handling required for pump function. We found that an inhibition of transient receptor potential canonical 3 (TRPC3) channel activity exhibited resistance to Rho-mediated maladaptive fibrosis in pressure-overloaded mouse hearts. Proteomic analysis revealed that microtubule-associated Rho guanine nucleotide exchange factor, GEF-H1, participates in TRPC3-mediated RhoA activation induced by mechanical stress in cardiomyocytes and transforming growth factor (TGF) $\beta$ stimulation in cardiac fibroblasts. We previously revealed that TRPC3 functionally interacts with microtubule-associated NADPH oxidase (Nox) 2, and inhibition of Nox2 attenuated mechanical stretch-induced GEF-H1 activation in cardiomyocytes. Finally, pharmacological TRPC3 inhibition significantly suppressed fibrotic responses in human cardiomyocytes and cardiac fibroblasts. These results strongly suggest that microtubule-localized TRPC3-GEF-H1 axis mediates fibrotic responses commonly in cardiac myocytes and fibroblasts induced by physico-chemical stimulation.
Keywords: reactive oxygen species, maladaptive cardiac remodeling, cardiomyocyte

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Hattori T, Watanabe-Takahashi M*[1], Shiina I*[2], Ohashi Y*[3], Dan S*[3], Nishikawa K*[1], Yamori T*[4], Naito M: M-COPA, a novel Golgi system disruptor, suppresses apoptosis induced by Shiga toxin.
*Genes Cells*. 2016;21:901-6

Shiga toxin (Stx) is a main virulence factor of Stx-producing Escherichia coli (STEC) that contributes to diarrhea and hemorrhagic colitis and occasionally to fatal systemic complications. Therefore, the development of an antidote to neutralize Stx toxicity is urgently needed. After internalization into cells, Stx is transferred to the Golgi apparatus via a retrograde vesicular transport system. We report here that 2-methylcoprophilinamide (M-COPA), a compound that induces disassembly of the Golgi apparatus by inactivating ADP-ribosylation factor 1 (Arf1), suppresses Stx-induced apoptosis. M-COPA inhibited transport of Stx from the plasma membrane to the Golgi apparatus and suppressed degradation of anti-apoptotic proteins and the activation of caspases. These findings suggest that inhibition of Stx retrograde transport by M-COPA could be a novel approach to suppress Stx toxicity.
Keywords: shiga toxin, apoptosis, M-COPA

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Furihata C, Watanabe T*[1], Suzuki T, Hamada S*[2], Nakajima M*[3]: Collaborative studies in toxicogenomics in rodent liver in JEMS·MMS; a useful application of principal component analysis on toxicogenomics.

*Genes and Environment*. 2016;38:15

As a collaborative study group of JEMS·MMS, we conducted studies on hepatocarcinogens in rodent liver in which 100 candidate marker genes were selected to discriminate genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens. Differential gene expression induced by 13 chemicals were examined using DNA microarray and quantitative real-time PCR (qPCR). We successfully showed discrimination of eight genotoxic hepatocarcinogens from four non-genotoxic hepatocarcinogens using qPCR and principal component analysis. The present review of these studies suggests that application of principal component analysis on the gene expression profile in rodent liver during the acute phase is useful to predict genotoxic hepatocarcinogens in comparison to non-genotoxic hepatocarcinogens and/or non-carcinogenic hepatotoxins.

Keywords: toxicogenomics, hepatocarcinogen

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Tsukumo Y, Alain T[*2], Fonseca BD[*2], Nadon R[*3], Sonenberg N[*1]: Translation control during prolonged mTORC1 inhibition mediated by 4E-BP3.

*Nat Commun*. 2016;7:11776

Targeting mTORC1 is a highly promising strategy in cancer therapy. Suppression of mTORC1 activity leads to rapid dephosphorylation of eIF4E-binding proteins (4E-BP1-3) and subsequent inhibition of mRNA translation. However, how the different 4E-BPs affect translation during prolonged use of mTOR inhibitors is not known. Here we show that the expression of 4E-BP3, but not that of 4E-BP1 or 4E-BP2, is transcriptionally induced during prolonged mTORC1 inhibition in vitro and in vivo. Mechanistically, our data reveal that 4E-BP3 expression is controlled by the transcription factor TFE3 through a cis-regulatory element in the EIF4EBP3 gene promoter. CRISPR/Cas9-mediated EIF4EBP3 gene disruption in human cancer cells mitigated the inhibition of translation and proliferation caused by prolonged treatment with mTOR inhibitors. Our findings show that 4E-BP3 is an important effector of mTORC1 and a robust predictive biomarker of therapeutic response to prolonged treatment with mTOR-targeting drugs in cancer.

Keywords: cancer, mTORC1, translation

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Tahmasebi S[*1], Jafarnejad SM[*1], Tam IS[*1], Gonatopoulos-Pournatzis T[*2], Matta-Camacho E[*1], Tsukumo Y, Yanagiya A[*1], Li W[*3], Atlasi Y[*4], Caron M[*5,6], Braunschweig U[*2], Pearl D[*1], Khoutorsky A[*7], Gkogkas CG[*8], Nadon R[*5,6], Bourque G[*5,6], Yang XJ[*1], Tian B[*3], Stunnenberg HG[*5], Yamanaka Y[*1], Blencowe BJ[*9], Giguère V[*1], Sonenberg N[*1]: Control of embryonic stem cell self-renewal and differentiation via coordinated alternative splicing and translation of YY2.

*Proc Natl Acad Sci U S A*. 2016;113:12360-7

Translational control of gene expression plays a key role during the early phases of embryonic development. Here we describe a transcriptional regulator of mouse embryonic stem cells (mESCs), Yin-yang 2 (YY2), that is controlled by the translation inhibitors, Eukaryotic initiation factor 4E-binding proteins (4E-BPs). YY2 plays a critical role in regulating mESC functions through control of key pluripotency factors, including Octamer-binding protein 4 (Oct4) and Estrogen-related receptor-$\beta$ (Esrrb). Importantly, overexpression of YY2 directs the differentiation of mESCs into cardiovascular lineages. We show that the splicing regulator Polypyrimidine tract-binding protein 1 (PTBP1) promotes the retention of an intron in the 5'-UTR of Yy2 mRNA that confers sensitivity to 4E-BP-mediated translational suppression. Thus, we conclude that YY2 is a major regulator of mESC self-renewal and lineage commitment and document a multilayer regulatory mechanism that controls its expression.

Keywords: 4E-BPs, embryonic stem cell, mRNA translation

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Yamamoto S*1, Hagiwara T*2, Horiuchi Y*2, Okui A*2, Wani S*1, Yoshida T, Inoue T, Tanaka A*1, Ito T*3, Hirose Y*1, Ohkuma Y*1: Mediator Cyclin-dependent kinases upregulate transcription of inflammatory genes in cooperation with NF-κB and C/EBPβ on stimulation of Toll-like receptor 9.
*Genes to Cells*. 2017;3:265-76

In eukaryotes, the Mediator complex has important roles in regulation of transcription by RNA polymerase II. Mediator is a large complex with more than 20 subunits that form head, middle, tail and CDK/cyclin modules. Among them, CDK8 and/or CDK19 (CDK8/19), and their counterpart cyclin C, form the CDK/cyclin module together with Mediator subunits MED12 and MED13. Despite evidences of both activation and repression, the precise functional roles of CDK8/19 in transcription are still elusive. Our previous results indicate that CDK8/19 recruits epigenetic regulators to repress immunoresponse genes. Here, this study focused on Toll-like receptors (TLRs), which exert innate immune responses through recognition of pathogen-associated molecular patterns and examined the functional roles of CDK8/19. As a result, CDK8/19 regulated transcription of inflammatory genes on stimulation of TLR9 in myeloma-derived RPMI8226 cells, which led to expression of inflammation-associated genes such as *IL8, IL10, PTX3* and *CCL2*. Mediator subunits CDK8/19 and MED1, inflammation-related transcriptional activator NF-κB and C/EBPβ, and general transcription factors TFIIE and TFIIB colocalized at the promoter regions of these genes under this condition. Our results show that CDK8/19 positively regulates inflammatory gene transcription in cooperation with NF-κB and C/EBPβ on stimulation of TLR9.
Keywords: NF-κB, C/EBPβ, TLR9

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Ohoka N, Nagai K*, Shibata N, Hattori T, Nara H*, Cho N*, Naito M: SNIPER (TACC3) induces cytoplasmic vacuolization and sensitizes cancer cells to Bortezomib.
*Cancer Sci*. 2017;in press:doi:10.1111/cas.13198

We previously developed a hybrid small molecule SNIPER (Specific and Nongenetic IAP-dependent Protein ERaser) against transforming acidic coiled-coil-3 (TACC3), SNIPER (TACC3), that induces proteasomal degradation of TACC3 protein. In this study, we found that SNIPER (TACC3) induces cytoplasmic vacuolization derived from endoplasmic reticulum (ER) and paraptosis-like cell death selectively in cancer cells. Mechanistic analysis suggests that accumulation of ubiquitylated protein aggregates that requires X-linked inhibitor of apoptosis protein (XIAP) induces ER stress, which results in ER-stress responses involving X-box binding protein-1 (XBP-1) and ER-derived vacuolization in cancer cells. Importantly, inhibition of proteasome enhanced the SNIPER (TACC3)-induced vacuolization, and the combination treatment of SNIPER (TACC3) and bortezomib exhibited a synergistic anticancer activity in several cancer cell lines. The induction of paraptosis-like cell death in cancer cells by SNIPER (TACC3) could be applied to treat cancer cells resistant to undergo apoptosis by overexpression of XIAP.
Keywords: XIAP, Bortezomib, SNIPER (TACC3)

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Ohoka N, Okuhira K*1, Ito M*2, Nagai K*2, Shibata N, Hattori T, Ujikawa O*2, Shimokawa K*2, Sano O*2, Koyama R*2, Fujita H*2, Teratani M*2, Matsumoto H*2, Imaeda Y*2, Nara H*2 Cho N*2, Naito M: *In Vivo* Knockdown of Pathogenic Proteins via Specific and Nongenetic Inhibitor of Apoptosis Protein (IAP)-dependent Protein Erasers (SNIPERs).
*J Biol Chem*. 2017;292:4556-70

Many diseases, especially cancers, result from aberrant or overexpression of pathogenic proteins. Specific inhibitors against these proteins have shown remarkable therapeutic effects, but these are limited mainly to enzymes. An alternative approach that may have utility in drug development relies on selective degradation of pathogenic proteins via small chimeric molecules linking an E3 ubiquitin ligase to the targeted protein for proteasomal degradation. To

this end, we recently developed a protein knockdown system based on hybrid small molecule SNIPERs (Specific and Nongenetic IAP-dependent Protein Erasers) that recruit inhibitor of the apoptosis protein (IAP) ubiquitin ligases to specifically degrade targeted proteins. Here, we extend our previous study to show a proof of concept of the SNIPER technology *in vivo*. By incorporating a high affinity IAP ligand, we developed a novel SNIPER against estrogen receptor α (ERα), SNIPER (ER)-87, that has a potent protein knockdown activity. The SNIPER (ER) reduced ERα levels in tumor xenografts and suppressed the growth of ERα-positive breast tumors in mice. Mechanistically, it preferentially recruits X-linked IAP (XIAP) rather than cellular IAP1, to degrade ERα via the ubiquitin-proteasome pathway. With this IAP ligand, potent SNIPERs against other pathogenic proteins, BCR-ABL, bromodomain-containing protein 4 (BRD4), and phosphodiesterase-4 (PDE4) could also be developed. These results indicate that forced ubiquitylation by SNIPERs is a useful method to achieve efficient protein knockdown with potential therapeutic activities and could also be applied to study the role of ubiquitylation in many cellular processes.

Keywords: LCL161, SNIPER, XIAP

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Okuhira K*1, Shoda T, Omura R*1, Ohoka N, Hattori T, Shibata N, Demizu Y, Sugihara R*1, Ichino A*1, Kawahara H*1, Itoh Y*2, Ishikawa M*2, Hashimoto Y*2, Kurihara M, Itoh S*3, Saito H*1, Naito M: Targeted Degradation of Proteins Localized in Subcellular Compartments by Hybrid Small Molecules.

*Mol Pharmacol.* 2017;91:159-66

Development of novel small molecules that selectively degrade pathogenic proteins would provide an important advance in targeted therapy. Recently, we have devised a series of hybrid small molecules named SNIPER (specific and nongenetic IAP-dependent protein ERaser) that induces the degradation of target proteins via the ubiquitin-proteasome system. To understand the localization of proteins that can be targeted by this protein knockdown technology, we examined whether SNIPER molecules are able to induce degradation of cellular retinoic acid binding protein II (CRABP-II) proteins localized in subcellular compartments of cells. CRABP-II is genetically fused with subcellular localization signals, and they are expressed in the cells. SNIPER (CRABP) with different IAP-ligands, SNIPER (CRABP)-4 with bestatin and SNIPER (CRABP)-11 with MV1 compound, induce the proteasomal degradation of wild-type (WT), cytosolic, nuclear, and membrane-localized CRABP-II proteins, whereas only SNIPER (CRABP)-11 displayed degradation activity toward the mitochondrial CRABP-II protein. The small interfering RNA-mediated silencing of cIAP1 expression attenuated the knockdown activity of SNIPER (CRABP) against WT and cytosolic CRABP-II proteins, indicating that cIAP1 is the E3 ligase responsible for degradation of these proteins. Against membrane-localized CRABP-II protein, cIAP1 is also a primary E3 ligase in the cells, but another E3 ligase distinct from cIAP2 and X-linked inhibitor of apoptosis protein (XIAP) could also be involved in the SNIPER (CRABP)-11-induced degradation. However, for the degradation of nuclear and mitochondrial CRABP-II proteins, E3 ligases other than cIAP1, cIAP2, and XIAP play a role in the SNIPER-mediated protein knockdown. These results indicate that SNIPER can target cytosolic, nuclear, membrane-localized, and mitochondrial proteins for degradation, but the responsible E3 ligase is different, depending on the localization of the target protein.

Keywords: SNIPER, localization, CRABP-II

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Haishima Y, Kawakami T, Fukui C, Tanoue A*1, Yuba T*2, Ozono S, Kumada H*3, Inoue K, Morikawa T, Takahashi M, Fujisawa A*4, Yamasaki K*5, Nomura Y, Isama K, Chung U*4, Ogawa K, Niimi S, Yoshida M: Characterization of alternative plasticizers in polyvinyl chloride sheets for blood containers.

*J Vinyl Add Technol.* 2016;22:520-8

This study aimed to optimize the ratio of dioctyl 4-cyclohexene-1,2-dicarboxylate (DOTH) and di-isononyl-cyclohexane-1,2-dicarboxylate (DINCH) for

use as plasticizers in poly（vinyl chloride）（PVC）sheets. We also evaluated the biological safety of DOTH for its potential to be part of a safe PVC-based blood container. The results suggest that DOTH/DINCH（25:33）is a promising candidate for the replacement of di（2-ethylhexyl）phthalate in blood containers.

Keywords: PVC medical device, blood container, hemolysis

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Watanabe S*1, Matsumura K*1, Iwai H*1, Funatogawa K*2, Haishima Y, Fukui C, Okumura K*3, Kato-Miyazawa M, Hashimoto M*4, Teramoto K*1, Kirikae F*1, Miyoshi-Akiyama T*5, Kirikae T*1 : A Mutation in the 16S rRNA Decoding Region Attenuates the Virulence of Mycobacterium tuberculosis.

*Infect Immun*. 2016;84:2264-73

We describe an rRNA mutation, U1406A, which was generated in vitro and confers resistance to kanamycin while highly attenuating M. tuberculosis virulence. The mutant showed an increase in 17S rRNA（precursor 16S rRNA）and a decrease in the ratio of 30S subunits to the 70S ribosomes, suggesting that the U1406A mutation in 16S rRNA attenuated M. tuberculosis virulence by affecting these processes.

Keywords: Mycobacterium tuberculosis, ribosome maturation, vaccine

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Haishima Y, Hasegawa C, Todoki K*1, Sasaki K*2, Niimi S, Ozono S: A biological study establishing the endotoxin limit of biomaterials for bone regeneration in cranial and femoral implantation of rats.

*J Biomed Mater Res. Part B* 2016;6 April

The purpose of this study was to accurately quantify the risk of endotoxin contamination in biomaterials for bone regeneration in order to establish the acceptable endotoxin limit. The results suggest that endotoxins may affect the process of osteoanagenesis. Additionally, the no-observed-adverse-effect level（NOAEL）was 9.6 EU/mg, corresponding to 255 EU/kg body weight in rats.

Keywords: endotoxin limit, bone regeneration, biomaterial

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Morishita Y, Yoshioka Y*1, Takimura Y*1, Shimizu Y*1, Namba Y*1, Nojiri N*1, Ishizaka T*1, Takao K*2,3, Yamashita F*4, Takuma K*1, Ago Y*1, Nagano K*1, Mukai Y*5,6, Kamada H*1,5, Tsunoda S *1,5,7, Saito S*3, Matsuda T*1, Hashida M*4, Miyakawa T*2,8, Higashisaka K*1, Tsutsumi Y*1,5: Distribution of Silver Nanoparticles to Breast Milk and Their Biological Effects on Breast-Fed Offspring Mice.

*ACS Nano*. 2016;10:8180-91

We used mice to investigate the safety of nanoparticle use during lactation. When Ag and Au nanoparticles were intravenously administered to lactating mice, the nanoparticles were distributed to breast milk without producing apparent damage to the mammary gland, and the amount of Ag nanoparticles distributed to breast milk increased with decreasing particle size. Orally administered Ag nanoparticles were also distributed to breast milk and subsequently to the brains of breast-fed pups.

Keywords: nanoparticle, breast milk, distribution

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Imai S[*1], Morishita Y, Hata T[*1], Kondoh M[*1], Yagi K[*1], Gao JQ[*2], Nagano K[*1], Higashisaka K[*1], Yoshioka Y[*1], Tsutsumi Y[*1,3]: Cellular internalization, transcellular transport, and cellular effects of silver nanoparticles in polarized Caco-2 cells following apical or basolateral exposure.

*Biochem Biophys Res Commun*. 2016;484:543-9

We examined cellular internalization and transcellular transport, and the effects of nanomaterials on Caco-2 monolayers after apical or basolateral exposure to Ag or Au nanoparticles with various sizes. The relationship between size of nanomaterials and cellular internalization or transcellular transfer was different between apical and basolateral exposure. Au nanoparticles showed different rules of internalization and transcellular transport compared with Ag nanoparticles. Basolateral exposure to Ag nanoparticles temporarily increased the paracellular permeability of Caco-2 monolayers.

Keywords: Caco-2, epithelial cell barrier, nanoparticle

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迫田秀行，新見伸吾，菅野伸彦[*]：抜去した人工関節超高分子量ポリエチレンコンポーネントに含まれる生体脂質の定量.

*臨床バイオメカニクス*　2016;37:9-13

Lipids such as squalene (SQ) have been reported to be absorbed in ultra-high molecular weight polyethylene (UHMWPE) components of joint prostheses during use in vivo. Since SQ absorption followed by accelerated aging has been reported to induce the degradation of UHMWPE in vitro, the in-vivo degradation of UHMWPE induced by lipids is suspected. In this study, 17 retrieved UHMWPE components from hip joint prostheses were used. Parameters such as the oxidation index (OI) and lipid index (LI) were obtained from the measurements using Fourier-transform infrared spectroscopy. Hexane extraction was carried out and the extracts from the UHMWPE components were weighed. Quantities of lipids such as triglycerides, cholesterols and cholesterol esters in the extracts were also measured using quantification kits.

LI was considered as a useful indicator of lipid absorption because there was a strong correlation between LI and the amount of the extracts. LI was not related to the duration of implantation or the patient body weight but it was significantly higher in components retrieved from osteoarthritis patients than those from osteonecrosis and fracture patients. It was considered, therefore, that the amount of lipids in the components is most affected by the constituents of individual synovial fluid, and that the lipid-induced degradation of UHMWPE is highly dependent on the condition of individual patients. However, only a portion of the extracts were identified and quantified in this study. Further studies to identify substances in the extracts and evaluate their effects on biological responses are considered to be necessary.

Keywords: joint prosthesis, UHMWPE, lipid-induced degradation

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Shinohara N[*1], Nakazato T[*1], Ohkawa K[*1], Tamura M[*1], Kobayashi N, Morimoto Y[*2], Oyabu T[*2], Myojo T[*2], Shimada M[*3], Yamamoto K[*1], Tao H[*1], Ema M[*1], Naya M[*1,4], Nakanishi J[*1]: Long-term retention of pristine multi-walled carbon nanotubes in rat lungs after intratracheal instillation.

*J Appl Toxicol*. 2016;36:501-9

We administered pristine MWCNTs well dispersed in 0.5 mg ml$^{-1}$ Triton-X solution to rats at doses of 0.20 or 0.55 mg via intratracheal instillation and investigated clearance over a 12-month observation period. The pristine MWCNTs pulmonary burden was determined 1, 3, 7, 28, 91, 175 and 364 days after instillation using a method involving combustive oxidation and infrared analysis.

Keywords: multi-walled carbon nanotube, intratracheal instillation, pulmonary clearance

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*環境科学会誌*　2016;29:147-58

水道水中のグルホシネート，グリホサートおよびAMPAのFMOC誘導体化－固相抽出－LC/MS/MS一斉分析法の妥当性を評価するため，9機関（衛生研究所3機関，登録検査機関3機関，水道事業体2機関および分析機器メーカー1機関）において，水道水を用いた添加回収試験を行った．各機関がそれぞれの所在地で採取した水道水に，3農薬をそれぞれ2.0および0.2 μg/Lとなるように添加した試料を本分析法に基づいて5回繰り返し分析し，各機関の分析条件を比較するとともに，検量線，選択性，真度，併行精度および室内精度について評価した．

試験の結果，1機関においては保有する装置の感度の問題から0.2 μg/L以下の濃度のグリホサートの定量が困難であったものの，他の8機関においては検量線試料の繰り返し測定の精度（RSD≦17%）および直線性（r2≧0.979）は良好であり，いずれの添加濃度の試験においても良好なピーク分離と定量に十分なピーク強度が得られた．真度および併行精度に関しては，グリホサートおよびAMPAについては全機関において，グルホシネートについては装置の感度が低かった1機関を除いて「水道水質検査方法の妥当性評価ガイドライン」の目標を満たした．さらに，いずれの試験においても室間精度が同ガイドラインの室内精度の目標を満たしたことから，本分析法は，水道水中のグルホシネート，グリホサートおよびAMPAの一斉分析用として有用と考えられる．

Keywords: glufosinate, glyphosate, AMPA

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Uchino T, Kuroda Y, Ishida S, Yamashita K[*1], Miyazaki H[*1], Oshikata A[*2], Shimizu K, Kojima H, Takezawa T[*2], Akiyama T, Ikarashi Y: Increase of β2-integrin on adhesion of THP-1 cells to collagen vitrigel membrane

*Biosci Biotechnol Biochem*. 2016;80:2271-6

When human monocyte-derived leukemia（THP-1）cells, which are floating cells, are stimulated with lipid peroxides, or Streptococcus suis, these cells adhere to a plastic plate or endothelial cells. However, it is unclear whether or not non-stimulated THP-1 cells adhere to collagen vitrigel membrane（CVM）. In this study, firstly, we investigated the rate of adhesion of THP-1 cells to CVM. When THP-1 cells were not stimulated, the rate of adhesion to CVM was high. Then, to identify adhesion molecules involved in adhesion of THP-1 cells to CVM, expressions of various cell adhesion molecules on the surface of THP-1 cells adhering to CVM were measured. β-actin, β-catenin, and β1-integrin expressions did not change in non-stimulated THP-1 cells cultured on CVM compared with those in cells cultured in a flask, but β2-integrin expression markedly increased.

Keywords: collagen vitrigel membrane, THP-1, β2-integrin

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*水道協会雑誌*　2016;85(10):7-21

水道水中のカルタップ，ピラクロニルおよびフェリムゾンのLC/MS/MSによる一斉分析法を確立するために，これら3農薬の一斉分析が可能な分析条件について検討した．

標準液を用いてLC/MS/MS分析条件を最適化した結果，3農薬とも良好なピーク形状と分離が得られる条件を確立することができた．また，アスコルビン酸ナトリウムあるいはチオ硫酸ナトリウムで脱塩素処理した水道水に，各農薬をそれぞれの目標値の1/10および1/100となるように添加した試料を分析した結果から，チオ硫酸ナトリウムで脱塩素処理することが適切であることが分かった．

さらに，本研究で確立した分析法の妥当性を評価するために，国立医薬品食品衛生研究所，水道事業体5機関，

登録検査機関3機関および分析機器メーカー2機関の合計11機関において，本分析法により水道水を用いた添加回収試験を行った．各機関が採取した水道水に，各農薬をそれぞれの目標値の1/10および1/100となるように添加した試料を分析し，各機関のLC/MS/MS分析条件を比較するとともに，検量線，選択性，真度，併行精度および室内精度について評価した．

試験の結果，検量線の直線性および繰り返し測定の精度は良好であった．また，選択性に関しても，いずれの添加濃度においても各農薬のピーク分離は良好であり，定量に十分なピーク強度が得られた．さらに，3農薬ともに全機関で真度および併行精度が「水道水質検査方法の妥当性評価ガイドライン」の目標を満たし，室間精度も同ガイドラインの室内精度の目標を満たした．以上のことから，本分析法は水道水中のカルタップ，ピラクロニルおよびフェリムゾンの一斉分析法として有用と考えられる．

Keywords: cartap, pyraclonil, ferimzone

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*水環境学会誌*　2016;39(6): 211-24

水道水中のホルムアルデヒドおよびアセトアルデヒドを迅速・簡便に分析するために，DNPHで誘導体化した試料をLC/UVあるいはLC/MS/MSにより測定する方法を検討した．検討の結果，水道水に塩化アンモニウムを加えて残留塩素を除去した後，リン酸とDNPHを加えて誘導体化した試料を測定した．いずれの測定機器を用いた場合も両誘導体のピークは短時間で良好に分離し，ホルムアルデヒドの基準値の1/10の濃度（0.008 mg $L^{-1}$）まで高精度に分析できた．さらに，本研究で確立した分析法が全国の水道水質検査に適用できるかどうかを検証するために，15機関において水道水を用いた添加回収試験を行った結果，いずれの測定機器を用いた場合も両物質について「水道水質検査方法の妥当性評価ガイドライン」の真度，併行精度および室内精度の目標を満たした．以上のことから，本分析法は水道水の標準検査法として利用可能と考えられる．

Keywords: formaldehyde, acetaldehyde, drinking water

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菅谷なえ子*，佐藤芳樹*，高橋美津子*，桜井克己*，河上強志：繊維製品に含まれるアゾ色素由来の特定芳香族アミンの分析及びその原因色素の探索．

*薬学雑誌*　2017;137:95-109

Twenty-four primary aromatic amines（PAAs）derived from azo colorants, which are controlled by the Act on Control of Household Products Containing Harmful Substances by the Japan Ministry of Health, Labour and Welfare, aniline and 1,4-phenylendiamine were analyzed in 86 samples of 40 textile products by GC-MS. Even though these PAAs detected in the samples did not exceed the regulation value (30 $\mu$g/g), 14 kinds of PAAs were detected that exceeded the limit of quantification. 4,4'-Methylenedianiline, in amounts that exceeded the limit of quantification, was detected in 20 textile samples containing synthesis fiber（16 samples made from polyurethane, two samples made from polyester, and two samples made from acryl); however, it was not detected in natural fiber textile samples. Of these samples, 4,4'-methylenedianiline was detected in 16 out of 19 samples（84%）made from polyurethane fiber. This

suggests that 4,4'-methylenedianiline is formed from polyurethane. The origin of 3,3′-dichlorobenzidine was investigated in three samples releasing more than 3 μg/g（3.9-15 μg/g）of 3,3'-dichlorobenzidine using atmospheric pressure solids analysis probe-mass spectrometry and Pigment Orange 13 was identified as the orange colorant in the textile printing parts. This result suggests that 3,3'-dichlorobenzidine detected in these three samples was generated by the reduction of Pigment Orange 13.

Keywords: Pigment Orange 13, textile, primary aromatic amine

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Saito-Shida S, Nemoto S, Matsuda R, Akiyama H: Simultaneous determination of seven anticoagulant rodenticides in agricultural products by gel permeation chromatography and liquid chromatography–tandem mass spectrometry.

*J Environ Sci Health B*. 2016;51:801-8

A sensitive and reliable method for the simultaneous determination of hydroxycoumarin-type（brodifacoum, bromadiolone, coumatetralyl, and warfarin）and indandione-type（chlorophacinone, diphacinone, and pindone）rodenticides in agricultural products by gel permeation chromatography（GPC）and liquid chromatography–tandem mass spectrometry（LC–MS/MS）was developed. The procedure involved extraction of the rodenticides from samples with acetone, followed by liquid–liquid partitioning with hexane/ethyl acetate（1:1, v/v）and 10% sodium chloride aqueous solution, then cleanup using GPC, and finally, analysis using LC–MS/MS. High recoveries from the GPC column were obtained for all rodenticides tested using a mobile phase of acetone/cyclohexane/triethylamine（400:1600:1, v/v/v）. An ODS column, which contains low levels of metal impurities, gave satisfactory peak shapes for both hydroxycoumarin- and indandione-type rodenticides in the LC–MS/MS separation. The average recoveries of rodenticides from eight agricultural foods（apple, eggplant, cabbage, orange, potato, tomato, brown rice, and soybean）fortified at 0.0005–0.001 mg/kg ranged from 76 to 116%, except for bromadiolone in orange（53%）and diphacinone in soybean（54%）, and the relative standard deviations ranged from 1 to 16%. The proposed method effectively removed interfering components, such as pigments and lipids, and showed high selectivity. In addition, the matrix effects were negligible for most of the rodenticide/food combinations. The results suggest that the proposed method is reliable and suitable for determining hydroxycoumarin- and indandione-type rodenticides in agricultural products.

Keywords: rodenticide, gel permeation chromatography, liquid chromatography–tandem mass spectrometry

小林麻紀＊，酒井奈穂子＊，上條恭子＊，大谷陽範＊，林真輝＊，小池裕＊，馬場糸子＊，笹本剛生＊，根本了，新藤哲也＊，高野伊知郎＊：LC-MS/MSによる畜水産物中のフルオピコリド分析法.

*食品衛生学雑誌* 2016;57(4):89-95

畜水産物中のフルオピコリド試験法について検討を行った．試料に塩化ナトリウムを加え，ギ酸酸性下でアセトン抽出し，ケイソウ土カラムで脱脂後，GC（グラファイトカーボン）およびPSA（エチレンジアミン-*N*-プロピルシリル化シリカゲル）カラムで精製して，LC-MS/MSで測定した．10種類の畜水産物（牛の筋肉，鶏の筋肉，牛の脂肪，牛の肝臓，鶏卵，牛乳，はちみつ，うなぎ，さけ，しじみ）を対象にして基準値濃度で添加回収試験を行った結果，真度（n=5）は96～100%，併行精度2.3～6.2%，定量限界は0.01 mg/kgを設定できた.

Keywords：フルオピコリド，畜水産物，LC-MS/MS

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Saito-Shida S, Nemoto S, Teshima R, Akiyama H: Determination of rodenticide tetramethylenedisulfotetramine（tetramine）in processed foods by gas chromatography-tandem mass spectrometry.

*Food Hyg Saf Sci*. 2016;57:72-5

A GC-MS/MS method for determination of the rodenticide tetramethylenedisulfotetramine was developed. Tetramethylenedisulfotetramine was extracted from the sample with ethyl acetate in the presence of anhydrous sodium sulfate. Then, an aliquot of the extract was evaporated under vaccum, followed by acetonitrile/hexane partitioning, and cleanup with a tandem graphitized carbon/primary secondary amine（PSA）column, prior to GC-MS/MS analysis. The recoveries from 10 processed foods, all of which were fortified at 0.1 mg/kg, were in the

range of 85–96%, and the relative standard deviations were less than 7%. The proposed method effectively removed co-extracted matrix components, and matrix effects were negligible for the GC-MS/MS analysis. In addition, no interfering peaks were found in the chromatograms of the blank samples at the retention time of tetramethylenedisulfotetramine, indicating that the method is highly selective. Overall results suggest that the proposed method is suitable for determining tetramethylenedisulfotetramine contained in processed foods.

Keywords: tetramethylenedisulfotetramine, rodenticide, GC-MS/MS

青柳光敏*，千葉真弘*，柿本洋一郎*，根本了：HPLC-FLによる農産物中のジフェニルアミンの分析法.

*食品衛生学雑誌*　2016;57(6):201-6

農産物中のジフェニルアミン分析法として，ジフェニルアミンを試料から酸性条件下アセトニトリルで抽出し，C18ミニカラムで精製した後*n*-ヘキサンに転溶，PSAミニカラムで精製した後，HPLC-FLで定量し，LC-MS/MSで確認する方法を開発した．開発した分析法を用いて，玄米，とうもろこし，大豆，ばれいしょ，キャベツ，なす，ほうれんそう，オレンジ，りんごおよび茶の10農産物に対し，残留基準値濃度での添加回収試験を行った結果，真度76.7～94.9%，併行精度0.6～5.8%の良好な結果が得られた．また，本試験法は一律基準値レベルの検出感度を有していたため，ジフェニルアミンの分析法として有用と思われた．

Keywords：ジフェニルアミン，農産物，蛍光検出器付き高速液体クロマトグラフ

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Kikuchi H, Sakai T, Teshima R, Nemoto S, Akiyama H: Total determination of chloramphenicol residues in foods by liquid chromatography-tandem mass spectrometry.

*Food Chemistry*. 2017;230:589-93

A simple and sensitive analytical method for the determination of chloramphenicol (CAP) and chloramphenicol glucuronide (CAPG) residues in foods such as livestock products, seafood, honey and royal jelly was developed. The method comprises solvent extraction with methanol, enzymatic hydrolysis with β-glucuronidase and clean-up using a hydrophilic lipophilic balanced copolymer solid phase extraction column. To determine the optimal conditions for the complete enzymatic hydrolysis of CAPG, we examined the effect of enzyme concentration and incubation time on the hydrolysis. The detection of CAP using LC-MS/MS was optimized and determined by SRM. The developed method was validated using ten food products at a spiked level of 0.5 μg/kg. The validation results show excellent recoveries (79-109%) and precision (<15%) for CAP and CAPG. The limit of quantification (S/N≥10) of the developed method was 0.5 μg/kg. The proposed method would be useful for the regulatory monitoring of CAP and CAPG residues in foods.

Keywords: chloramphenicol, chloramphenicol glucuronide, β-Glucuronidase

Uekusa Y, Takatsuki S, Tsutsumi T, Akiyama H, Matsuda R, Teshima R, Hachisuka A, Watanabe T: Determination of polychlorinated biphenyls in marine fish obtained from tsunami-stricken areas of Japan.

*PLOS ONE*. 2017;12:e0174961

We determined the polychlorinated biphenyl (PCB) congeners in 101 marine fish obtained from tsunami-stricken areas following the Great East Japan Earthquake in 2011. In particular, to determine the degree of PCB contamination in the fish, we investigated the concentration of total PCB ( ΣPCB) and the proportions of 209 individual PCB congeners by high-resolution gas chromatography/high-resolution mass spectrometry. The ΣPCB concentration was 1.7–33 ng/g in fat greenling (n = 29), 0.44–25 ng/g in flounder (n = 36), and 1.6–86 ng/g in mackerel (n = 36), all values being much lower than the provisional regulatory limit in Japan. In the congener analysis, tetra-, penta-, hexa-, and hepta-chlorinated PCB congeners dominated in all samples (comprising over 86% of the ΣPCB). The proportions of the chlorinated PCB congeners were similar to the contamination patterns derived from Kanechlor in the environment, implying that the marine fish were not contaminated with fresh PCBs.

Keywords: polychlorinated biphenyls, the Great East Japan Earthquake, fish

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Kamada H[*2,3], Tsunoda S[*2,3], Gao J[*4], Higashisaka K[*1], Yoshioka Y[*1,5,6], Tsutsumi Y[*1,3]: Modifying the Surface of Silica Nanoparticles with Amino or Carboxyl Groups Decreases Their Cytotoxicity to Parenchymal Hepatocytes.

*Biol Pharm Bull*. 2017;40:726-8

We previously reported that unmodified silica nanoparticles with diameters of 70 nm (nSP70) induced liver damage in mice, whereas nSP70 modified with carboxyl or amino groups did not. In addition, we have found that both unmodified and modified nSP70s localize in both Kupffer cells and parenchymal hepatocytes. We therefore evaluated the contributions of nSP70 uptake by these cell populations to liver damage. To this end, we pretreated mice with gadolinium (III) chloride hydrate (GdCl3) to prevent nSP70 uptake by Kupffer cells, subsequently injected the mice with either type of nSP70, and then assessed plasma levels of alanine aminotransferase (ALT). In mice given GdCl3, unmodified nSP70 increased ALT levels. From these data, we hypothesized that in GdCl3-treated mice, the unmodified nSP70 that was prevented from entering Kupffer cells was shunted to parenchymal hepatocytes, where it induced cytotoxicity and increased liver damage. In contrast, GdCl3 pretreatment had no effect on ALT levels in mice injected with surface-modified nSP70s, suggesting that modified nSP70s spared parenchymal hepatocytes and thus induced negligible liver damage. In cytotoxicity analyses, the viability of a parenchymal hepatocyte line was greater when exposed to surface-modified nSP70s than to unmodified nSP70s. These findings imply that the decreased liver damage associated with surface-modified compared with unmodified nSP70 is attributable to decreased cytotoxicity to parenchymal hepatocytes.

Keywords: surface-modification, liver damage, safety assessment

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片岡洋平，渡邉敬浩，松田りえ子，林智子，穐山浩，手島玲子：ミネラルウォーター類中の元素類一斉分析法の妥当性確認と実態調査.

*食品衛生学雑誌* 2017;58:59-64

ミネラルウォーター中の元素（B, Cr, Mn, Cu, Zn, As, Se, Cd, Ba, Pb）をICP-MSを用いて同時に分析する公定法の妥当性確認を行った．すべての分析対象元素において本法の真度は95～106%，併行精度は0.2～1.4%，室内精度は0.4～4.2%の範囲にあると推定され，本分析法は厚生労働省が示すガイドラインの目標値を満たす性能を有することが確認された．また，2013（平成25）年度と2014（平成26）年度に日本の市場に流通するミネラルウォーター類を買い上げ，それらに含まれる各種元素濃度を調査した．調査の結果，すべての試料における分析値は基準値より低い値であった．また，各種元素濃度には入手年や生産国との明らかな関係は見られなかった．

Keywords：ミネラルウォーター類，元素類，実態調査

渡邉敬浩，林智子，松田りえ子，穐山浩，手島玲子：食品として流通する魚の総水銀およびメチル水銀濃度の実態調査.

*食品衛生学雑誌* 2017;58(2):80-5

多くの魚にはメチル水銀が含まれるが，濃度は魚種によって異なる．そのため，魚種を選び適量を摂食することが肝要である．本研究では，妥当性を確認した分析法を用い，食品として流通していた19種（計210試料）の魚における総水銀およびメチル水銀濃度の実態を調査した．その結果，大型の捕食魚であるメカジキとクロマグロの一部試料において，総水銀とメチル水銀濃度がともに1 mg/kgを超えることが確認された．天然魚と養殖魚との濃度を比較した結果，クロマグロとブリの両魚種で，養殖魚における濃度がより低かった．魚種によらず，総水銀とメチル水銀濃度との間には正の相関があることが明らかとなり，効率的かつ見逃しのない検査には，0.3 mg/kgを閾値とする，総水銀濃度によるスクリーニングが有効と考えられた．

Keywords：メチル水銀，魚，実態調査

Akiyama H, Nose M[*1], Ohtsuki N, Hisaka S[*1], Takiguchi H, Tada A, Sugimoto N, Fuchino H[*2], Inui T[*2], Kawano N[*2], Hayashi S[*2], Hishida A[*2], Kudo T[*3], Sugiyama K, Abe Y, Mutsuga M, Kawahara N[*2], Yoshimatsu K[*2]: Evaluation of the safety and efficacy of *Glycyrrhiza uralensis* root extracts produced using artificial hydroponic and artificial hydroponic-field hybrid cultivation systems.

*J Na. Med*. 2017;71:265-71

*Glycyrrhiza uralensis* roots used in this study were produced using novel cultivation systems, including artificial hydroponics and artificial hydroponic-field hybrid cultivation. The equivalency between G. *uralensis* root extracts produced by hydroponics and/or hybrid cultivation and a commercial *Glycyrrhiza* crude drug were evaluated for both safety and efficacy, and there were no significant differences in terms of mutagenicity on the Ames tests. The levels of cadmium and mercury in both hydroponic roots and crude drugs were less than the limit of quantitation. Arsenic levels were lower in all hydroponic roots than in the crude drug, whereas mean lead levels in the crude drug were not significantly different from those in the hydroponically cultivated G. *uralensis* roots. Both hydroponic and hybrid-cultivated root extracts showed antiallergic activities against contact hypersensitivity that were similar to those of the crude drug extracts. These study results suggest that hydroponic and hybrid-cultivated roots are equivalent in safety and efficacy to those of commercial crude drugs. Further studies are necessary before the roots are applicable as replacements for the currently available commercial crude drugs produced from wild plant resources.

Keywords: *Glycyrrhiza uralensis*, Licorice, Glycyrrhizic acid

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Kodama H[*1], Tamura Y[*1], Kamei[*2], Sato K, Akiyama H: The solubility of microcrystalline cellulose in sodium hydroxide solution is inconsistent with international specifications.

*Biol Pharm Bull*. 2017;40:68-72

Microcrystalline cellulose (MCC) is used globally as an inactive ingredient in food and nutraceutical products and is commonly used as a food additive. To confirm the conformity of MCC to the solubility requirements stipulated in international specifications, the solubilities of commercially available MCC products were tested in sodium hydroxide (NaOH) solution. All of the samples were insoluble in NaOH solution, which is inconsistent with the descriptions provided in international specifications. We also prepared celluloses with different degree of polymerization (DP) values by acid hydrolysis. Celluloses with lower DP were prepared using a three-step process, and their solubilities were tested in NaOH solution. These celluloses were found to be insoluble, which is inconsistent with the descriptions provided in international specifications. The present study suggests that the descriptions of the solubility of the celluloses in NaOH solution found in the current international specifications should be revised.

Keywords: microcrystalline cellulose, international specification, solubility

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Nagai Y[*1], Kawano S[*1], Motoda K[*2], Tomida M[*1], Tatebe C, Sato K, Akiyama H: Solubility testing of sucrose esters of fatty acids in international food additive specifications.

*Biol Pharm Bull*. 2017;40:284-9

We investigated the solubility of 10 samples of sucrose esters of fatty acids (SEFA) products that are commercially available worldwide as food additives (emulsifiers). Although one sample dissolved transparently in both water and ethanol, other samples produced white turbidity and/or precipitates and did not meet the solubility criterion established by the Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives (JECFA). When the sample solutions were heated, the solubility in both water and ethanol increased. All of the samples dissolved transparently in ethanol, and dispersed and became white without producing precipitates in water. The present study suggests that the current solubility criterion of the JECFA SEFA specifications needs to be revised.

Keywords: sucrose fatty acid ester, solubility, emulsifier

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Suzuki I, Kubota H, Terami S, Hara T*1, Hirakawa Y*1, Iizuka T*1, Tatebe C, Ohtsuki T, Yano T*2, Sato K, Akiyama H: Development of an analytical method for copper chlorophyll and sodium copper chlorophyllin in processed foods.

*Jpn J Food Chem Safety*. 2016;23:55-62

The food colourants copper chlorophyll (CuCh) and sodium copper chlorophyllin (CuCh-Na) are used worldwide in a wide range of processed foods. We developed an analytical method for the determination of CuCh/CuCh-Na levels in processed foods to effectively monitor the appropriate use of these colourants. The proposed analytical method involves simultaneous extraction and parallel analysis of hydrophobic CuCh and hydrophilic CuCh-Na without harmful solvents. CuCh/CuCh-Na were extracted from processed foods with 1-butanol and ethyl acetate. CuCh-Na was extracted from the initial extraction solvent with 0.15 mol/L NaOH, and then the residual extraction solvent and alkaline water layers were dried. Finally, the samples were carbonized with anhydrous sulphate. The carbonized samples were ashed in a muffle furnace at 480°C. The residue was dissolved in 0.1 mol/L $HNO_3$, and the level of copper in the samples was determined using atomic absorption spectrophotometry to indirectly quantify the levels of CuCh and CuCh-Na. Recoveries of CuCh and CuCh-Na from spiked samples were in the range of 70.7%-80.8% and 55.6%-72.3% (except for white chocolate, at 50%), respectively, with standard deviations in the range of 1.7%-5.0% and 1.5%-7.8%, respectively. In commercial processed foods, the levels of CuCh and CuCh-Na were in the range not detected ND-3.7 mg/kg and ND-8.0 mg/kg as copper, respectively.

Keywords: copper chlorophyll, copper chlorophyllin, food colorant

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Suzuki I, Kubota H, Ohtsuki T, Tatebe C, Tada A, Yano T*, Akiyama H, Sato K: An IC-MS/MS method for the determination of 1-hydroxyethylidene-1,1-diphosphonic acid on uncooked foods treated with peracetic acid–based sanitizers.

*Chem Pharm Bull*. 2016;64:1713-9

A rapid, sensitive, and specific analytical method for the determination of 1-hydroxyethylidene-1,1-diphosphonic acid (HEDP) on uncooked foods after treatment with a peracetic acid–based sanitizer (PAS) was developed. The method involves simple sample preparation steps and analysis using ion chromatography (IC) coupled with tandem mass spectrometry (MS/MS). The quantification limits of HEDP on uncooked foods are 0.007 mg/kg for vegetables and fruits and 0.2 mg/kg for meats. The recovery and relative standard deviation (RSD) of HEDP analyses of uncooked foods ranged from 73.9 to 103.8% and 1.9 to 12.6%, respectively. The method's accuracy and precision were evaluated by inter-day recovery tests. The recovery for all samples ranged from 93.6 to 101.2%, and the within-laboratory repeatability and reproducibility were evaluated based on RSD values, which were less than 6.9 and 11.5%, respectively. Analyses of PAS-treated fruits and vegetables using the developed method indicated levels of HEDP ranging from 0.008 to 0.351 mg/kg. Therefore, the results of the present study suggest that the proposed method is an accurate, precise, and reliable way to determine residual HEDP levels on PAS-treated uncooked foods.

Keywords: 1-hydroxyethylidene-1,1-diphosphonic acid, peracetic acid, food additive

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Nishizaki Y, Ishizuki K, Akiyama H, Tada A, Sugimoto N, Sato K: Preparation of a ammonia-treated lac dye and structure elucidation of its main component.

*Food Hyg Saf Sci*. 2016;57:193-200

Lac dye and cochineal extract contain laccaic acids and carminic acid as the main pigments, respectively. Both laccaic acids and carminic acid are anthraquinone derivatives. 4-Aminocarminic acid (acid-stable carmine), an illegal colorant, has been

detected in several processed foods. 4-Aminocarminic acid is obtained by heating cochineal extract (carminic acid) in ammonia solution. We attempted to prepare ammonia-treated lac dye and to identify the structures of the main pigment components. Ammonia-treated lac dye showed acid stability similar to that of 4-aminocarminic acid. The structures of the main pigments in ammonia-treated lac dye were analyzed using LC/MS. One of the main pigments was isolated and identified as 4-aminolaccaic acid C using various NMR techniques, including 2D-INADEQUATE. These results indicated that ammonia-treatment of lac dye results in the generation of 4-aminolaccaic acids.

Keywords: lac dye, 4-aminolaccaoc acid, acid-stable carmine

Nakajima N*, Sugimoto N, Ohki K*, Kamiya M*: Diversity of phlorotannin profiles among sargassasacean species affecting variation and abundance of epiphytes.

*Eur J Phycol*. 2016;70:797-802

In general, epiphytes have detrimental effects on the growth of their basiphytes due to competition for light and nutrients. Therefore, basiphyte species must expend energy suppressing epiphytes. Some studies suggest that phlorotannins, i.e. brown algal polyphenols, prevent colonization by epiphytes, whereas others question their allelopathic function because there is not necessarily a negative correlation between epiphyte abundance and the phlorotannin content of the basiphyte algae. Various phlorotannin components are found in brown algal species, thus we hypothesized that the antifouling activities of polyphenolic compounds may differ and that the analysis of phlorotannin profiles could be useful for estimating their ecological functions. We surveyed the epiphyte richness in the apical portions of 373 thalli from 15 sargassacean species, demonstrating that the variation and abundance of epiphyte species differed remarkably among the basiphyte species. However, there was a weak negative correlation between the density and total phlorotannin content of the basiphyte algae in only one of the 18 epiphyte species. The interspecific differences in the phlorotannin profile were characterized by quantitative 1H nuclear magnetic resonance spectroscopy (qNMR), and four major groups were categorized based on cluster and principal component analyses of polyphenolic signals in the qNMR spectra. The epiphyte Neosiphonia harveyi was more abundant on Sargassum hemiphyllum, S. patens and S. piluliferum than on other basiphyte species, and these three species were similar according to the cluster analysis. These results suggest that some phlorotannin components may be more effective for antifouling; thus interspecific differences in the phlorotannin profile could affect the variation and abundance of epiphytes.

Keywords: allelopathy, antifouling effect, basiphyte

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Takahashi M*, Nishizaki Y, Sugimoto N, Takeuchi H*, Nakagawa K*, Akiyama H, Sato K, Inoue K*: Determination and purification of sesamin and sesamolin in sesame seed oil unsaponified matter using reversed-phase liquid chromatography coupled with photodiode array and tandem mass spectrometry and high-speed countercurrent chromatography.

*J Sep Sci*. 2016;39:3898-905

In Asian countries, sesame seed oil unsaponified matter is used as a natural food additive due to its associated antioxidant effects. We determined and purified the primary lignans sesamin and sesamolin in sesame seed oil unsaponified matter using reversed-phase liquid chromatography coupled with photodiode array and tandem mass spectrometry and high-speed countercurrent chromatography. Calibration curves showed good correlation coefficients (r2 > 0.999, range 0.08 and/or 0.15 to 5 μg/mL) with a limit of detection (at 290 nm) of 0.02 μg/mL for sesamin and 0.04 μg/mL for sesamolin. Sesame seed oil unsaponified matter contained 2.82% sesamin and 2.54% sesamolin, respectively. Direct qualitative analysis of sesamin and sesamolin was achieved using quadrupole mass spectrometry with positive-mode electrospray ionization. Pure (>99%) sesamin and sesamolin standards were obtained using high-speed countercurrent chromatographic purification (hexane/ethyl acetate/methanol/water; 7:3:7:3). An effective method for determining and purifying sesamin and sesamolin from sesame seed oil unsaponified matter was developed by combining these separation

techniques for standardized food additives.
Keywords: food additive, high-speed countercurrent chromatography, sesamin

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阿部裕，山口未来，六鹿元雄，穐山浩，河村葉子：ポリウレタン，ナイロンおよび布製玩具中の芳香族第一級アミン類および着色料の調査.
*日本食品衛生学雑誌* 2016;57:23-31

国内で流通するポリウレタンおよびナイロン製玩具中の芳香族第一級アミン類（PAAs）28成分の残存量および溶出量をLC-MS/MSを用いて測定した．また布製玩具のPAAsおよび着色料15成分については，欧州規格EN71を参考にLC-MS/MSもしくはLC-TOF/MSを用いて溶出量および残存量を測定した．ポリウレタン製玩具34検体では，12検体から2,6-ジアミノトルエンおよび2,4-ジアミノトルエンが同時に検出され，残存量はそれぞれ2.1～19.7および7.6～39.6 μg/gであった．また9検体から4,4'-ジアミノジフェニルメタン（4,4'-MDA），1検体からアニリンが検出され，残存量はそれぞれ0.2～8.7および0.4 μg/gであった．ナイロン8検体ではいずれのPAAsも検出されなかった．ポリウレタン製玩具について水を用いた溶出試験を行った結果，3検体から4,4'-MDAの溶出が認められ，溶出量は0.4～2.5 μg/gであった．一方，布製玩具43検体ではいずれのPAAsも溶出は認められなかったが，1検体から着色料のSolvent Yellow 1およびBasic Red 9が検出され，残存量は各0.02 μg/gであった．本研究で残存が認められたPAAsおよび着色料はいずれも欧州連合における濃度限度値よりも低い値であった.
Keywords：おもちゃ，第一級芳香族アミン類，着色料

Abe Y, Mutsuga M, Ohno H*, Kawamura Y, Akiyama H: Isolation and quantification of polyamide cyclic oligomers in kitchen utensils and their migration into various food simulants.
*PLOS ONE*. 2016;11:e0159547

Small amounts of cyclic monomers and oligomers are present in polyamide (PA)-based kitchen utensils. In this study, we isolated eight PA-based cyclic monomers and oligomers from kitchen utensils made from PA6 (a polymer of ε-caprolactam) and PA66 (a polymer of 1,6-diaminohexane and adipic acid). Their structures were identified using high-resolution mass spectrometry and 1H- and $^{13}$C-nuclear magnetic resonance spectroscopy, and their residual levels in PA-based kitchen utensils and degree of migration into food simulants were quantified by high-performance liquid chromatography/mass spectrometry using purchased PA6 monomer and isolated PA66 monomers, and isolated PA6 and PA66 oligomers as calibration standards. Their total residual levels among 23 PA-based kitchen utensils made from PA6, PA66, and copolymers of PA6 and PA66 (PA6/66) ranged from 7.8 to 20 mg/g. Using water, 20% ethanol, and olive oil as food simulants, the total migration levels of the PA monomers and oligomers ranged from 0.66 to 100 μg/cm$^2$ under most examined conditions. However, the total migration levels of the PA66 monomer and oligomers from PA66 and PA6/66 kitchen utensils into 20% ethanol at 95°C were very high (1,700 and 2,200 μg/cm$^2$, respectively) due to swelling by high-temperature ethanol.
Keywords: polyamide, oligomer migration

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阿部裕，六鹿元雄，河﨑裕美，山口未来，佐藤恭子，穐山浩：器具・容器包装の溶出試験における試験溶液調製時の温度制御に関する検討.
*日本食品化学学会誌* 2016;23:81-9

食品用器具および容器包装の溶出試験における試験溶液の調製における，種々の加温・加熱条件での浸出用液の温度を温度データロガーにより測定した．4種類の水浴を60℃に設定したときの浸出用液の温度は，装置が異なっていてもほぼ同じであり，15分後には60±2℃の範囲に収まった．同様に4種類の乾燥器を60℃に設定した場合，設定温度への温度上昇が水浴に比べ遅いだけではなく，設定温度に到達しないものもあった．また，浸出用液の温度が装置や加温する場所によっても異なっていた．浸漬法における60℃30分間の溶出操作では，試料を投入する際に浸出用液の温度が一時的に56.5~58.0℃まで低下した．その後，60℃の水浴を用いて加温した場合は速やかに温度が上昇し，60±2℃の範囲内に保持することができた．また，60℃の乾燥器を用いた場合，温度上昇が遅く，60±2℃に到達するのに15分以上かかった．しかしながら，65℃の乾燥機を用いた場合と，65℃にあらかじめ加温した浸出溶液を用いて60℃の乾燥器を用いた場合は60±2℃の範囲内に保持することができた．95℃30分間の溶出操作では，試料を投入する際に浸出用液の温度が一時的に85.8~90.9℃まで低下した．しかしながら，95℃の水浴および105，110および115℃の乾燥器を用いると95±5℃の範囲内に保持可能であった．一

方，片面溶出法における60℃30分間の溶出操作では，片面溶出器に浸出用液を注ぎ入れた際に浸出用液の温度が44.4~51.3℃まで大きく低下し，その後20分間以上経過しても60±2℃に到達しなかった．しかしながら，片面溶出器をあらかじめ60℃に加温してから浸出溶液を注ぎ入れた場合，温度は59.1~59.7℃とわずかに低下しただけだった．その後60℃の水浴もしくは70℃の乾燥器を用いた場合は60±2℃の範囲内に保持することができた．95℃30分間の溶出操作では，あらかじめ95℃に加温した片面溶出器に浸出用液を注ぎ入れた際に浸出用液の温度は89.2~90.1℃まで一時的に低下したが，その後95℃の水浴もしくは115℃の乾燥器を用いると95±5℃の範囲内に保持可能であった．試料としてゴム製手袋2検体，浸出用液として4%酢酸，試験溶液の調製法として浸漬法を用いて種々の加温条件で溶出試験を行い，蒸発残留物量，亜鉛，カルシウムの溶出量を比較した結果，浸出用液の温度が60±2℃または95±5℃の範囲内であれば，蒸発残留物量および溶出量に大きな差は生じなかった．

Keywords：容器包装，溶出試験，温度管理

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*食品衛生学雑誌*　2016;57:169-78

食品衛生法におけるポリスチレン製器具・容器包装の揮発性物質試験の性能を評価するため，ポリスチレン，アクリロニトリル・スチレン共重合樹脂，アクリロニトリル・ブタジエン・スチレン共重合樹脂のペレットを検体として試験室間共同試験を行った．当試験には21機関が参加し，3検体（各2測定）について規制対象であるスチレン，トルエン，エチルベンゼン，イソプロピルベンゼンおよびプロピルベンゼンの含有量をGC-FID，GC-MSおよびヘッドスペース（HS）-GCにより定量した．GC-FIDを用いた方法による併行精度（$RSD_r$）は1.0～2.6%，室間再現精度（$RSD_R$）は2.5～5.8%であり，その性能は目標値を満たしており，規格試験法として十分であった．GC-MSにおける$RSD_r$は1.4～7.8%，$RSD_R$は4.9～13%，HS-GCにおける$RSD_r$は2.0～2.6%，$RSD_R$は3.3～6.9%であり，それらの定量値はGC-FIDとほぼ同等であった．そのため，これらは規格試験法の代替法として適用可能であった．

Keywords：器具・容器包装，揮発性物質，試験室間共同試験

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*食品衛生学雑誌*　2016;57:222-9

食品衛生法ではナイロン製器具・容器包装からのカプロラクタムの溶出量が規制されている．そこで，公定法であるGC-FID法とその代替法であるGC-MS法の性能を評価するため，20機関で試験室間共同試験を行った．各試験機関は，濃度非明示の20%エタノール溶液（3検体，各2測定）中のカプロラクタムをGC-FIDまたはGC-MSにより定量した．公定法（GC-FIDを用いた絶対検量線による定量）における真度は96～97%，併行精度（$RSD_r$）は3.3～5.4%，室間再現精度（$RSD_R$）は4.0～6.7%であり，これらの値は目標値（真度：80～110%，$RSD_r$: 10%，

$RSD_R$: 25%）を満たしていた．さらに，ヘプタラクタムを用いて内標準補正を行うといずれの性能パラメーターも向上した．GC-MS法では，絶対検量線法において一部の$RSD_r$が目標値の10%を超えた．しかし，内標準補正を行うと真度は94～96%，$RSD_r$は2.0～4.4%，$RSD_R$は7.0～9.4%であり，規格試験法の代替法として適用可能であった．

Keywords：器具・容器包装，カプロラクタム試験，試験室間共同試験

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Asakura H, Ikeda T*1, Yamamoto S, Kabeya H*2, Sugiyama H*3, Takai S*4: Draft genome sequence of five Shiga toxin-producing *Escherichia coli* strains isolated from wild deer in Japan.

*Genome Announc*. 2017;5:e01455-16

Shiga toxin-producing Escherichia coli（STEC）is one of the major foodborne pathogens. Having observed the wide distribution of this pathogen in wild deer, we report here the draft genome sequence of five STEC strains isolated from wild deer（Cervus nippon yesoensis）in Hokkaido, Japan.

Keywords: Shiga toxin-producing Escherichia coli（STEC）, wild deer, draft genome sequence

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Taguchi M*1, Kanki M*1, Yamaguchi Y*2, Inamura H*2, Koganei Y*3, Sano T*4, Nakamura H*1, Asakura H: Prevalence of *Listeria monocytogenes* in retail lightly pickled vegetables and its successful control at processing plants.

*J Food Prot*. 2017;80:467-75

Incidences of food poisoning traced to nonanimal food products have been increasingly reported. One of these was a recent large outbreak of Shiga toxin-producing Escherichia *coli*（STEC）O157 infection from the consumption of lightly pickled vegetables, indicating the necessity of imposing hygienic controls during manufacturing. However, little is known about the bacterial contamination levels in these minimally processed vegetables. Here we examined the prevalence of STEC, *Salmonella* spp., and *Listeria monocytogenes* in 100 lightly pickled vegetable products manufactured at 55 processing factories. Simultaneously, we also performed quantitative measurements of representative indicator bacteria（total viable counts, coliform counts, and beta-glucuronidase-producing E. coli counts）. STEC and *Salmonella* spp. were not detected in any of the samples; *L. monocytogenes* was detected in 12 samples manufactured at five of the factories. Microbiological surveillance at two factories（two surveys at factory A and three surveys at factory B）between June 2014 and January 2015 determined that the areas predominantly contaminated with *L. monocytogenes* included the refrigerators and packaging rooms. Genotyping provided further evidence that the contaminants found in these areas were linked to those found in the final products. Taken together, we demonstrated the prevalence of *L. monocytogenes* in lightly pickled vegetables sold at the retail level. Microbiological surveillance at the manufacturing factories further clarified the sources of the contamination in the retail products. These data indicate the necessity of implementing adequate monitoring programs to minimize health risks attributable to the consumption of these minimally processed vegetables.

Keywords: *Listeria monocytogenes*, light pickles, hygienic control

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Ishihara K*1, Chuma T*2, Andoh M*2, Yamashita M*1, Asakura H, Yamamoto S*3: Effect of climatic elements on Campylobacter colonization in broiler flocks reared in southern Japan from 2008 to 2012.
*Poult Sci*. 2017;96:931-7

To demonstrate the effect of climatic elements on *Campylobacter* colonization in broiler chickens reared in Japan, the correlation between *Campylobacter* isolated from chickens (191 of 236 flocks, 80.9%) between 2008 and 2012 and climatic elements was analyzed by logistic regression. We divided the rearing process into 13 terms of 5 d each (total: 65 d). Terms were numbered backwards, wherein a 0-term lag was considered as the sampling day plus 4 d before sampling; 1-term lag was the 5-d term before the 0-term lag, and so on, until the 12-term lag. We obtained climatic data tracing back from the 0-term to the 12-term lags. For evaluation in each season, we divided chickens reared during periods of rising temperature (spring, summer) and decreasing temperature (autumn, winter) . Air temperature showed a positive correlation with *Campylobacter* colonization from the 0- to 12-term lags in chickens reared during the period of rising temperature (odds ratio [OR], 1.069 to 1.104) , and from the 0- to 4- and 6-term lags (OR, 1.079 to 1.105) in chickens reared during the period of decreasing temperature. The strong positive effect of air temperature on *Campylobacter* colonization, particularly during the period of rising temperature, may be associated with the effect on the *Campylobacter* environmental sources and/or vectors. A positive correlation was observed between *Campylobacter* colonization and humidity when chicken houses were empty and new chicks were introduced (from the 9- to 12-term lags) during the period of decreasing temperature (OR, 1.076 to 1.141). Thus, high humidity would be an important factor causing carry-over of *Campylobacter* infection during the period of decreasing temperature. We also found that solar radiation increased *Campylobacter* colonization during the period of decreasing temperature, from the 2- to 8-term lags, except for the 4- and 5-term lags, in Japan. The results of this study demonstrate the effects of air temperature, humidity, and solar radiation on *Campylobacter* colonization in broiler chickens, and are potentially important for developing strategies to reduce the risk of *Campylobacter* contamination in broiler chickens.
Keywords: *Campylobacter*, broiler chicken, climate

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山本詩織，朝倉宏，五十君靜信*：基質特異性拡張型βラクタマーゼ（ESBL）産生菌に関わる最近の動向とその拡散に関する考察～食品汚染実態とその危害性について～.
*食品衛生学雑誌*　2017;58:1-11

近年国内外において健康被害が増加の一途をたどる，基質特異性拡張型ラクタマーゼ（Extended Spectrum Beta-Lactamases；以下ESBL）産生菌に着目し，ヒト・動物・食品からの検出状況，ならびに耐性伝播機構に関する近年の知見について概説した.
Keywords：ESBL産生菌，食品汚染実態，伝播経路

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Suzuki H: Differences in susceptibility of mouse strains to tetrodotoxin.
*Toxicon*. 2016;119:168-70

In this study, we investigated the response of various mouse strains to tetrodotoxin. Tetrodotoxin solution was injected intraperitoneally into male mice of 5 inbred strains (A/J, BALB/c, C3H/He, C57BL/6, and DBA/2) and male and female mice of 2 non-inbred strains (ddY and ICR). Significant differences in susceptibility to tetrodotoxin were found among the mouse strains tested. In comparison to the ddY male mice, which are designated to be used in the Japanese reference method, the 5 inbred strains of mice tested were significantly more resistant to tetrodotoxin. However, no significant differences in tetrodotoxin susceptibility were observed between ddY male and female mice or between ddY male mice and ICR male

and female mice.
Keywords: mouse, strain, tetrodotoxin

荻原博和*，渡邊嵩之*，堀川俊暢*，古川壮一*，岡田由美子，五十君靜信：非加熱喫食調理済み食品に接種した*Listeria monocytogenes*の低温保存中における挙動.
*日本食品保蔵科学会誌* 2016;42:155-63

In this study, the growth characteristics of *Listeria monocytogenes* in commercially supplied ready-to-eat foods (RTEFs) were investigated. The tested RTEFs were a seafood product, Kanifumi-kamaboko (imitation crab surimi product; KK), and a vegetable product, Asazuke-Hakusai (lightly pickled Chinese cabbage; AH) both these foods are available in Japan. Four strains were used for inoculation in these RTEFs: *L. monocytogenes* ATCC19115, 99023, LC-8, and LC-21. These strains produced upto $10^7$-$10^9$ CFU/ml in tryptic soy broth at 20, 10, and 4 ºC. The viable cell count and L. monocytogenes cell count in KK were more than $10^7$ CFU/g at 4, 10, and 20 ºC in 20, 6, and 2 days, respectively. The viable cell count in AH were more than $10^7$ CFU/ml in 4 days at 20 ºC and in 8 days at 10 ºC, but there was no significant change in the viable cell count at 4 ºC. In contrast, the viable *L. monocytogenes* counts in AH decreased to about 2 log CFU/g after 4 days at 20 ºC, and to about 1 log CFU/g after 10 and 20 days at 10 ºC and 4 ºC, respectively. These results show that while viable count increased during preservation, *L. monocytogenes* count decreased. These findings indicate that lactic acid bacteria proliferate during preservation and they produce organic acids that repress the growth of *Listeria* in AH.
Keywords：*Listeria monocytogenes*，低温，非加熱喫食調理済み食品

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Sato H*[1], Yokoyama M*[1], Nakamura H*[1], Oka T*[1], Katayama K*[1,2], Takeda N*[3,4], Noda M, Tanaka T*[5], Motomura K*[1,3,4]: Evolutionary Constraints on the Norovirus Pandemic Variant GII.4_2006b over the Five-Year Persistence in Japan.
*Front Microbiol.* 2017;8:410

Norovirus GII.4 is a major cause of global outbreaks of viral gastroenteritis in humans, and has evolved by antigenic changes under the constantly changing human herd immunity. Major shift in the pandemic GII.4 strain periodically occurs concomitant with changes in the antigenic capsid protein VP1. However, how the newly emerged strain evolves after the onset of pandemic remains unclear. To address this issue, we examined molecular evolution of a pandemic lineage, termed the GII.4_2006b, by using the full-length viral genome and VP1 sequences (n = 317) from stools collected at 20 sites in Japan between 2006 and 2011. Phylogenetic tree showed a radial diversification of the genome sequences of GII.4_2006b, suggesting a rapid genetic diversification of the GII.4_2006b population from a few ancestral variants. Impressively, amino acid sequences of the variable VP1 in given seasons remained as homogeneous as those of viral enzymes under annual increase in the nucleotide diversity in the VP1 coding region. The Hamming distances between the earliest and subsequent variants indicate strong constraints on amino acid changes even for the highly variable P2 subdomain. These results show the presence of evolutionary constraints on the VP1 protein and viral enzymes, and suggest that these proteins gain near maximal levels of fitness benefits in humans around the onset of the outbreaks. These findings have implications for our understanding of molecular evolution, mechanisms of the periodic shifts in the pandemic NoV GII.4 strains, and control of the NoV GII.4 pandemic strain.
Keywords: GII.4, molecular evolution, norovirus

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Shigemoto N*[1], Hisatsune Y*[1], Toukubo Y*[1], Tanizawa Y*[1], Shimazu Y*[1], Takao S*[1], Tanaka T*[2], Noda M, Fukuda S*[3]：Detection of gastroenteritis viruses among pediatric patients in Hiroshima Prefecture, Japan, between 2006 and 2013 using multiplex reverse transcription PCR assays involving fluorescent dye-labeled primers.
*J Med Virol.* 2016;doi:10.1002/jmv.24714

Multiplex reverse transcription (RT)-polymerase

chain reaction (PCR)-based assays involving fluorescent dye-labeled primers were modified to detect 10 types of gastroenteritis viruses by adding two further assays to a previously developed assay. Then, these assays were applied to clinical samples, which were collected between January 2006 and December 2013. All 10 types of viruses were effectively detected in the multiplex RT-PCR-based assays. In addition, various viral parameters, such as the detection rates and age distributions of each viral type, were examined. The frequency and types of mixed infections were also investigated. Among the 186 virus-positive samples, genogroup II noroviruses were found to be the most common type of virus (32.7%), followed by group A rotaviruses (10.6%) and parechoviruses (10.3%). Mixed infections were observed in 37 samples, and many of them were detected in patients who were less than 2 years old. These observations showed that the multiplex RT-PCR-based assays involving fluorescent dye-labeled primers were able to effectively detect the viruses circulating among pediatric acute gastroenteritis patients and contributed to the highly specific and sensitive diagnosis of gastroenteritis.

Keywords: gastroenteritis viruses, multiplex RT-PCR, pediatric patients

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Takahashi H*, Takahashi M*, Ohshima C*, Izawa Y*, Uema M, Kuda T*, Kimura B*, Noda M: Differences in the viability of murine norovirus in different aquatic locations.
*Mar Pollut Bull*. 2016;112:313-7

Norovirus is detected from shellfish and environmental water more frequently in winter than in other seasons. However, there is no report regarding its viability in actual seawater in situ. We investigated the viability of murine norovirus strain 1 (MNV-1), a surrogate for human norovirus, in 2 types of aquatic locations, a seawater pool carrying oceanic water and inner bay carrying brackish water. Sterilized seawater was inoculated with MNV-1 and enclosed in dialysis tubes, which were placed at the 2 locations. MNV-1 exhibited higher level of viability in brackish than in oceanic water. Factors that influenced the viability of MNV-1 included salt concentration as well as temperature of the seawater. Therefore, based on our findings, coastal brackish water that is routinely used for harvesting or cleaning seafood at fishing ports may promote the viability of norovirus.

Keywords: MNV-1, salt concentration, water temperature

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Motomura K*1, Boonchan M*1, Noda M, Tanaka T*2, Takeda N*1: Norovirus Surveillance Group of Japan: Norovirus epidemics caused by new GII.2 chimera viruses in 2012-2014 in Japan.
*Infect Genet Evol*. 2016;42:49-52

The new GII.2 variant collected from May 2012-March 2014 consisted of GII.15 and GII.2 genomes, in which the putative recombination points found in the boundary region between ORF1 and ORF2. These findings suggested that the swapping of structural and non-structural proteins is a common mechanism for generating new epidemic variants in nature.

Keywords: GII.2, new chimera virus, norovirus

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Hara-Kudo Y, Konishi N*1, Ohtsuka K*2, Iwabuchi K*3, Kikuchi R*4, Isobe J*5, Yamazaki T*6, Suzuki F*7, Nagai Y*8, Yamada H*9, Tanouchi A*10, Mori T*11, Nakagawa H*12, Ueda Y*13, Terajima J: An interlaboratory study on efficient detection of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O121, O145, and O157 in food using real-time PCR assay and chromogenic agar.
*Int J Food Microbiol*. 2016;230:81-8

To establish an efficient detection method for Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O26, O103, O111, O121, O145, and O157 in food, an interlaboratory study using all the serogroups of detection targets was firstly conducted. We employed a series of tests including enrichment, real-time PCR assays, and concentration by immunomagnetic separation, followed by plating onto selective agar

media (IMS-plating methods). This study was particularly focused on the efficiencies of real-time PCR assays in detecting stx and O-antigen genes of the six serogroups and of IMS-plating methods onto selective agar media including chromogenic agar. Ground beef and radish sprouts sampleswere inoculatedwith the six STEC serogroups either at 4–6 CFU/25 g (lowlevels) or at 22–29 CFU/25 g (high levels). The sensitivity of stx detection in ground beef at both levels of inoculationwith all six STEC serogroupswas 100%. The sensitivity of stx detection was also 100% in radish sprouts at high levels of inoculationwith all six STEC serogroups, and 66.7%–91.7% at lowlevels of inoculation. The sensitivity of detection of O-antigen genes was 100% in both ground beef and radish sprouts at high inoculation levels, while at low inoculation levels, it was 95.8%–100% in ground beef and 66.7%–91.7% in radish sprouts. The sensitivity of detection with IMS-platingwas either the same or lower than those of the real-time PCR assays targeting stx and O-antigen genes. The relationship between the results of IMS-plating methods and Ct values of real-time PCR assays were firstly analyzed in detail. Ct values in most samples that tested negative in the IMS-plating method were higher than the maximumCt values in samples that tested positive in the IMS-plating method. This study indicates that all six STEC serogroups in food contaminatedwith more than 29 CFU/25 gwere detected by real-time PCR assays targeting stx and O-antigen genes and IMS-plating onto selective agar media. Therefore, screening of stx and Oantigen genes followed by isolation of STECs by IMS-plating methods may be an efficient method to detect the six STEC serogroups.

Keywords: Shiga toxin-producing *Escherichia coli*, detection method, food

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Wang L*1,2, Nakamura H*3, Kage-Nakadai E*2, Hara-Kudo Y, Nishikawa Y*2: Comparison by multi-locus variable-number tandem repeat analysis and antimicrobial resistance among atypical enteropathogenic *Escherichia coli* strains isolated from foods and human and animal faecal specimens. *Journal of Applied Microbiology*. 2017;122:268-78

Aim: This study assessed whether multi-locus variable-number tandem repeat analysis (MLVA) and antimicrobial susceptibility testing discriminated diarrhoeagenic atypical enteropathogenic *Escherichia coli* (aEPEC) from aEPEC indigenous to domestic animals or healthy people. Methods and Results: MLVA genotyping of 142 aEPEC strains isolated from foods and faecal samples of domestic animals and humans revealed 126 distinct MLVA profiles that distributed to four clusters, yielding a Simpson's index of diversity (D) of 99.8%. Cluster 2 included 87% of cattle isolates and 67% of patient isolates. The plurality (15/34, 44%) of strains from healthy humans mapped to Cluster 1, while half (18/41, 44%) of the swine strains belonged to Cluster 4. Testing for antimicrobial susceptibility revealed that 52 strains (37%) of aEPEC were resistant to one or more agents; only 10 strains (7%) exhibited resistance to more than three agents. Strains isolated from swine or food exhibited a wider variety of resistance phenotypes than bovine or human strains. Conclusions: MLVA assigned the aEPEC isolates from cattle and patients to Cluster 2, distinct from aEPEC from other sources. Hog yards may be a larger source of drug-resistant strains than are cattle ranches.

Keywords: antimicrobial resistance, multi-locus variable-number tandem repeat analysis, enteropathogenic *Escherichia coli*

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Kanayasu-Toyoda T, Tanaka T*1, Kikuchi Y, Uchida E, Matsuyama A*2, Yamaguchi T: Cell-surface MMP-9 protein is a novel functional marker to identify and separate proangiogenic cells from early endothelial progenitor cells derived from CD133（+）cells.
*Stem Cells*. 2016;34:1251-62

To develop cell therapies for ischemic diseases, endothelial progenitor cells（EPCs）have been expected to play a pivotal role in vascular regeneration. It is desirable to use a molecular marker that is related to the function of the cells. Here, a quantitative polymerase chain reaction array revealed that early EPCs derived from CD133（+）cells exhibited significant expression of MMP-9. Some populations of early EPCs expressed MMP-9 on the cell surface and others did not. We also attempted to separate the proangiogenic fraction from early EPCs derived from CD133（+）cells using a functional cell surface marker, and we then analyzed the MMP-9（+）and MMP-9（-）cell fractions. The MMP-9（+）cells not only revealed higher invasion ability but also produced a high amount of IL-8. Moreover, the stimulative effect of MMP-9（+）cells on angiogenesis in vitro and in vivo was prohibited by anti-IL-8 antibody. These data indicate that MMP-9 is one of the useful cell surface markers for the separation of angiogenic cells. Our treatment of early EPCs with hyaluronidase caused not only a downregulation of cell-surface MMP-9 but also a decrease in invasion ability, indicating that membrane-bound MMP-9, which is one of the useful markers for early EPCs, plays an important role in angiogenesis.
Keywords: angiogenesis, CD133, cell therapy

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菊池裕，酘島由二，福井千恵，村井敏美*1，中川ゆかり*1，海老澤亜樹子*1，松村佳代子*2，大内和幸*3，小田俊男*4，向井基樹*4，益田多満喜*5，甲藤夕佳*5，高須賀禎浩*6，高岡文*6：平成27 年度「日本薬局方の試験法等に関する研究」研究報告 エンドトキシン試験法に用いる組換え試薬の評価に関する研究.
*医薬品医療機器レギュラトリーサイエンス*　2017;48:252-60

カブトガニの保護，試薬の安定供給，製品ロット間差の解消及び試験の安定性の向上を目的として，Factor Cの組換えタンパク質からなるエンドトキシン測定試薬（組換え試薬）が開発された．本研究では，各種組換え試薬の感度，反応性及び特異性を既存のカブトガニ血球成分由来のライセート試薬と比較検討することを目的として，7機関で共同研究班を組織し，各種菌株由来精製LPSからなるエンドトキシンパネル及び精製されていない天然に存在するLPS（Naturally occurring endotoxin，NOE）を測定対象として，国内で入手可能な組換え試薬及び代表的なライセート試薬で測定し，各試薬間で整合した測定値が得られるかどうかを調べることにより，組換え試薬使用の妥当性を評価した.
Keywords：エンドトキシン試験法，ライセート試薬，組換え試薬

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*6 和光純薬工業（株）

Ohnishi T, Fujiwara M*, Tomaru A, Yoshinari T, Sugita-Konishi Y*: Survivability of *Kudoa septempunctata* in human intestinal conditions.
*Parasitol Res*. 2016;115(6):2519-22

To elucidate whether *Kudoa septempunctata* was able to live in the human intestine, we assessed viability of *K. septempunctata* sporoplasms under conditions that mimicked human and ragworm digestive tracts. To study the effect of osmotic pressure on viability, sporoplasms were incubated in 0.9 or 3.4 % sodium chloride solutions, which roughly corresponded to the osmotic pressure in human or ragworm tissues, respectively. While viability in 3.4 % sodium chloride did not change after 72 h, it dropped to 21 % in 0.9 % sodium chloride. To study the effect of temperature on viability, sporoplasms were incubated at 37, 15, or 25 °C, which were representative of human, winter ragworm, or summer ragworm temperatures, respectively. Viability decreased sharply to 8.4 % after 48 h at 37 °C, but remained essentially unchanged at 15 and 25 °C. In addition, sporoplasms showed strong susceptibility to bile. These results

indicate that K. septempunctata could not live in the human intestine for a long time.
Keywords: Kudoa, parasite, food-borne disease

* Azabu University

Ohnishi T, Lim B[*1], Nojima N[*2], Ogasawara K[*2], Inagaki S[*3], Makitsuru K[*3], Sasaki M[*4], Nakane K[*5], Tsuchioka H[*6], Horikawa K[*7], Kwabe M[*7], Minegishi Y[*8], Miyazaki N[*9], Sugita-Konishi Y[*10]: Inter-Laboratory Study to Validate New Rapid Screening Methods for Kudoa septempunctata.
*Biocontrol Science*. 2016;21(2):135-8

*Kudoa septempunctata* is the causative agent of a food-borne disease associated with the ingestion of raw olive flounder. As the current qRT-PCR method for its detection is time-consuming, a rapid and simple method is required. Recently, a new real-time loop-mediated isothermal amplification (LAMP) method and an immunochromatography method, whose sensitivities are intended to be compatible with that designated in the official analytical method (10 (5) spores/g olive flounder), have been developed. To validate these new methods, we performed an inter-laboratory study across seven laboratories. Both methods could not detect less than 10 (4) spores/g; however, these methods were able to detect more than 10 (5) spores/g in olive flounder samples. These results demonstrated that the sensitivities of these methods were compatible with the designated level in the official analytical method. We concluded that these new methods were acceptable as the screening methods for *K. septempunctata*.
Keywords: *Kudoa*, parasite, food-borne disease

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Ohnishi T, Fujiwara M*, Tomaru A, Yoshinari T, Sugita-Konishi Y*: Cryopreservation of *Kudoa septempunctata* sporoplasm using commercial freezing media.
*Parasitol Res*. 2017;116(1):425-7

Cryopreservation methods for *Kudoa septempunctata* have not been established. This prevents an effective study of *K. septempunctata*, which cannot be artificially cultivated in the laboratory. In this study, we attempted to establish a cryopreservation method for *K. septempunctata* sporoplasm using Cellbanker® 1, a commercial preservation medium for mammalian cells. Spores were purified from the meat of Paralichthys olivaceus (olive flounder). These purified spores were suspended in Cellbanker® 1 and were stored at -80 °C for up to 16 months. Although the spores stored at -80 °C for 16 months were damaged, the sporoplasms maintained its amoeba-like indeterminate morphology, and their motility was well preserved. The viability of sporoplasms was variable among vials but was not below 70 %. In addition, the sporoplasms stored at -80 °C for 16 months could decrease the transepithelial electrical resistance of Caco-2 cells. These results indicate that this cryopreservation method using Cellbanker® 1 could preserve the viability and pathogenesis of *K. septempunctata* sporoplasm.
Keywords: Kudoa, parasite, food-borne disease

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大西貴弘，都丸亜希子，吉成知也，鎌田洋一[*1]，小西良子[*2]：生鮮魚介類の生食に関連した有症苦情事例残品に含まれる粘液胞子虫の検出.
*日本食品微生物学会雑誌* 2016;33(3):150-4

2010年4月から2016年3月までに発生した生鮮魚介類の生食に関連した有症苦情事例の内，44事例の喫食残品65検体を収集した．これらの事例の症状は下痢，嘔吐，腹痛などで重症例はなかった．また，多くの事例では初発の潜伏時間が6時間以内と短く，*Kudoa septempunctata* による食中毒症状と類似していた．喫食残品中の粘液胞子虫の検出を行ったところ44事例中，粘液胞子虫のDNA が検出されたのは31事例（70%）で，その内，顕微鏡検査で胞子を確認できたのは23事例（52%）だった．検出された粘液胞子虫の内，*Unicapsula seriolae*が最も多く，カンパチ，ヒラマサ合わせて20検体中，15検体（75%）から分離され，カンパチの生食に伴う事例と*U. seriolae*の間に関連性が示唆された.
Keywords: *Kudoa*, parasite, food-borne disease

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Furukawa M*1,2, Minegishi Y*3, Izumiyama S*4, Yagita K*5, Mori H*5, Uemura T*6, Etoh Y*7, Maeda E*7, Sasaki M*8, Ichinose K*8, Harada S*1, Kamata Y*9, Otagiri M*2, Sugita-Konishi Y*10, Ohnishi T: The Development of a Novel, Validated, Rapid and Simple Method for the Detection of *Sarcocystis fayeri* in Horse Meat in the Sanitary Control Setting.
*Biocontrol Sci*. 2016;21(2):131-4

*Sarcocystis fayeri*（*S. fayeri*）is a newly identified causative agent of foodborne disease that is associated with the consumption of raw horse meat. The testing methods prescribed by the Ministry of Health, Labour and Welfare of Japan are time consuming and require the use of expensive equipment and a high level of technical expertise. Accordingly, these methods are not suitable for use in the routine sanitary control setting to prevent outbreaks of foodborne disease. In order to solve these problems, we have developed a new, rapid and simple testing method using LAMP, which takes only 1 hour to perform and which does not involve the use of any expensive equipment or expert techniques. For the validation of this method, an inter-laboratory study was performed among 5 institutes using 10 samples infected with various concentrations of *S. fayeri*. The results of the inter-laboratory study demonstrated that our LAMP method could detect *S. fayeri* at concentrations greater than 10（4）copies/g. Thus, this new method could be useful in screening for *S. fayeri* as a routine sanitary control procedure.
Keywords: *Sarcocystis fayeri*, parasite, food-borne disease

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Yoshinari T, Ohnishi T, Terajima J: Evaluation of Four Commercial Kits Based on Immunochromatography for Screening Aflatoxin M1 in Milk.
*Shokuhin Eiseigaku Zasshi*. 2016;57:76-9

In order to confirm whether commercial immunochromatographic kits for detecting AFM1 satisfy these criteria, the performance of four kits was evaluated by performing spike-and-recovery experiments using AFM1-free milk samples and milk samples spiked at seven levels（100-700 ng/kg）. With the two qualitative kits, determinations of blank samples were all negative and those of the samples spiked at 500 ng/kg were all positive. With the two quantitative kits, the measured values of the blank samples were all less than 100 ng/kg and the recoveries of the samples spiked at 500 ng/kg were all more than 70%. These results indicate that all four immunochromatographic kits meet the criteria and can be used to screen $AFM_1$ in milk.
Keywords: aflatoxin, milk, mycotoxin

Yoshinari T, Suzuki Y*, Sugita-Konishi Y*, Ohnishi T, Terajima J: Occurrence of beauvericin and enniatins in wheat flour and corn grits on the Japanese market, and their co-contamination with type B trichothecene mycotoxins.
*Food Addit Contam Part A*. 2016;33:1620-6

The contamination levels of beauvericin and four enniatins, A, $A_1$, B and $B_1$, in 207 samples of wheat flour and corn grits on the Japanese market were determined by an analytical method based on LC-MS/MS. Deoxynivalenol and nivalenol in the same samples were determined by a method using an immunoaffinity column. Co-contamination of deoxynivalenol and enniatins was observed in 61% of the imported wheat samples and in 58% of the domestic wheat samples. These results suggest the need for a risk assessment for cyclic depsipeptide mycotoxins in Japan and a study on the synergistic effect of deoxynivalenol and enniatins.
Keywords: mycotoxin, enniatin, wheat

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Koba Y*1, Hirata Y*1, Ueda A*1, Oba M*1, Doi M*2, Demizu Y, Kurihara M, Tanaka M*1: Synthesis of chiral five-membered carboxylic ring amino acids

with an acetal moiety and helical conformations of its homo-chiral homopeptides.
*Biopolymer*. 2016;106:555-62

Chiral five-membered carbocyclic ring amino acids bearing various diol acetal moieties were synthesized starting from l-malic acid, and homo-chiral homopeptides composed of cyclic amino acid (S)-Ac5c$^{3EG}$ bearing an ethylene glycol acetal, up to an octapeptide, were prepared. A conformational analysis revealed that (S)-Ac5c$^{3EG}$ homopeptides formed helical structures. (S)-Ac5c$^{3EG}$ homopeptides, up to hexapeptides, formed helical structures without controlling the helical screw direction, while (S)-Ac5c$^{3EG}$ hepta- and octapeptides formed helical structures with a preference for the left-handed (M) helical-screw direction.
Keywords: conformation, cyclic amino acid, helix

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Okuhira K*, Demizu Y, Hattori T, Ohoka N, Shibata N, Kurihara M, Naito M: Molecular Design, Synthesis, and Evaluation of SNIPER (ER) That Induces Proteasomal Degradation of ER α.
*Methods Mol Biol*. 2016;1366:549-60

Manipulation of protein stability using small molecules has a great potential for both basic research and clinical therapy. Based on our protein knockdown technology, we recently developed a novel small molecule SNIPER (ER) that targets the estrogen receptor alpha (ER α) for degradation via the ubiquitin-proteasome system. This chapter describes the design and synthesis of SNIPER (ER) compounds, and methods for the evaluation of their activity in cellular system.
Keywords: cell death, estrogen receptor, protein knockdown

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Demizu Y, Ohoka N, Nagakubo T, Yamashita H, Misawa T, Okuhira K*, Naito M, Kurihara M: Development of a peptide-based inducer of nuclear receptors degradation.
*Bioorg Med Chem Lett*. 2016;26:2655-8

A peptide-based protein knockdown system for inducing nuclear receptors degradation via the ubiquitin–proteasome system was developed. Specifically, the designed molecules were composed of two biologically active scaffolds: a peptide that binds to the estrogen receptor α (ERα) surface and an MV1 molecule that binds to cellular inhibitors of apoptosis proteins (IAP: cIAP1/cIAP2/XIAP) to induce ubiquitylation of the ERα. The hybrid peptides induced IAP-mediated ubiquitylation followed by proteasomal degradation of the ERα. Those peptides were also applicable for inducing androgen receptor (AR) degradation.
Keywords: nuclear receptor, helical peptide, protein knockdown

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Kato T*, Yamashita H, Misawa T, Nishida K*, Kurihara M, Tanaka M*, Demizu Y, Oba M*: Plasmid DNA delivery by arginine-rich cell-penetrating peptides containing unnatural amino acids.
*Bioorg Med Chem*. 2016;24:2681-7

Cell-penetrating peptides (CPPs) have been developed as drug, protein, and gene delivery tools. In the present study, arginine (Arg)-rich CPPs containing unnatural amino acids were designed to deliver plasmid DNA (pDNA). The transfection ability of one of the Arg-rich CPPs examined here was more effective than that of the Arg nonapeptide, which is the most frequently used CPP. The transfection efficiencies of Arg-rich CPPs increased with longer post-incubation times and were significantly higher at 48-h and 72-h post-incubation than that of the commercially available transfection reagent TurboFect. These Arg-rich CPPs were complexed with pDNA for a long time in cells and effectively escaped from the late endosomes/lysosomes into the cytoplasm. These results will be helpful for designing novel CPPs for pDNA delivery.
Keywords: cell penetrating peptide, plasmid DNA delivery, unnatural amino acid

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Shoda T, Kato M*, Fujisato T*, Misawa T, Demizu Y, Inoue H*, Naito M, Kurihara M: Synthesis and evaluation of raloxifene derivatives as a selective

estrogen receptor down-regulator.

*Bioorg Med Chem*. 2016;24:2914-9

Estrogen receptors (ERs) play a major role in the growth of human breast cancer cells. A selective estrogen receptor down-regulator (SERD) that acts as not only an inhibitor of ligand binding, but also induces the down-regulation of ER, would be useful for the treatment for ER-positive breast cancer. We previously reported that tamoxifen derivatives, which have a long alkyl chain, had the ability to down-regulate ER $\alpha$. With the aim of expanding range of the currently available SERDs, we designed and synthesized raloxifene derivatives, which had various lengths of the long alkyl chains, and evaluated their SERD activities. All compounds were able to bind ER $\alpha$, and RC10, which has a decyl group on the amine moiety of raloxifene, was shown to be the most potent compound. Our findings suggest that the ligand core was replaceable, and that the alkyl length was important for controlling SERD activity. Moreover, RC10 showed antagonistic activity and its potency was superior to that of 4,4'-(heptane-4,4-diyl) bis (2-methylphenol) (18), a competitive antagonist of ER without SERD activity. These results provide information that will be useful for the development of promising SERDs candidates.

Keywords: selective estrogen receptor down-regulator, raloxifene, estrogen receptor positive breast cancer

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Demizu Y, Doi M[*1], Yamashita H, Misawa T, Oba M[*2], Kurihara M, Suemune H[*3], Tanaka M[*2]: Development of a peptide-based inducer of nuclear receptors degradation.

*Biopolymers*. 2016;106:757-68

A single chiral cyclic $\alpha,\alpha$-disubstituted amino acid with side-chain methoxymethyl (MOM) protecting groups, (3S,4S)－1-amino-(3,4-dimethoxymethoxy) cyclopentanecarboxylic acid [(S, S)-$Ac_5c^{dOMOM}$], or side-chain hydroxy groups, (3S,4S)－1-amino-(3,4-dihydroxy) cyclopentanecarboxylic acid [(S, S)-$A_c5c^{dOH}$], was attached to the N-terminal or C-terminal position of $\alpha$-aminoisobutyric acid (Aib) tetrapeptide segments; i.e., we designed and synthesized four pentapeptides, Cbz [(S, S)-$Ac_5c^{dOMOM}$]-$(Aib)_4$-OEt (1), Cbz-[(S, S)-$Ac_5c^{dOH}$]-$(Aib)_4$-OEt (2), Cbz-$(Aib)_4$-[(S, S)-$Ac_5c^{dOMOM}$]-OMe (3), and Cbz-$(Aib)_4$-[(S, S)--$Ac_5c^{dOH}$]-OMe (4). We then analyzed the peptides' structures in the crystalline state. The four peptides all folded into $3_{10}$-helical structures; 1 formed a left-handed (M) $3_{10}$-helix, 2 formed a mixture of right-handed (P) and (M) $3_{10}$-helices, 3 formed a mixture of (P) and (M) $3_{10}$-helices, and 4 formed a (P) $3_{10}$-helix, respectively. In packing mode, the molecules of peptides 1 and 3, which both possessed an $Ac_5c^{dOMOM}$ residue, were connected by intermolecular hydrogen bonds along the peptide backbone (N[BOND]H···O type). On the other hand, the packing of peptides 2 and 4, which both contained an $Ac_5c^{dOH}$ residue, was based on intermolecular hydrogen bonds derived from both the peptide backbone and the side-chain hydroxy groups of the amino acid $Ac_5c^{dOH}$ (O[BOND]H···O type).

Keywords: nuclear receptor, helical peptide, protein knockdown

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Sugiyama T[*1], Kuwata K[*2], Imamura Y[*3], Demizu Y, Kurihara M, Takano M[*1], Kittaka A[*1]: Peptide Nucleic Acid with a Lysine Side Chain at the $\beta$-Position: Synthesis and Application for DNA Cleavage.

*Chem Pharm Bull*. 2016;64(7):817-23

This paper reports the synthesis of new $\beta$-Lys peptide nucleic acid (PNA) monomers and their incorporation into a 10-residue PNA sequence. PNA containing $\beta$-Lys PNA units formed a stable hybrid duplex with DNA. However, incorporation of $\beta$-Lys PNA units caused destabilization of PNA–DNA duplexes to some extent. Electrostatic attractions between $\beta$-PNA and DNA could reduce this destabilization effect. Subsequently, bipyridine-conjugated $\beta$-Lys PNA was prepared and exhibited sequence selective cleavage of DNA. Based on the structures of the cleavage products and molecular modeling, we reasoned that bipyridine moiety locates within the minor groove of the PNA–DNA duplexes. The lysine side chain of $\beta$-PNA is a versatile handle for attaching various functional molecules.

Keywords: peptide nuclear acid, chirality, duplex

stability

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Akiyama N＊1, Takizawa N＊1, Miyauchi M＊1, Yanai H＊1, Tateishi R＊1, Shinzawa M＊1, Yoshinaga R＊1, Kurihara M, Demizu Y, Yasuda H＊3, Yagi S＊4, Wu G＊4, Matsumoto M＊5, Sakamoto R＊2, Yoshida N＊2, Penninger J M＊6, Kobayashi Y＊7, Inoue J＊1, Akiyama T＊1: Identification of embryonic precursor cells that differentiate into thymic epithelial cells expressing autoimmune regulator.

*J Exp Med*. 2016;213:1441-58

Medullary thymic epithelial cells (mTECs) expressing autoimmune regulator (Aire) are critical for preventing the onset of autoimmunity. However, the differentiation program of Aire-expressing mTECs (Aire$^+$ mTECs) is unclear. Here, we describe novel embryonic precursors of Aire$^+$ mTECs. We found the candidate precursors of Aire$^+$ mTECs (pMECs) by monitoring the expression of receptor activator of nuclear factor-$\kappa$B (RANK), which is required for Aire$^+$ mTEC differentiation. pMECs unexpectedly expressed cortical TEC molecules in addition to the mTEC markers UEA-1 ligand and RANK and differentiated into mTECs in reaggregation thymic organ culture. Introduction of pMECs in the embryonic thymus permitted long-term maintenance of Aire+ mTECs and efficiently suppressed the onset of autoimmunity induced by Aire$^+$ mTEC deficiency. Mechanistically, pMECs differentiated into Aire$^+$ mTECs by tumor necrosis factor receptor-associated factor 6-dependent RANK signaling. Moreover, nonclassical nuclear factor-$\kappa$B activation triggered by RANK and lymphotoxin-$\beta$ receptor signaling promoted pMEC induction from progenitors exhibiting lower RANK expression and higher CD24 expression. Thus, our findings identified two novel stages in the differentiation program of Aire$^+$ mTECs.

Keywords: autoimmune regulator, medullary thymic epithelial cell, receptor activator of nuclear factor KB

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Yamashita H, Kato T＊, Oba M＊, Misawa T, Hattori T, Ohoka N, Tanaka M＊, Naito M, Kurihara M, Demizu Y: Development of a Cell-penetrating Peptide that Exhibits Responsive Changes in its Secondary Structure in the Cellular Environment.

*Sci Rep*. 2016;6: Article number 33003

Cell-penetrating peptides (CPP) are received a lot of attention as an intracellular delivery tool for hydrophilic molecules such as drugs, proteins, and DNAs. We designed and synthesized nona-arginine analogues 1–5 [FAM-$\beta$-Ala-(L-Arg-L-Arg-L-Pro)$_3$-(Gly)$_3$-NH2 (1), FAM-$\beta$-Ala-(L-Arg-L-Arg-L-Pro$^{NH2}$)$_3$-(Gly)$_3$-NH2 (2), FAM-$\beta$-Ala- (L-Arg-L-Arg-L-Pro$^{Gu}$)$_3$-(Gly)$_3$-NH2 (3), FAM-$\beta$-Ala-(L-Arg)$_2$-(L-Pro$^{Gu}$)$_2$-(L-Arg)$_4$-L-Pro$^{Gu}$-(Gly)$_3$-NH$_2$ (4), and FAM-$\beta$-Ala-(L-Arg)$_6$-(L-ProGu)$_3$-(Gly)$_3$-NH$_2$ (5)] containing L-proline (L-Pro) or cationic proline derivatives (L-Pro$^{NH2}$ and L-Pro$^{Gu}$), and investigated their cell-penetrating abilities. Interestingly, only peptide 3 having the side-chain guanidinyl L-Pro$^{Gu}$ exhibited a secondary structural change in cellular environment. Specifically, peptide 3 formed a random structure in hydrophilic conditions, whereas it formed a helical structure under amphipathic conditions. Furthermore, during cellular permeability tests, peptide 3 demonstrated greater cell-penetrating activity than other peptides and effectively transported plasmid DNA into HeLa cells. Thus, L-Pro$^{Gu}$-containing peptide 3 may be a useful candidate as a gene delivery carrier.

Keywords: drug delivery, peptide

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Ueda A＊1, Oba M＊1, Izumi Y＊1, Sueyoshi Y＊1, Doi M＊2, Demizu Y, Kurihara M, Tanaka M＊1: D Helical structures of homo-chiral isotope-labeled :-aminoisobutyric acid peptides.

*Tetrahedron*. 2016;39:5864-58

The chiral deuterium- and $^{13}$C-isotope-labeled $\alpha$-aminoisobutyric acids $CD_3$-Aib and $^{13}CH_3$-Aib were enantioselectively synthesized from L-Ala aldimine

using simplified Maruoka chiral phase-transfer catalysts. Homo-chiral (*S*)-$CD_3$-Aib homopeptides, up to decamers, were prepared. A (*R*)-$CD_3$-Aib polymer and (*S*)-$^{13}CH_3$-Aib polymer were also prepared. Conformational studies on homopeptides using CD spectra and an X-ray crystallographic analysis revealed that the preferred conformations were $3_{10}$-helical structures comprising equal amounts of right-handed (*P*) and left-handed (*M*) helical-screw structures. The $\alpha$-carbon chiral centers induced by the D- or $^{13}C$-isotope substitution of Aib were incapable of controlling the helical-screw directions of their oligopeptides and short polymers.

Keywords: helix, conformation, peptide

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Demizu Y, Shibata N, Hattori T, Ohoka N, Motoi H, Misawa T, Shoda T, Naito M, Kurihara M: Development of BCR-ABL degradation inducers via the conjugation of an imatinib derivative and a cIAP1 ligand.

*Bioorg Med Chem Lett*. 2016;26:4865-9

The manipulation of protein stability with small molecules has great potential as a technique for aiding the development of clinical therapies, including treatments for cancer. In this study, BCR-ABL protein degradation inducers called SNIPER (ABL) (Specific and Non-genetic inhibitors of apoptosis protein [IAP]-dependent Protein Erasers) were developed. The designed molecules contained two biologically active scaffolds: one was an imatinib derivative that binds to BCL-ABL and the other was a methyl bestatin that binds to cellular IAP 1 (cIAP1) . The hybrid molecules, SNIPER (ABL) , were expected to recruit BCR-ABL to cIAP1 for removal by proteasomes. In fact, SNIPER (ABL) induced the degradation of BCR-ABL protein and a subsequent reduction in cell growth. Thus, the degradation of BCR-ABL by SNIPER (ABL) is one potential strategy for treating BCR-ABL driven chronic myelogenous leukemia.

Keywords: bestatin, imatinib, protein knockdown

Takuji Shoda, Masashi Kato*, Takuma Fujisato*, Yosuke Demizu, Hideshi Inoue*, Mikihiko Naito, Masaaki Kurihara: Tamoxifen and fulvestrant hybrid showed potency as a selective estrogen receptor down-regulator.

*Med Chem*. 2017;13(3):206-13

BACKGROUND: Estrogen receptors (ERs) are an important target for the management of breast cancers. Selective estrogen receptor down-regulators (SERDs) block ER activity, as well as reduce ERa protein levels in cells, and therefore are promising therapeutic agents for the treatment of breast cancers.
OBJECTIVE: In order to develop potent SERDs, we prepared tamoxifen and fulvestrant hybrids and evaluated their binding activity and down-regulation of ERa.
METHODS: We designed and synthesized tamoxifen derivatives, which had a 4,4,5,5,5-pentafluoropentyl group on the terminal alkyl chain. The oxidation state of the sulfur atom and alkyl length between the sulfur and nitrogen atoms were varied. Western blotting was performed to determine the ability to down-regulate ERa. Binding affinities of synthesized compounds were evaluated by a fluorescence polarization-based competitive binding assay.
RESULTS: We successfully prepared nine compounds. Treatment with 11, 14, and 17 effectively reduced ERa protein levels in MCF-7 cells in a concentration-dependent manner. This reduction was inhibited by a proteasome inhibitor. The ability of 14 to down-regulate the ERa protein level was equal to fulvestrant. All compounds showed a largely equal affinity for ERa.
CONCLUSION: As indicated by Western blots, the ERa degradation activity was observed only in the series of butyl linker derivatives, namely, 11, 14, and 17. These findings suggest that the specific length of the alkyl chain is an important factor in controlling the down-regulation of ER. These results provide useful information for designing promising SERD candidates.

Keywords: tamoxifen, fulvestrant, estrogen receptor, selective estrogen receptor down-regulator, ER-positive breast cancer

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Misawa T, Fujisato T, Kanda Y, Ohoka N, Shoda T, Yorioka M, Makishima M*, Sekino Y, Naito M, Demizu Y, Kurihara M: Design and synthesis of novel selective estrogen receptor degradation

inducers based on the diphenylheptane skeleton.
*MedChemComm*. 2017;8:239-46

Estrogen receptors（ERs）are a family of nuclear receptors（NRs）that regulate physiological effects such as reproduction and bone homeostasis. It has been reported that approximately 70% of human breast cancers are hormone-dependent and ERα-positive. Recently, novel anti-breast cancer drugs based on different mechanisms of action have received significant attention. In this article, we have designed and synthesized a selective ER degradation inducer based on the diphenylheptane skeleton. Western blotting analysis revealed that PBP-NC10 degraded ERα through the ubiquitin–proteasome system. We also performed computational docking analysis to predict the binding mode of PBP-NC10 to ERα.
Keywords: estrogen receptor, protein knockdown, diphenylmethane skeleton

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Yamashita H, Misawa T, Oba M*, Tanaka M*, Naito M, Kurihara M, Demizu Y: Development of helix-stabilized cell-penetrating peptides containing cationic α,α-disubstituted amino acids as helical promoters.
*Bioorg Med Chem*. 2017;25:1846-51

Cell-penetrating peptides（CPP）have attracted many scientists' attention as intracellular delivery tools due to their high cargo molecule transportation efficiency and low cytotoxicity. Therefore, in many research fields CPP, such as HIV-Tat and oligoarginine（Rn）, are used to deliver hydrophilic drugs and biomolecules, including proteins, DNA, and RNA. We designed four types of CPP that contained cationic α,α-disubstituted amino acids（$Api^{C2Gu}$ and $Api^{C4Gu}$）as helical promoters; i.e., 1–4 [FAM-β-Ala-$(_L$-Arg-$_L$-Arg-Xaa$)_3$-$(Gly)_3$-$NH_2$（1: Xaa = $Api^{C2Gu}$, 2: Xaa = $Api^{C4Gu}$）, 3: FAM-β-Ala-$(_L$-Arg$)_8$-ApiC2Gu-$(Gly)_3$-$NH_2$, and 4: FAM-β-Ala-$(_L$-Arg$)_5$-$Api^{C2Gu}$-$(_L$-Arg$)_2$-$Api^{C2Gu}$-$(Gly)^3$-$NH_2$]，and investigated their preferred secondary structures and cell membrane-penetrating ability. As a result, we found that the permeation efficiency of the CPP was affected by the number of helical promoters in their sequences. Specially, peptide 1, which contained three $Api^{C2Gu}$ residues, formed a stable helical structure and passed through the cell membrane more efficiently than the other peptides. Moreover, it was demonstrated that the spatial arrangement of the peptides' side chains also influenced their permeability and the helical stabilization of their main chains.
Keywords: cell penetrating peptide, helical structure, plasmid DNA delivery

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Misawa T, Tanaka K, Demizu Y, Kurihara M: Efficient synthesis of a multi-substituted diphenylmethane skeleton as a steroid mimetic.
*Bioorg Med Chem Lett*. 2017;DOI:10.1016/j.bmcl.2017.03.066

Steroids are important components of cell membranes and are involved in several physiological functions. A diphenylmethane（DPM）skeleton has recently been suggested to act as a mimetic of the steroid skeleton. However, difficulties are associated with efficiently introducing different substituents between two phenyl rings of the DPM skeleton, and, thus, further structural development based on the DPM skeleton has been limited. We herein developed an efficient synthetic method for introducing different substituents into two phenyl rings of the DPM skeleton. We also synthesized DPM-based estrogen receptor（ER）modulators using our synthetic method and evaluated their ER transcriptional activities.
Keywords: estrogen receptor, diphenylmethane skeleton, multi substituted

三澤隆史，出水庸介，谷内出友美*1，大岡伸通，野尻久雄*2，橘高敦史*2，槇島誠*3，内藤幹彦，栗原正明：長鎖アルキル基を有するノンセコステロイド型VDRリガンドの創製.
*ビタミン学会誌* 2017;2:113-20

Vitamin D receptor（VDR）is the superfamily of nuclear receptors（NRs）. The receptor regulates physiological functions in the immune system, calcium homeostasis, cell proliferation, and so on. 1α,25- Dihydroxyvitamin D3（1α,25$(OH)_2D_3$）, which is the physiologically active form of vitamin D, acts as an endogenous VDR ligand. Most of VDR ligands identified to date have a secosteroid skeleton, which is a steroid backbone, and cause hypercalcemia. On the other hand, several non-secosteroidal VDR ligands have been developed for the aim to avoid this disadvantage.

We synthesized non-secosteroidal VDR ligands bearing a long alkyl chain based on the diphenylpentane skeleton (KM derivatives). The VDR-mediated transcriptional activity of the KM derivatives was evaluated using the reporter gene assay and HL-60 cell differentiation-inducing assay. We herein described the structure-activity relationship and the effect of alkyl-chain length on VDR-mediated transcriptional activity for the KM derivatives. Furthermore, we performed a computational docking analysis to investigate the binding mode of the KM derivatives to VDR. These results suggest that the affinity of synthesized VDR ligands not only for ligand binding regions, but also for the surrounding solvent is important for increasing the biological activity of the synthesized VDR ligands and that this affinity may be a key information for drug design.

Keywords: vitamin D receptor, long alkyl chain, docking analysis

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曽我慶介，亀井俊之，蜂須賀暁子，最上（西巻）知子：
食品中自由水に含まれるトリチウムの共沸蒸留による分離・分析法.
*食品衛生学雑誌*　2016;57(4):81-8

福島第一原子力発電所事故以後，トリチウム（$^{3}$H）を含んだ汚染水の環境中への放流の危険性が浮上し，食品の$^{3}$H安全性評価が求められている．本研究では，食品中自由水に存在する$^{3}$Hの実用的な分析法を確立するため，利便性と検出感度を指標に液体シンチレーション法の最適化を行い，食品中の自由水単離法として共沸蒸留法の検討を行った．検出下限値は年間1 mSvの約0.01％となる10 Bq/Lを満たすように$^{3}$H測定条件を設定した．$^{3}$H添加回収実験では，$^{3}$H回収率が果実・野菜・肉・魚介類で85～90％，米や穀類で75～85％であった．一方，含水量の低い菓子類では，$^{3}$H回収率が50％以下であったが，蒸留前に加水処理を行うことによって，$^{3}$H回収率と精度が向上した．その結果，用いた全13食品群で，$^{3}$H回収率75％以上，RSDが10％以内であった．したがって，本分析法は食品の$^{3}$H安全性評価のための感度，精度を有し，広範囲の食品に適用可能と考えられる．本分析法を用いて流通食品42種類の$^{3}$H分析を実施したところ，すべて検出下限値以下であった．

Keywords：放射性物質汚染食品，トリチウム，液体シンチレーション

Noguchi A, Nakamura K, Sakata K, Sato-Fukuda N, Ishigaki T, Mano J*, Takabatake R*, Kitta K*, Teshima R, Kondo K, Nishimaki-Mogami T: Development and interlaboratory validation of a simple screening method for genetically modified maize using ΔΔCq-based multiplex real-time PCR.
*Analytical Chemistry*. 2016;88(8):4285-93

A number of genetically modified (GM) maize events have been developed and approved worldwide for commercial cultivation. A screening method is needed to monitor GM maize approved for commercialization in countries that mandate the labeling of foods containing a specified threshold level of GM crops. In Japan, a screening method has been implemented to monitor approved GM maize since 2001. However, the screening method currently used in Japan is time-consuming and requires generation of a calibration curve and experimental conversion factor (C(f)) value. We developed a simple screening method that avoids the need for a calibration curve and C(f) value. In this method, ΔC(q) values between the target sequences and the endogenous gene are calculated using multiplex real-time PCR, and the ΔΔC(q) value between the analytical and control samples is used as the criterion for determining analytical samples in which the GM organism content is below the threshold level for labeling of GM crops. An interlaboratory study indicated that the method is applicable independently with at least two models of PCR instruments used in this study.

Keywords: genetically modified maize, screening, PCR

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Nakamura K, Kondo K, Akiyama H, Ishigaki T, Noguchi A, Katsumata H*1, Takasaki K*1, Futo S*1, Sakata K, Fukuda N, Mano J*2, Kitta K *2, Tanaka H*3, Akashi R*3, Nishimaki-Mogami T: Whole genome sequence analysis of unidentified genetically modified papaya for development of a specific detection method.
*Food Chemistry*. 2016;205:272-9

Identification of transgenic sequences in an unknown genetically modified (GM) papaya (*Carica papaya* L.) by whole genome sequence analysis was demonstrated.

Whole genome sequence data were generated for a GM-positive fresh papaya fruit commodity detected in monitoring using real-time polymerase chain reaction (PCR). The sequences obtained were mapped against an open database for papaya genome sequence. Transgenic construct- and event-specific sequences were identified as a GM papaya developed to resist infection from a Papaya ringspot virus. Based on the transgenic sequences, a specific real-time PCR detection method for GM papaya applicable to various food commodities was developed. Whole genome sequence analysis enabled identifying unknown transgenic construct- and event-specific sequences in GM papaya and development of a reliable method for detecting them in papaya food commodities.

Keywords: *Carica papaya* L., genetically modified, genome sequence

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*Data in Brief*. 2016;7:1165-70

This article is referred to research article entitled "Whole genome sequence analysis of unidentified genetically modified papaya for development of a specific detection method" (Nakamura et al., 2016). Real-time polymerase chain reaction (PCR) detection method for unauthorized genetically modified (GM) papaya (*Carica papaya* L.) line PRSV-YK (PRSV-YK detection method) was developed using whole genome sequence data (DDBJ Sequenced Read Archive under accession No. PRJDB3976). Interlaboratory validation datasets for PRSV-YK detection method were provided. Data indicating homogeneity of samples prepared for interlaboratory validation were included. Specificity and sensitivity test data for PRSV-YK detection method were also provided.

Keywords: *Carica papaya* L., genetically modified, real-time PCR

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Miyahara T*1, Miyake N*1, Sawahuji K*1, Kitta K*2, Nakamura K, Kondo K, Ozeki Y*1: Wheat DNA fragmentation of commercial processed foods.

*Journal of Food Chemistry and Safety*. 2016;23:141-8

Recent advances in plant biotechnology have established transgenic wheat lines, which are almost ready to be cultivated for commercial production in the field. Wheat flours are used as ingredients in many food products. Here, in order to detect genetically modified wheat in processed foods, the yield and fragmentation of genomic DNA prepared from processed foods were investigated. Qualitative PCR using primer sets that gave 96-755 bp PCR products at 100 bp intervals showed that in fermenting processes by yeast and baking processes for breads and buns, including steaming and frying, DNA fragmentation of less than 430 bp did not occur. Amplicons longer than 755 bp were found in all noodles, but roasting and retort processes to produce stews caused severe degradation of genomic DNA leading to fragmentation and reduced yields. A Japanese traditional sweet, kuzumochi, which is processed by *Lactobacillus* fermentation with kneaded flours for a year, gave amplicons shorter than 323 bp. These results indicated that PCR detection methods for transgenes in wheat processed foods should be established using primer pairs that target DNA sequences shorter than 200 bp.

Keywords: DNA fragmentation, wheat processed food, qualitative PCR

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Takabatake R*1, Masubuchi T*1, Futo S*2, Minegishi Y*3, Noguchi A, Kondo K, Teshima R, Kurashima T*1, Mano J*1, Kitta K*1: Selection of suitable DNA extraction methods for genetically modified maize 3272, and development and evaluation of an event-specific quantitative PCR method for 3272.

*Shokuhin Eiseigaku Zasshi*. 2016;57:1-6

A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) maize, 3272. We first attempted to obtain genome DNA from this maize using a DNeasy Plant Maxi kit and a DNeasy Plant Mini kit, which have been widely utilized in our previous studies, but DNA extraction yields from 3272 were markedly lower than those from non-GM maize seeds. However, lowering of DNA extraction yields was not observed with GM quicker or Genomic-tip 20/G. We chose GM quicker for evaluation of the quantitative method. We prepared a standard plasmid for 3272 quantification. The conversion factor (Cf), which is required to calculate the amount of a genetically modified organism (GMO), was experimentally determined for two real-time PCR instruments, the Applied Biosystems 7900HT (the ABI 7900) and the Applied Biosystems 7500 (the ABI7500). The determined Cf values were 0.60 and 0.59 for the ABI 7900 and the ABI 7500, respectively. To evaluate the developed method, a blind test was conducted as part of an interlaboratory study. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSDr). The determined values were similar to those in our previous validation studies. The limit of quantitation for the method was estimated to be 0.5% or less, and we concluded that the developed method would be suitable and practical for detection and quantification of 3272.

Keywords: 3272, event-specific, genetically modified (GM)

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Nakajima O, Nishimaki-Mogami T, Kondo K: Cas9 in genetically modified food is unlikely to cause food allergy.

*Biological and Pharmaceutical Bulletin*. 2016;39 (11):1876-80

Genome editing has undergone rapid development during the last three years. It is anticipated that genetically modified organisms (GMOs) for food purposes will be widely produced using the clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR)/Cas9 system in the near future. However, the Cas9 gene may then enter the genomes of GMOs for food if the breeding process is not strictly managed, which could lead to the Cas9 protein or associated peptides being produced within these organisms. A variety of peptides could theoretically be produced from the Cas9 gene by using open reading frames different from that of Cas9 in the GMOs. In this study, Cas9 and the peptides potentially encoded by Cas9 genes were studied regarding their immunogenicity, in terms of the digestibility of Cas9 and the homology of the peptides to food allergens. First, the digestibility and thermal stability of Cas9 were studied. Digestibility was tested with natural or heat-denatured Cas9 in simulated gastric fluid in vitro. The two types of Cas9 were digested rapidly. Cas9 was also gradually degraded during heat treatment. Second, the peptides potentially encoded by Cas9 genes were examined for their homology to food allergens. Specifically, an 8-mer exact match search and a sliding 80-mer window search were performed using allergen databases. One of the peptides was found to have homology with a food allergen.

Keywords: food safety, genetically modified organism, Cas9

Tsukahara K*1, Takabatake R*1, Masubuchi T*1, Futo S*2, Minegishi Y*3, Noguchi A, Kondo K, Nishimaki-Mogami T, Kurashima T*1, Mano J*1, Kitta K*1: Development and evaluation of event-specific quantitative PCR method for genetically modified soybean MON87701.

*Shokuhin Eiseigaku Zasshi*. 2016;57:187-92

A real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) soybean event, MON87701. First, a standard plasmid for MON87701 quantification was constructed. The conversion factor (Cf) required to calculate the amount of genetically modified organism (GMO) was experimentally determined for a real-time PCR instrument. The determined Cf for the real-time PCR instrument was 1.24. For the evaluation of the developed method, a blind test was carried out in an inter-laboratory trial. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDr), respectively.

The determined biases and the RSDr values were less than 30 and 13%, respectively, at all evaluated concentrations. The limit of quantitation of the method was 0.5%, and the developed method would thus be applicable for practical analyses for the detection and quantification of MON87701.

Keywords: MON87701, event-specific, genetically modified (GM)

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Mano J*1, Nishitsuji Y*2, Kikuchi Y*2, Fukudome S*2, Hayashida T*3, Kawakami H*3, Kurimoto Y*3, Noguchi A, Kondo K, Teshima R, Takabatake R*1, Kitta K*1: Quantification of DNA fragmentation in processed foods using real-time PCR.

*Food Chemistry*. 2017;226:149-55

DNA analysis of processed foods is performed widely to detect various targets, such as genetically modified organisms (GMOs). Food processing often causes DNA fragmentation, which consequently affects the results of PCR analysis. In order to assess the effects of DNA fragmentation on the reliability of PCR analysis, we investigated a novel methodology to quantify the degree of DNA fragmentation. We designed four real-time PCR assays that amplified 18S ribosomal RNA gene sequences common to various plants at lengths of approximately 100, 200, 400, and 800 base pairs (bp). Then, we created an indicator value, "DNA fragmentation index (DFI)", which is calculated from the Cq values derived from the real-time PCR assays. Finally, we demonstrated the efficacy of this method for the quality control of GMO detection in processed foods by evaluating the relationship between the DFI and the limit of detection.

Keywords: DNA, fragmentation, processed food

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Ito K*, Yamamoto T*, Oyama Y*, Tsuruma R*, Saito E*, Saito Y*, Ozu T*, Honjoh T*, Adachi R, Sakai S, Akiyama H, Shoji M*: Food allergen analysis for processed food using a novel method to eliminate harmful reagents for both ELISA and lateral-flow tests.

*Anal Bioanal Chem*. 2016;408(22):5973-84

Enzyme-linked immunosorbent assay (ELISA) is commonly used to determine food allergens in food products. However, a significant number of ELISAs give an erroneous result, especially when applied to highly processed food. Accordingly, an improved ELISA, which utilizes an extraction solution comprising the surfactant sodium lauryl sulfate (SDS) and reductant 2-mercaptoethanol (2-ME), has been specially developed to analyze food allergens in highly processed food by enhancing analyte protein extraction. Recently, however, the use of 2-ME has become undesirable. In the present study, a new extraction solution containing a human- and eco-friendly reductant, which is convenient to use at the food manufacturing site, has been established. Among three chemicals with different reducing properties, sodium sulfite, tris (3-hydroxypropyl) phosphine, and mercaptoethylamine sodium sulfite was selected as a 2-ME substitute. The protein extraction ability of SDS/0.1 M sodium sulfite solution was comparable to that of SDS/2-ME solution. Next, the ELISA performance for egg, milk, wheat, peanut, and buckwheat was evaluated by using model-processed foods and commercially available food products. The data showed that the SDS/0.1 M sulfite ELISA significantly correlated with the SDS/2-ME ELISA for all food allergens examined ($p < 0.01$), thereby establishing the validity of the SDS/0.1 M sulfite ELISA performance. Furthermore, the new SDS/0.1 M sulfite solution was investigated for its applicability to the lateral-flow (LF) test. The result demonstrated the successful analysis of food allergens in processed food, showing consistency with the SDS/0.1 M sulfite ELISA results. Accordingly, a harmonized analysis system for processed food comprising a screening LF test and a quantitative ELISA with identical extraction solution has been established. The ELISA based on the SDS/0.1 M sulfite extraction solution has now been authorized as the revised official method for food allergen analysis in Japan.

Keywords: ELISA, processed food, food allergen analysis

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Hasunuma T*[1], Tohkin M*[2], Kaniwa N, Jang IJ*[3], Yimin C*[4], Kaneko M*[5], Saito Y, Takeuchi M*[6], Watanabe H*[7], Yamazoe Y*[8], Uyama Y*[9], Kawai S*[1]: Absence of ethnic differences in the pharmacokinetics of moxifloxacin, simvastatin, and meloxicam among three East Asian populations and Caucasians.

*Br J Clin Pharmacol*. 2016;81:1078-90

To examine whether strict control of clinical trial conditions could reduce apparent differences of pharmacokinetic (PK) parameters among ethnic groups.

Open-label, single dose PK studies of moxifloxacin, simvastatin and meloxicam were conducted in healthy male subjects from three East Asian populations (Japanese, Chinese and Koreans) and one Caucasian population as a control. These three drugs were selected because differences in PK parameters have been reported, even though the backgrounds of these East Asian populations are similar. Moxifloxacin (400 mg) was administered orally to 20 subjects, and plasma and urine levels of moxifloxacin and its metabolite (M2) were measured. Simvastatin (20 mg) was given to 40 subjects, and plasma levels of simvastatin and simvastatin acid were measured. Meloxicam (7.5 mg) was given to 30 subjects and its plasma concentration was determined. Intrinsic factors (polymorphism of UGT1A1 for moxifloxacin, SLCO1B1 for simvastatin, and CYP2C9 for meloxicam) were also examined.

AUCinf values for moxifloxacin, simvastatin and meloxicam showed no significant differences among the East Asian groups. Cmax values of moxifloxacin and simvastatin, but not meloxicam, showed significant differences. There were no significant differences of data for M2 or simvastatin acid. Genetic analysis identified significant differences in the frequencies of relevant polymorphisms, but these differences did not affect the PK parameters observed.

Although there were some differences in PK parameters among the three East Asian groups, the present study performed under strictly controlled conditions did not reproduce the major ethnic differences observed in previous studies.

Keywords: east asia, pharmacokinetics, population difference

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Okemoto K, Maekawa K, Tajima Y*, Tohkin M*, Saito Y: Cross-Classification of Human Urinary Lipidome by Sex, Age, and Body Mass Index.

*PLOS ONE*. 2016;11:e0168188

Technological advancements in past decades have led to the development of integrative analytical approaches to lipidomics, such as liquid chromatography-mass spectrometry (LC/MS), and information about biogenic lipids is rapidly accumulating. Although several cohort-based studies have been conducted on the composition of urinary lipidome, the data on urinary lipids cross-classified by sex, age, and body mass index (BMI) are insufficient to screen for various abnormalities. To promote the development of urinary lipid metabolome-based diagnostic assay, we analyzed 60 urine samples from healthy white adults (young (c.a., 30 years) and old (c.a., 60 years) men/women) using LC/MS. Women had a higher urinary concentration of omega-3 12-lipoxygenase (LOX)-generated oxylipins with anti-inflammatory activity compared to men. In addition, young women showed increased abundance of poly-unsaturated fatty acids (PUFAs) and cytochrome P450 (P450)-produced oxylipins with anti-hypertensive activity compared with young men, whereas elderly women exhibited higher concentration of 5-LOX-generated anti-inflammatory oxylipins than elderly men. There were no significant differences in urinary oxylipin levels between young and old subjects or between subjects with low and high BMI. Our findings suggest that sex, but neither ages nor BMI could be a confounding factor for measuring the composition of urinary lipid metabolites in the healthy population. The information showed contribute to the development of

reliable biomarker findings from urine.

Keywords: background factor, metabolomics, human urine

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Sai K, Kajinami K*[1], Akao H*[1], Iwadare M*[1], Sato-Ishida R*[1], Kawai Y*[1], Takeda K*[1], Tanimoto T*[2], Yamano T*[2], Akasaka T*[2], Ishida T*[3], Hirata KI*[3], Saku K*[4], Yagi S*[5], Soeki T*[5], Sata M*[5], Ueno M*[6], Miyazaki S*[6], Shiraki A*[7], Oyama JI*[7], Node K*[7], Sugamura K*[8], Ogawa H*[8], Kurose K, Maekawa K, Matsuzawa Y, Imatoh T, Hasegawa R, Japanese Pharmacogenomics Data Science Consortium*[9], Saito Y: A possible role for *HLA-DRB1**04:06 in statin-related myopathy in Japanese patients.

*Drug Metab Pharmacokinet*. 2016;31:467-70

This study was aimed to identify clinically important genetic markers associated with Statin-Related Myopathy (SRM) in Japanese SRM patients (n = 52) and healthy Japanese subjects (n = 2878 or 86) as controls. No significant association of three reported makers, i.e., RYR2, SLCO1B1 and GATM variants, with SRM were observed, but a significant association was detected for *HLA-DRB1**04:06 with SRM (odds ratio: 3.19; 95% confidence interval: 1.53-6.66). This study suggested that *HLA-DRB1**04:06 might be associated with SRM onset in a Japanese population.

Keywords: genetic polymorphism, HLA, myopathy

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Saito K, Ueta M*, Maekawa K, Sotozono C*, Kinoshita S*, Saito Y: Plasma Lipid Profiling of Patients with Chronic Ocular Complications Caused by Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis.

*PLOS ONE*. 2016;11:e0167402

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are drug-induced acute inflammatory vesiculobullous reactions of the skin and mucous membranes, including the ocular surface. Even after recovery from skin symptoms, some SJS/TEN patients continue to suffer with severe ocular complications (SOCs). Therefore, this study aims to understand the pathophysiology of chronic SOCs. Because plasma lipid profiling has emerged as a useful tool to understand pathophysiological alterations in the body, we performed plasma lipid profiling of 17 patients who suffered from SJS/TEN-associated chronic SOCs. A lipidomics approach yielded 386 lipid molecules and demonstrated that plasma levels of inflammatory oxylipins increased in patients with SJS/TEN-associated chronic SOCs. In addition, oxidized phosphatidylcholines and ether-type diacylglycerols increased in the patients with chronic SOCs, while phosphoglycerolipids decreased. When we compared these lipidomic profiles with those of patients with atopic dermatitis, we found that patients with chronic SOCs, specifically, had decreased levels of ether-type phosphatidylcholines (ePCs) containing arachidonic acid (AA), such as PC(18:0e/20:4) and PC(20:0e/20:4). To confirm our finding, we recruited additional patients, who suffered from SOC associated with SJS/TEN (up to 51 patients), and validated the decreased plasma levels of AA-containing ePCs. Our study provides insight into the alterations of plasma lipidomic profiles in chronic SOCs and into the pathophysiology of SJS/TEN-associated chronic SOCs.

Keywords: lipidomics, ether-phosphatidylcholine, sever ocular complications

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Saito K, Maekawa K, Kinchen JM*[1], Tanaka R*[2], Kumagai Y*[2], Saito Y: Gender- and Age-Associated Differences in Serum Metabolite Profiles among Japanese Populations.

*Biol Pharm Bull*. 2016;39:1179-86

Serum metabolites can reflect the diffusion/export of biochemicals from various organs. They can serve as biomarkers related to diseases and therapeutic efficacy/toxicity. While studies in Caucasians suggested that subject gender and age can affect circulating metabolite profiles, the Japanese population has not

been surveyed. Our objective was to delineate gender- and age-associated differences in serum metabolite profiles among Japanese populations. Using a mass spectrometry-based global metabolomics approach, 516 endogenous metabolites were detected in sera from Japanese individuals. The principal component analysis identified gender as the primary component, followed by age, suggesting that these two criteria were key contributors to variations in the dataset. Gender-associated differences were observed in 31 and 25% of metabolites in the young (age 25-35) and old (ages 55-65) populations, respectively, in redox homeostasis, and in steroid and purine nucleotide metabolism pathways. Age-associated differences were observed in 24 and 23% of metabolites in men and women, respectively. No pathway was commonly highlighted. Thus, gender and age impact on metabolite profiles in the Japanese population. Our results provide useful information to explore biomarkers for clinical applications in the Japanese population and to assess the applicability of known biomarkers identified in other populations to the Japanese population.

Keywords: metabolomics, Japanese populations, serum metabolite

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Saito K, Arai E＊1,2, Maekawa K, Ishikawa M, Fujimoto H＊3, Taguchi R, Matsumoto K＊4, Kanai Y＊1,2, Saito Y: Lipidomic Signatures and Associated Transcriptomic Profiles of Clear Cell Renal Cell Carcinoma.

*Sci Rep*. 2016;6:28932

Renal cell carcinoma (RCC) is the most common histological type of adult kidney cancer. In this study, we obtained lipidomic profiles of clear cell RCC (ccRCC), a major RCC subtype, by performing a lipidomic analysis of specimens of cancerous tissue and the surrounding normal renal cortex obtained from the same patients (N＝49). We also compared the lipidomic profiles with the lipogenic transcriptome of specimens of cancerous tissue and the surrounding normal renal cortex for an additional set of patient samples (N＝95). Overall, we detected 326 lipids, including phospholipids, sphingolipids, neutral lipids, and eicosanoids. The levels of more than 70% of the detected lipids were significantly different ($P < 0.01$, corrected by the false discovery rate). The cancerous tissue was distinguished by higher levels of ether-type phospholipids, cholesterol esters, and triacylglycerols, as well as by lower levels of phospholipids (except for phosphatidylcholines) and polyunsaturated fatty acids. Characteristic changes in the levels of mRNAs and metabolites suggested that the phosphatidylethanolamine (PE) synthesis pathway is suppressed in ccRCC and associated with cell proliferation. The present study represents the lipidomic profiles of ccRCC, which provides novel information about the metabolic changes in renal cancerous tissue and RCC pathophysiology.

Keywords: lipidomics, renal cancer, transomics

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Nishimura M＊1, Toyoda M＊1, Takenaka K＊1, Imamura Y＊1, Chayahara N＊1, Kiyota N＊1, Mukohara T＊1,2, Kotake T＊3,4, Tsuji A＊3,5, Saito K, Saito Y, Minami H＊1,2: The combination of HLA-B＊15:01 and DRB1＊15:01 is associated with gemcitabine plus erlotinib-induced interstitial lung disease in patients with advanced pancreatic cancer.

*Cancer Chemother Pharmacol*. 2016;77:1165-70

PURPOSE: In a phase III study of gemcitabine plus erlotinib for advanced pancreatic cancer conducted in Canada, the incidence of interstitial lung disease (ILD) was 3.5 %. However, the incidence of ILD was reported as high as 8.5 % in a Japanese phase II study. These results suggest the influence of ethnic factors in the association of the use of gemcitabine plus erlotinib with the incidence of ILD. Here, we conducted a prospective study to analyze the relationship between human leukocyte antigen (HLA) alleles and ILD in Japanese patients with advanced pancreatic cancer receiving gemcitabine plus erlotinib. METHODS: Patients were treated with gemcitabine (1000 mg/m (2); administered by intravenous infusion on days 1, 8, and 15 every 4 weeks) and erlotinib (given orally at 100 mg/day). We compared the frequencies of HLA alleles in patients who did and did not develop ILD.

RESULTS: A total of 57 patients were treated, and 4 patients (7.0 %) developed ILD. The combination of HLA-B*15:01 and DRB1*15:01 was observed in 2 of 4 patients (50 %) with ILD and in only 1 of 53 patients without ILD (2 %) resulting in odds ratio of 52.0 (95 % CI 3.2-842.5; p = 0.011). CONCLUSION:These results suggest that the combination of HLA-B*15:01 and DRB1*15:01 is associated with ILD in Japanese patients with advanced pancreatic cancer receiving gemcitabine plus erlotinib.
Keywords: gemcitabine, erlotinib, HLA analysis

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Ishikawa M, Saito K, Yamada H*, Nakatsu N*, Maekawa K, Saito Y: Plasma lipid profiling of different types of hepatic fibrosis induced by carbon tetrachloride and lomustine in rats.
*Lipids Health Dis.* 2016;15:74

BACKGROUND: Plasma lipid profiling has emerged as a useful tool for understanding the pathophysiology of hepatic injury and disease. Hepatic fibrosis results from chronic, progressive damage to the liver and can lead, in turn, to more serious conditions such as hepatic cirrhosis and hepatocellular carcinoma. Thus, the present study aimed to investigate the plasma lipid profiles of two types of hepatic fibrosis in order to aid the understanding of the pathophysiology of hepatic fibrosis. METHODS: A liquid chromatography and mass spectrometry platform was used to reveal and compare the plasma lipid profiles of two types of chemical-induced hepatic fibrosis. Rat models of centrilobular fibrosis and bile duct fibrosis were established via chronic exposure to the known fibrogenic hepatotoxins, carbon tetrachloride (CCl4) or lomustine (LS), respectively, over a 28-day period. To delineate the specific alterations in the lipid profiles as a result of the hepatic fibrosis, we also employed non-fibrogenic hepatotoxicants (2-acetamidofluorene, N-nitrosodiethylamine, and ethambutol) as well as 3-day treatment of CCl4 and LS, which did not induce fibrosis. RESULTS: Our assay platform identified 228 lipids in the rat plasma, and the global lipid profile clearly distinguished these models from the control via principal component analysis. In addition, the alteration of the plasma lipid profile caused by CCl4 and LS were clearly different. Furthermore, a number of lipids were identified as specific alterations caused by fibrosis induced only by CCl4 and LS, respectively. Three lysophosphatidylcholines (LPC[18:3], LPC[20:4], and LPC[22:6]), and three phosphatidylcholines (PC[18:2/20:4], PC[40:8], and PC[20:4/22:6]) are specific circulating lipids, the levels of which were altered by both CCl4 and LS treatment; however, their levels were decreased by chronic exposure to CCl4 and increased by chronic exposure to LS. CONCLUSIONS: These results suggest that different types of chemical-induced hepatic fibrosis demonstrate clear differences in their plasma lipid profiles. Our study provides insights into the alteration of plasma lipidomic profiles as a result of the fibrosis of different parts of the hepatic lobule, and may help to understand the pathophysiology of different types of hepatic fibrosis.
Keywords: lipidomics, liver fibrosis, hepatic zonation

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Okamoto-Uchida Y, Yu R*1, Miyamura N*1, Arima N*1, Ishigami-Yuasa M*1, Kagechika H*1, Penninger JM*2, Nishina S*3, Azuma N*3, Nishina H*1: The mevalonate pathway regulates primitive streak formation *via* protein farnesylation.
*Sci Rep.* 2016;6:37697

The primitive streak in peri-implantation embryos forms the mesoderm and endoderm and controls cell differentiation. The metabolic cues regulating primitive streak formation remain largely unknown. Here we utilised a mouse embryonic stem (ES) cell differentiation system and a library of well-characterised drugs to identify these metabolic factors. We found that statins, which inhibit the mevalonate metabolic pathway, suppressed primitive streak formation in vitro and in vivo. Using metabolomics and pharmacologic approaches we identified the downstream signalling pathway of mevalonate and revealed that primitive streak formation requires protein farnesylation but not cholesterol synthesis. A tagging-via-substrate approach revealed that nuclear lamin B1 and small G proteins were farnesylated in

embryoid bodies and important for primitive streak gene expression. In conclusion, protein farnesylation driven by the mevalonate pathway is a metabolic cue essential for primitive streak formation.

Keywords: statin, embryotoxicity, primitive streak

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Okamoto-Uchida Y, Nakamura R, Matsuzawa Y, Soma M*, Kawakami H*, Ishii-Watabe A, Nishimaki-Mogami T, Teshima R, Saito Y: Different results of IgE binding- and crosslinking-based allergy tests caused by allergen immobilization.
*Biol Pharm Bull*. 2016;39:1662-6

The physicochemical nature of allergen molecules differ from the liquid phase to the solid phase. However, conventional allergy tests are based on the detection of immunoglobulin (Ig) E binding to immobilized allergens. We recently developed an in vitro allergy testing method using a luciferase-reporting humanized rat mast cell line to detect IgE crosslinking-induced luciferase expression (EXiLE test). The aim of the present study was to evaluate the effects of antigen immobilization on the results of different in vitro allergy tests using two anti-ovalbumin (OVA) antibodies (Abs), E-C1 and E-G5, with different properties in the OVA-induced allergic reaction. Both Abs showed clear binding to OVA with an enzyme-linked immunosorbent assay and by BIAcore analysis. However, only E-C1 potentiated EXiLE response for the liquid-phase OVA. On the other hand, OVA immobilized on solid-phase induced EXiLE responses in both E-C1 Ab- and E-G5 Ab-sensitized mast cells. Western blotting of OVA indicated that E-C1 Ab binds both to OVA monomers and dimers, unlike E-G5 Ab, which probably binds only to the OVA dimer. These results suggest that antigen immobilization enhanced IgE crosslinking ability through multimerization of allergen molecules in the solid phase, resulting in an increase in false positives in IgE binding-based conventional in vitro allergy tests. These findings shed light on the physicochemical nature of antigens as an important factor for the development and evaluation of in vitro allergy tests and suggest that mast cell activation-based allergy testing with liquid-phase allergens is a promising strategy to evaluate the physiological interactions of IgE and allergens.

Keywords: allergy test, EXiLE, allergen immobilization

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Furukawa Y, Tanemura K*1, Igarashi K*2, Ideta-Otsuka M*2, Aisaki K, Kitajima S, Kitagawa M*3, Kanno J*4: Learning and memory deficits in male adult mice treated with a benzodiazepine sleep-inducing drug during the juvenile period.
*Front Neurosci*. 2016;10:339

Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, is also known to be important for brain development. Therefore, disturbances of GABA receptor (GABA-R) mediated signaling (GABA-R signal) during brain development may influence normal brain maturation and cause late-onset brain malfunctions. In this study, we examined whether the stimulation of the GABA-R signal during brain development induces late-onset adverse effects on the brain in adult male mice. To stimulate the GABA-R signal, we used either the benzodiazepine sleep-inducing drug triazolam (TZ) or the non-benzodiazepine drug zolpidem (ZP). We detected learning and memory deficits in mice treated with TZ during the juvenile period, as seen in the fear conditioning test. On the other hand, ZP administration during the juvenile period had little effect. In addition, decreased protein expression of GluR1 and GluR4, which are excitatory neurotransmitter receptors, was detected in the hippocampi of mice treated with TZ during the juvenile period. We measured mRNA expression of the immediate early genes (IEGs), which are neuronal activity markers, in the hippocampus shortly after the administration of TZ or ZP to juvenile mice. Decreased IEG expression was detected in mice with juvenile TZ administration, but not in mice with juvenile ZP administration. Our findings demonstrate that TZ administration during the juvenile period can induce irreversible learning and memory deficits in adult mice. It may need to take an extra care for the prescription of benzodiazepine sleep-inducing drugs to

juveniles because it might cause learning and memory deficits.

Keywords: GABA receptor signal, behavioral battery test, sleep-inducing drug

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Tsuboi I*, Harada T*, Hirabayashi Y, Kanno J, Aizawa S*: Differential Regulation of Lympho-Myelopoiesis by Stromal Cells in the Early and Late Phases in BALB/c Mice Repeatedly Exposed to Lipopolysaccharide.

*Biol Pharm Bull*. 2016;39:1939-47

Chronic lipopolysaccharide (LPS) exposure to mice reduces the lymphoid compartment and skews the hematopoietic cell compartment toward myeloid-cells, which is considered to be a direct effect of LPS on hematopoietic stem cells. However, the effect of chronic LPS exposure on stromal-cells, which compose the hematopoietic microenvironment, has not been elucidated. Here, we investigated early- and late-phase effects of repeated LPS exposure on stromal-cells. During the early phase, when mice were treated with 5 or 25 microg LPS three times at weekly intervals, the numbers of myeloid-progenitor (colony forming unit-granulocyte macrophage (CFU-GM)) cells and B lymphoid-progenitor (CFU-preB) cells in the bone-marrow (BM) rapidly decreased after each treatment. The number of CFU-GM cells recovered from the initial decrease and then increased to levels higher than pretreatment levels, whereas the number of CFU-preB cells remained lower than pretreatment levels. In the BM, expression of genes for positive-regulators of myelopoiesis including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin (IL) -6 and negative-regulators of B lymphopoiesis including tumor necrosis factor (TNF)-alpha was up-regulated, whereas expression of positive-regulators of B lymphopoiesis including stromal cell-derived factor (SDF)-1, IL-7, and stem cell factor (SCF) was down-regulated. During the late phase, the number of CFU-preB cells remained lower than pretreatment levels 70 d after the first treatments with 5 and 25 microg LPS, whereas the number of CFU-GM cells returned to pretreatment levels. IL-7 gene expression in the BM remained down-regulated, whereas gene-expression levels of SDF-1 and SCF were restored. Thus, chronic LPS exposure may impair stromal-cell function, resulting in prolonged suppression of B lymphopoiesis, which may appear to be senescence similar to the hematological phenotype.

Keywords: gene Expression, lipopolysaccharides, stromal cell

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Takeda K*1,2, Kou I*1, Kawakami N*3, Iida A*1, Nakajima M*1, Ogura Y*1,2, Imagawa E*4, Miyake N*4, Matsumoto N*4, Yasuhiko Y, Sudo H*5, Kotani T*6, Nakamura M*2, Matsumoto M*2, Watanabe K*2, Ikegawa S*1: Compound Heterozygosity for Null Mutations and a Common Hypomorphic Risk Haplotype in TBX6 Causes Congenital Scoliosis.

*Hum Mutat*. 2017;38(3):317-23

Congenital scoliosis (CS) occurs as a result of vertebral malformations and has an incidence of 0.5-1/1,000 births. Recently, TBX6 on chromosome 16p11.2 was reported as a disease gene for CS; about 10% of Chinese CS patients were compound heterozygotes for rare null mutations and a common haplotype defined by three SNPs in TBX6. All patients had hemivertebrae. We recruited 94 Japanese CS patients, investigated the TBX6 locus for both mutations and the risk haplotype, examined transcriptional activities of mutant TBX6 in vitro, and evaluated clinical and radiographic features. We identified TBX6 null mutations in nine patients, including a missense mutation that had a loss of function in vitro. All had the risk haplotype in the opposite allele. One of the mutations showed dominant negative effect. Although all Chinese patients had one or more hemivertebrae, two Japanese patients did not have hemivertebra. The compound heterozygosity of null mutations and the common risk haplotype in TBX6 also causes CS in Japanese patients with similar incidence. Hemivertebra was not a specific type of spinal malformation in TBX6-associated CS (TACS). A heterozygous TBX6 loss-of-function mutation has been reported in a family with autosomal-dominant spondylocostal dysostosis,

but it may represent a spectrum of the same disease with TACS.

Keywords: TBX6, compound heterozygosity, congenital scoliosis

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Sato K, Takahashi K, Shigemoto-Mogami Y, Chujo K, Sekino Y: Glypican 6 increases the level of functional N-Methyl-D-aspartate receptors in human induced pluripotent stem cell derived neurons.
*Front Cell Neurosci*, 2016;10:259

The in *vitro* use of neurons that are differentiated from human induced pluripotent stem cells (hiPSC-neurons) is expected to improve the prediction accuracy of preclinical tests for both screening and safety assessments in drug development. To achieve this goal, hiPSC neurons are required to differentiate into functional neurons that form excitatory networks and stably express N-methyl-D-aspartate receptors (NMDARs). Recent studies have identified some astrocyte-derived factors that are important for the functional maturation of neurons. We therefore examined the effects of the astrocyte-derived factor glypican 6 (GPC6) on hiPSC-neurons. When we pharmacologically examined which receptor subtypes mediate L-glutamate (L-Glu)-induced changes in the intracellular $Ca^{2+}$ concentrations in hiPSC neurons using fura-2 $Ca^{2+}$ imaging, NMDAR-mediated responses were not detected through 7 days in vitro (DIV). These cells were also not vulnerable to excitotoxicity at 7DIV. However, a 5-d treatment with GPC6 from 3DIV induced an NMDAR-mediated $Ca^{2+}$ increase in hiPSC-neurons and increased the level of NMDARs on the cell surface. We also found that GPC6-treated hiPSC-neurons became responsive to excitotoxicity. These results suggest that GPC6 increases the level of functional NMDARs in hiPSC-neurons. Glial factors may play a key role in accelerating the functional maturation of hiPSC neurons for drug-development applications.

Keywords: human induced pluripotent stem cell, neuron, glypican 6

Gao M*, Igata H*, Takeuchi A*, Sato K, Ikegaya Y*: Machine learning-based prediction of adverse drug effects: an example of seizure-inducing compounds.
*J Pharmacol Sci*. 2017;133:70-8

Various biological factors have been implicated in convulsive seizures, involving side effects of drugs. For the preclinical safety assessment of drug development, it is difficult to predict seizure-inducing side effects. Here, we introduced a machine learning-based in vitro system designed to detect seizure-inducing side effects. We recorded local field potentials from the CA1 alveus in acute mouse neocortico-hippocampal slices, while 14 drugs were bath-perfused at 5 different concentrations each. For each experimental condition, we collected seizure-like neuronal activity and merged their waveforms as one graphic image, which was further converted into a feature vector using Caffe, an open framework for deep learning. In the space of the first two principal components, the support vector machine completely separated the vectors (i.e., doses of individual drugs) that induced seizure-like events and identified diphenhydramine, enoxacin, strychnine and theophylline as "seizure-inducing" drugs, which indeed were reported to induce seizures in clinical situations. Thus, this artificial intelligence-based classification may provide a new platform to detect the seizure-inducing side effects of preclinical drugs.

Keywords: artificial intelligence, side effect, epilepsy

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Shigemoto-Mogami Y, Hoshikawa K, Hirose A, Sato K: Phagocytosis-dependent and independent mechanisms underlie the microglial cell damage caused by carbon nanotube agglomerates.
*J Toxicol Sci*. 2016;41:501-9

Although carbon nanotubes (CNTs) are used in many fields, including energy, healthcare, environmental technology, materials, and electronics, the adverse effects of CNTs in the brain are poorly understood. In this study, we investigated the effects of CNTs on cultured microglia, as microglia are the first responders to foreign materials. We compared

the effects of sonicated suspensions of 5 kinds of CNTs and their flow-through filtered with a 0.22- m membrane filter on microglial viability. We found that sonicated suspensions caused microglial cell damage, however, their flow-through did not. The number of microglial aggregates was well correlated with the extent of the damage. We also determined that the CNT agglomerates consisted of two groups: one was phagocytosed by microglia and caused microglial cell damage, and the other caused cell damage without phagocytosis. These results suggest that phagocytosis-dependent and independent mechanisms underlie the microglial cell damage caused by CNT agglomerates and it is important to conduct studies about the relationships between physical properties of nanomaterial-agglomerates and cell damage.

Keywords: carbon nanotube, microglia, phagocytosis

Kanda Y, Yamazaki D, Kurokawa J[*1,2], Inutsuka T[*1,3], Sekino Y: Points to consider for a validation study of iPS cell-derived cardiomyocytes using a multi-electrode array system.

*J Pharmacol Toxicol Methods*. 2016;81:196-200

Human induced pluripotent stem cell-derived cardiomyocytes (iPS-CMs) provide a novel assay system to assess cardiac safety in drug development to overcome a problem of species difference in non-clinical testing during drug development. Using the multi-electrode array (MEA) platform, electrophysiological activities of iPS-CMs can be recorded easily to assess QT prolongation and proarrhythmic potential of drug candidates. Here we have established a standardized protocol to evaluate the possibility of iPS-CMs, and shared the protocol with an international consortium. To obtain reproducible and reliable experimental data from these cells, we determined the optimal experimental conditions, such as cell density, MEA coating, culture conditions, high-pass filter frequency, definition of early afterdepolarization or triggered activity, and calibration compounds. Based on the protocol, our validation study using 60 compounds is in progress. Thus, MEA-based experiments using iPS-CMs would be a standard testing method to evaluate QT prolongation and proarrhythmic potentials.

Keywords: cardiac safety pharmacology, human iPS cell, JiCSA

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Asanagi M[*1], Yamada S, Hirata N, Itagaki H[*1], Kotake Y[*2], Sekino Y, Kanda Y: Tributyltin induces G2/M cell cycle arrest via NAD+-dependent isocitrate dehydrogenase in human embryonic carcinoma cells.

*J Toxicol Sci*. 2016;41:207-15

Organotin compounds, such as tributyltin (TBT), are well-known endocrine-disrupting chemicals (EDCs). We have recently reported that TBT induces growth arrest in the human embryonic carcinoma cell line NT2/D1 at nanomolar levels by inhibiting NAD+-dependent isocitrate dehydrogenase (NAD-IDH), which catalyzes the irreversible conversion of isocitrate to α-ketoglutarate. However, the molecular mechanisms by which NAD-IDH mediates TBT toxicity remain unclear. In the present study, we examined whether TBT at nanomolar levels affects cell cycle progression in NT2/D1 cells. Propidium iodide staining revealed that TBT reduced the ratio of cells in the G1 phase and increased the ratio of cells in the G2/M phase. TBT also reduced cell division cycle 25C (cdc25C) and cyclin B1, which are key regulators of G2/M progression. Furthermore, apigenin, an inhibitor of NAD-IDH, mimicked the effects of TBT. The G2/M arrest induced by TBT was abolished by NAD-IDHα knockdown. Treatment with a cellpermeable α-ketoglutarate analogue recovered the effect of TBT, suggesting the involvement of NADIDH. Taken together, our data suggest that TBT at nanomolar levels induced G2/M cell cycle arrest via NAD-IDH in NT2/D1 cells. Thus, cell cycle analysis in embryonic cells could be used to assess cytotoxicity associated with nanomolar level exposure of EDCs.

Keywords: embryonic carcinoma cell, tributyltin, cell cycle

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Hirata N, Yamada S, Asanagi M[*], Sekino Y, Kanda

Y: Nicotine induces mitochondrial fission through mitofusin degradation in human multipotent embryonic carcinoma cells.

*Biochem Biophys Res Commun*. 2016;470:300-5

Nicotine is considered to contribute to the health risks associated with cigarette smoking. Nicotine exerts its cellular functions by acting on nicotinic acetylcholine receptors (nAChRs), and adversely affects normal embryonic development. However, nicotine toxicity has not been elucidated in human embryonic stage. In the present study, we examined the cytotoxic effects of nicotine in human multipotent embryonal carcinoma cell line NT2/D1. We found that exposure to 10 μM nicotine decreased intracellular ATP levels and inhibited proliferation of NT2/D1 cells. Because nicotine suppressed energy production, which is a critical mitochondrial function, we further assessed the effects of nicotine on mitochondrial dynamics. Staining with MitoTracker revealed that 10 μM nicotine induced mitochondrial fragmentation. The levels of the mitochondrial fusion proteins, mitofusins 1 and 2, were also reduced in cells exposed to nicotine. These nicotine effects were blocked by treatment with mecamylamine, a nonselective nAChR antagonist. These data suggest that nicotine degrades mitofusin in NT2/D1 cells and thus induces mitochondrial dysfunction and cell growth inhibition in a nAChR-dependent manner. Thus, mitochondrial function in embryonic cells could be used to assess the developmental toxicity of chemicals.

Keywords: embryonic cell, cigarette smoking, nicotine

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Yamada S, Asanagi M*, Hirata N, Itagaki H*, Sekino Y, Kanda Y: Tributyltin induces mitochondrial fission through Mfn1 degradation in human induced pluripotent stem cells.

*Toxicol In Vitro*. 2016;34:257-63

Organotin compounds, such as tributyltin (TBT), are well-known endocrine disruptors. TBT is also known to cause various forms of cytotoxicity, including neurotoxicity and immunotoxicity. However, TBT toxicity has not been identified in normal stem cells. In the present study, we examined the effects of TBT on cell growth in human induced pluripotent stem cells (iPSCs). We found that exposure to nanomolar concentrations of TBT decreased intracellular ATP levels and inhibited cell viability in iPSCs. Because TBT suppressed energy production, which is a critical function of the mitochondria, we further assessed the effects of TBT on mitochondrial dynamics. Staining with MitoTracker revealed that nanomolar concentrations of TBT induced mitochondrial fragmentation. TBT also reduced the expression of mitochondrial fusion protein mitofusin 1 (Mfn1), and this effect was abolished by knockdown of the E3 ubiquitin ligase membrane-associated RING-CH 5 (MARCH5), suggesting that nanomolar concentrations of TBT could induce mitochondrial dysfunction via MARCH5-mediated Mfn1 degradation in iPSCs. Thus, mitochondrial function in normal stem cells could be used to assess cytotoxicity associated with metal exposure.

Keywords: induced pluripotent stem cells, mitochondrial fission, mitofusin

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Yalikun Y*[1], Kanda Y, Morishima K*[1,2]: Hydrodynamic vertical rotation method for a single cell in an open space.

*Microfluidic and Nanofluidic*. 2016;20:74

Rotation of a single cell is an indispensable cell manipulation technique for genetic studies and clinical applications. Conventional contact manipulation methods for rotation of a cell use complex control systems and tools, while conventional non-contact manipulation methods have limitations regarding the operating space or range of the rotated cell size. Here, we report on a convenient, non-contact, and open space method for a wide range of single cell sizes (micrometer scale to millimeter scale) rotating in a vertical plane (out-of-plane) of an open space. This method uses a vertical microscale recirculation zone for capturing and rotating the cell. We fabricated a micro-orifice on the surface of a microfluidic chip to generate the micro-recirculation zone and then carried out experiments on vertical rotations of *Xenopus* oocyte, embryoid body, brine shrimp oocyte, and zebrafish oocyte using this chip. We demonstrated the rotation of four types of cells

in the vertical plane between the air–liquid interface and the top surface of the microfluidic chip; then, we conducted a simulation to analyze the dynamics of the vertical rotation of the *Xenopus* oocyte qualitatively. Our results indicated rotation speed of the four types of cells was controllable by the micro-recirculation zone. The size and density of oocytes also affected the process of capturing and rotation. We expect this method opens new research opportunities in three-dimensional cell manipulation, imaging, and analysis.

Keywords: hydrodynamic, micro-recirculation zone, vertical rotation

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Yalikun Y*1,2, Kanda Y, Morishima K*1,3: A method of three-dimensional micro-rotational flow generation for biological applications.
*Micromachines*. 2016;7:140

We report a convenient method to create a three-dimensional micro-rotational fluidic platform for biological applications in the direction of a vertical plane (out-of-plane) without contact in an open space. Unlike our previous complex fluidic manipulation system, this method uses a micro-rotational flow generated near a single orifice when the solution is pushed from the orifice by using a single pump. The three-dimensional fluidic platform shows good potential for fluidic biological applications such as culturing, stimulating, sorting, and manipulating cells. The pattern and velocity of the micro-rotational flow can be controlled by tuning the parameters such as the flow rate and the liquid-air interface height. We found that bio-objects captured by the micro-rotational flow showed self-rotational motion and orbital motion. Furthermore, the path length and position, velocity, and pattern of the orbital motion of the bio-object could be controlled. To demonstrate our method, we used embryoid body cells. As a result, the orbital motion had a maximum length of 2.4 mm, a maximum acceleration of 0.63 $m/s^2$, a frequency of approximately 0.45 Hz, a maximum velocity of 15.4 mm/s, and a maximum rotation speed of 600 rpm. The capability to have bio-objects rotate or move orbitally in three dimensions without contact opens up new research opportunities in three-dimensional microfluidic technology.

Keywords: three-dimensional microfluidic platform, micro-rotational flow, non-contact

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Yamamoto W*1,2, Asakura K*1,3,4, Ando H*1,3,5, Taniguchi T*1,6, Ojima A*1,6, Uda T*1,5, Osada T*1,3,7, Hayashi S*1,3,4, Kasai C*1,3,8, Miyamoto N*1,6, Tashibu H*1,3,9, Yoshinaga T*1,3,6, Yamazaki D, Sugiyama A*1,3,10, Kanda Y, Sawada K*1,3,6, Sekino Y: Electrophysiological Characteristics of Human iPSC-Derived Cardiomyocytes for the Assessment of Drug-Induced Proarrhythmic Potential.
*PLOS ONE*. 2016;11:e0167348

The aims of this study were to (1) characterize basic electrophysiological elements of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) that correspond to clinical properties such as QT-RR relationship, (2) determine the applicability of QT correction and analysis methods, and (3) determine if and how these in-vitro parameters could be used in risk assessment for adverse drug-induced effects such as Torsades de pointes (TdP). Field potential recordings were obtained from commercially available hiPSC-CMs using multi-electrode array (MEA) platform with and without ion channel antagonists in the recording solution. Under control conditions, MEA-measured interspike interval and field potential duration (FPD) ranged widely from 1049 to 1635 ms and from 334 to 527 ms, respectively and provided positive linear regression coefficients similar to native QT-RR plots obtained from human electrocardiogram (ECG) analyses in the ongoing cardiovascular-based Framingham Heart Study. Similar to minimizing the effect of heart rate on the QT interval, Fridericia's and Bazett's corrections reduced the influence of beat rate on hiPSC-CM FPD. In the presence of E-4031 and cisapride, inhibitors of the rapid delayed rectifier potassium current, hiPSC-CMs showed reverse use-dependent FPD prolongation.

Categorical analysis, which is usually applied to clinical QT studies, was applicable to hiPSC-CMs for evaluating torsadogenic risks with FPD and/or corrected FPD. Together, this results of this study links hiPSC-CM electrophysiological endpoints to native ECG endpoints, demonstrates the appropriateness of clinical analytical practices as applied to hiPSC-CMs, and suggests that hiPSC-CMs are a reliable models for assessing the arrhythmogenic potential of drug candidates in human.
Keywords: hiPSC-CM, MEA, TdP

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Yamada S, Kubo Y, Yamazaki D, Sekino Y, Kanda Y: Chlorpyrifos inhibits neural induction via Mfn1-mediated mitochondrial dysfunction in human induced pluripotent stem cells.
*Sci Rep*. 2016;7:40925

Organophosphates, such as chlorpyrifos (CPF), are widely used as insecticides in agriculture. CPF is known to induce cytotoxicity, including neurodevelopmental toxicity. However, the molecular mechanisms of CPF toxicity at early fetal stage have not been fully elucidated. In this study, we examined the mechanisms of CPF-induced cytotoxicity using human induced pluripotent stem cells (iPSCs). We found that exposure to CPF at micromolar levels decreased intracellular ATP levels. As CPF suppressed energy production that is a critical function of the mitochondria, we focused on the effects of CPF on mitochondrial dynamics. CPF induced mitochondrial fragmentation via reduction of mitochondrial fusion protein mitofusin 1 (Mfn1) in iPSCs. In addition, CPF reduced the expression of several neural differentiation marker genes in iPSCs. Moreover, knockdown of Mfn1 gene in iPSCs downregulated the expression of PAX6, a key transcription factor that regulates neurogenesis, suggesting that Mfn1 mediates neural induction in iPSCs. Taken together, these results suggest that CPF induces neurotoxicity via Mfn1-mediated mitochondrial fragmentation in iPSCs. Thus, mitochondrial dysfunction in iPSCs could be used as a possible marker for cytotoxic effects by chemicals.
Keywords: induced pluripotent stem cells, chlorpyrifos, neural induction

Ando H*1,2,3, Yoshinaga T*1,2,4, Yamamoto W*1,5, Asakura K*1,2,6, Uda T*1,3, Taniguchi T*1,4, Ojima A*1,4, Shinkyo R*4, Kikuchi K*4, Osada T*1,2,7, Hayashi S*1,2,6, Kasai C*1,2,8, Miyamoto N*1,4, Tashibu H*1,2,9, Yamazaki D, Sugiyama A*1,2,10, Kanda Y, Sawada K*1,2,4, Sekino Y*1,11: A new paradigm for drug-induced torsadogenic risk assessment using human iPS cell-derived cardiomyocytes.
*J Pharmacol Toxicol Methods*. 2017;84:111-27

INTRODUCTION: Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are anticipated to be a useful tool for conducting proarrhythmia risk assessments of drug candidates. However, a torsadogenic risk prediction paradigm using hiPSC-CMs has not yet been fully established.
METHODS: Extracellular field potentials (FPs) were recorded from hiPSC-CMs using the multi-electrode array (MEA) system. The effects on FPs were evaluated with 60 drugs, including 57 with various clinical torsadogenic risks. Actual drug concentrations in medium were measured using the equilibrium dialysis method with a Rapid Equilibrium Dialysis device. Relative torsade de pointes (TdP) scores were determined for each drug according to the degree of FP duration prolongation and early afterdepolarization occurrence. The margins were calculated from the free concentration in medium and free effective therapeutic plasma concentration. Each drug's results were plotted on a two-dimensional map of relative TdP risk scores versus margins.
RESULTS: Each drug was categorised as high, intermediate, or low risk based on its location within predefined areas of the two-dimensional map. We categorised 19 drugs as high risk; 18 as intermediate risk; and 17 as low risk. We examined the concordance between our categorisation of high and low risk drugs against the torsadogenic risk categorisation in CredibleMeds®. Our system demonstrated high

concordance, as reflected in a sensitivity of 81%, specificity of 87%, and accuracy of 83%.
DISCUSSION: These results indicate that our torsadogenic risk assessment is reliable and has a potential to replace the hERG assay for torsadogenic risk prediction, however, this system needs to be improved for the accurate of prediction of clinical TdP risk. Here, we propose a novel drug induced torsadogenic risk categorising system using hiPSC-CMs and the MEA system.
Keywords: concordance, early afterdepolarization, field potential duration

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Zhao C*1, Ichimura A*1, Qian N*1, Iida T*1, Yamazaki D, Noma N*2, Asagiri M*2, Yamamoto K*2, Komazaki S*3, Sato C*4, Aoyama F*5, Sawaguchi A*5, Kakizawa S*1, Nishi M*1, Takeshima H*1: Mice lacking the intracellular cation channel TRIC-B have compromised collagen production and impaired bone mineralization.
*Sci Signal. 2016*;9:ra49

The trimeric intracellular cation（TRIC）channels TRIC-A and TRIC-B localize predominantly to the endoplasmic reticulum（ER）and likely support Ca(2+) release from intracellular stores by mediating cationic flux to maintain electrical neutrality. Deletion and point mutations in TRIC-B occur in families with autosomal recessive osteogenesis imperfecta. Tric-b knockout mice develop neonatal respiratory failure and exhibit poor bone ossification. We investigated the cellular defect causing the bone phenotype. Bone histology indicated collagen matrix deposition was reduced in Tric-b knockout mice. Osteoblasts, the bone-depositing cells, from Tric-b knockout mice exhibited reduced Ca（2+）release from ER and increased ER Ca（2+）content, which was associated with ER swelling. These cells also had impaired collagen release without a decrease in collagen-encoding transcripts, consistent with a defect in trafficking of collagen through ER. In contrast, osteoclasts, the bone-degrading cells, from Tric-b knockout mice were similar to those from wild-type mice. Thus, TRIC-B function is essential to support the production and release of large amounts of collagen by osteoblasts, which is necessary for bone mineralization.
Keywords: TRIC-B, osteogenesis imperfecta, osteoblast

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Ogaki S*1, Omori H*2, Morooka M*2, Shiraki N*1, Ishida S, Kume S*1: Late stage definitive endodermal differentiation can be defined by Daf1 expression.
*BMC Dev Biol*. 2016;16:19-27

Definitive endoderm（DE）gives rise to the respiratory apparatus and digestive tract. Sox17 and Cxcr4 are useful markers of the DE. Previously, we identified a novel DE marker, Decay accelerating factor 1（Daf1/CD55）, by identifying DE specific genes. Daf1 is expressed in a subpopulation of E-cadherin$^+$Cxcr4$^+$ DE cells. In this report, we utilized the ESC differentiation system to examine the characteristics of Daf1-expressing DE cells. We found that Daf1 expression could discriminate late DE from early DE. We also found that Daf1$^+$ late DE cells show low proliferative and low cell matrix adhesive characteristics. Furthermore, the purified SOX17（low）early DE cells gave rise to Daf1$^+$ Sox17（high）late DE cells. Daf1-expressing late definitive endoderm proliferates slowly and show low adhesive capacity.
Keywords: definitive endoderm marker, Daf1, differentiation

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Kuroda Y, Kim SR, Kanaki T*, Horikawa M*, Sekino Y, Ishida S: Suspension Culture Improves cryopreservation-induced damage of liver-derived cells.

*AATEX*. 2016;21:63-70

The biotransformation activity of the liver is a major concern of medical and chemical production processes. Human cryopreserved hepatocytes are widely used for in vitro analysis of metabolites identification and toxicity evaluation of drug candidates or chemical compounds. However, cryopreservation causes cell damage and leads to decreased cell functionality and viability after thawing. To overcome such disadvantages, a variety of cryopreservation and culture methods have been developed. In this study, cryopreserved human hepatocytes and HepG2 cells were cultured under suspension conditions with FP001, a newly developed gellan gum-based cell culture material, after thawing. Under suspension conditions, cell damage was decreased compared with that under monolayer conditions. Combined with gene expression analysis of cell adhesion-related genes, our results suggested that FP001 contributed to decreased damage and increased viability of cryopreserved cells by altering the expression of genes involved in cell-cell adhesion.

Keywords: FP001, suspension culture, cryopreservation-induced damage suppression.

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Kubo T, Kuroda Y, Hojyo M, Kim SR, Horiuchi S, Sekino Y, Morel F*, Corlu A*, Ishida S: Maintenance of hepatic progenitor-like characteristics of HepaRG cells by cultivation on VECELL Inserts.

*AATEX*. 2016;21:71-9

Cell shape influences cell functionality. We investigated the possibility that the differentiation state of HepaRG, a bipotential hepatic progenitor cell line, was changed by cultivation on VECELL Inserts, which consist of type I collagen-coated expanded polytetrafluoroethylene (ePTFE) mesh. HepaRG cells plated on VECELL Inserts possessed a round shape. Gene expression patterns obtained from HepaRG cells cultured on VECELL Inserts suggested that the cells maintained their progenitor cell-like characteristics, while not losing the capacity to differentiate into hepatocytes. Retaining cell stemness is important for the expansion of progenitor cells to maintain their differentiation potency. VECELL Inserts are a novel culture apparatus that easily retains the characteristics of hepatic progenitor cells. It may be useful for the culture and expansion of hepatic progenitor cells, and thus, provide fundamental cell source for in vitro toxicity and biotransformation assays.

Keywords: VECELL Insert, 3D-culture, progenitor cell maintenance, HepaRG cells

* INSERM

Toyoda T, Shi L*1, Takasu S, Cho YM, Kiriyama Y*2, Nishikawa A, Ogawa K, Tatematsu M*3, Tsukamoto T*2: Anti-inflammatory effects of capsaicin and piperine on *Helicobacter pylori*-induced chronic gastritis in Mongolian gerbils.

*Helicobacter*. 2016;21:131-42

Spices have been used for thousands of years, and recent studies suggest that certain spices confer beneficial effects on gastric disorders. The purpose of this study was to evaluate possible chemopreventive effects of spice-derived compounds on *Helicobacter pylori*- (*H. pylori*)-induced gastritis. We examined the inhibitory effects of curcumin, capsaicin and piperine on *H. pylori in vitro* by determining the colony forming units and real-time RT-PCR in *H. pylori*-stimulated AGS gastric cancer cells. For *in vivo* analysis, 6-week-old SPF male Mongolian gerbils were infected with *H. pylori*, fed diets containing 5,000 ppm curcumin, 100 ppm capsaicin or 100 ppm piperine and sacrificed after 13 weeks. All three compounds inhibited *in vitro* proliferation of *H. pylori*, with curcumin being the most effective. Infiltration of neutrophils and mononuclear cells was suppressed by piperine both in the antrum and corpus of *H. pylori*-infected gerbils. Capsaicin also decreased neutrophils in the antrum and corpus and mononuclear cell infiltration and heterotopic proliferative glands in the corpus. mRNA expression of *Tnf-α* and formation of phospho-I$\kappa$B-*α* in the antrum were reduced by both capsaicin and piperine. In addition, piperine suppressed expression of *Il-1β*, *Ifn-γ*, *Il-6* and *iNos*, while *H. pylori UreA* and other virulence factors were not significantly attenuated by any compounds. These results suggest that capsaicin

and piperine have anti-inflammatory effects on *H. pylori*-induced gastritis in gerbils independent of direct antibacterial effects, and may thus have potential for use in the chemoprevention of *H. pylori*-associated gastric carcinogenesis.

Keywords: *Helicobacter pylori*, capsaicin, piperine

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Tamura K, Inoue K, Takahashi M, Matsuo S, Kodama Y, Yoshida M*: A crucial role of constitutive androstane receptor (CAR) in liver tumor development by imazalil in mice.

*J Toxicol Sci*. 2016;41:801-11

To clarify the major pathway of liver tumor development induced by imazalil (IMA), an imidazole fungicide, male constitutive androstane receptor (CAR)-knockout (CARKO) and wild-type (WT) mice were treated with IMA at 500 ppm in the diet up to 27 weeks after initiation by diethylnitrosamine. After 27 weeks of treatment, neither altered foci nor adenomas were significantly increased in CARKO mice, whereas both eosinophilic altered foci and adenomas were increased in WT mice. After 4 or 13 weeks of IMA treatment, liver hypertrophy was observed at the tumor-inducible dose without differences among genotypes or durations. Analysis of hepatic drug metabolite enzymes, performed after administration of multiple doses during a 1-week period, indicated that pregnane X receptor might be involved in liver hypertrophy because IMA markedly elevated Cyp3a11 and Cyp2b10 expression levels in a dose-dependent manner in both genotypes. Our results demonstrated that the CAR pathway was the main mechanism of liver tumor development induced by IMA. The carcinogenic pathway was different from that of liver hypertrophy.

Keywords: imazalil, constitutive androstane receptor, liver hypertrophy

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* Food Safety Commission

Toyoda T, Cho YM, Akagi J, Mizuta Y, Matsushita K, Nishikawa A, Imaida K*, Ogawa K: Altered susceptibility of an obese rat model to 13-week subchronic toxicity induced by 3-monochloropropane-1,2-diol.

*J Toxicol Sci*. 2017;42:1-11

3-Monochloropropane-1,2-diol (3-MCPD) is a heat-induced food contaminant that has been shown to be a nongenotoxic renal carcinogen. Although the toxicity of 3-MCPD has been widely investigated for decades, there is a further concern that 3-MCPD might exert more potent toxicity in high-risk population with underlying diseases such as hyperlipidemia associated with obesity. In the present study, we performed a 13-week subchronic toxicity study for 3-MCPD using an obesity rat model to investigate the differences in susceptibility between obese and normal individuals. Male F344 and obese Zucker (lean and fatty) rats were administered 0, 9, 28.5, 90, 285, or 900 ppm 3-MCPD in drinking water for 13 weeks. 3-MCPD treatment decreased body weight gain, increased relative kidney weights, induced anemia, and induced epithelial cell necrosis in epididymal ducts in all 3 strains. The degrees of epididymal damage were higher in F344 and lean rats than in fatty rats, while renal toxicity was most potent in F344 rats and comparable in lean and fatty rats. In contrast, the hematology data indicated that anemia was worse in fatty rats than in F344 and lean rats, and a significant decrease in hematopoietic cells in the bone marrow was observed only in fatty rats. The no-observed-adverse-effect level was estimated to be 28.5 ppm in all 3 strains for 3-MCPD. These results suggested that obese Zucker rats may be more susceptible to 3-MCPD-dependent toxicity in the hematopoietic tissues than their lean counterparts.

Keywords: obese Zucker rat, obesity, 3-monochloropropane-1,2-diol

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Suzuki I, Cho YM, Hirata T, Toyoda T, Akagi J, Nakamura Y*1, Park EY*1, Sasaki A*1, Nakamura T*1, Okamoto S*2, Shirota K*3, Suetome N*3, Nishikawa A, Ogawa K: 4-Methylthio-3-butenyl isothiocyanate (Raphasatin) exerts chemopreventive effects against esophageal carcinogenesis in rats.

*J Toxicol Pathol*. 2016;29:237-46

To examine the effects of 4-methylthio-3-butenyl isothiocyanate on esophageal carcinogenesis,

male 6-week-old F344 rats were subcutaneously injected with 0.5 mg/kg body weight *N*-nitrosomethylbenzylamine three times per week for 5 weeks and fed a diet supplemented with 80 ppm 4-methylthio-3-butenyl isothiocyanate, equivalent to 6.05 mg/kg body weight/day for the initiation stage, 4.03 mg/kg body weight/day for the promotion stage, or 4.79 mg/kg body weight/day for all stages. Although the incidence of lesions was not affected by 4-methylthio-3-butenyl isothiocyanate treatment, the multiplicity of squamous cell papilloma in the esophagus was significantly decreased in rats in the 4-methylthio-3-butenyl isothiocyanate initiation stage group (1.13 ± 0.74), 4-methylthio-3-butenyl isothiocyanate promotion stage group (1.47 ± 0.99), and 4-methylthio-3-butenyl isothiocyanate all stage group (1.47 ± 1.13) as compared with rats treated with *N*-nitrosomethylbenzylamine alone (3.00 ± 1.46). Immunohistochemical analysis revealed that 4-methylthio-3-butenyl isothiocyanate induced apoptosis, suppressed cell proliferation, and increased p21 expression when administered in the promotion phase. These modifying effects were not observed in the rats treated with 4-methylthio-3-butenyl isothiocyanate alone. Our results indicated that 4-methylthio-3-butenyl isothiocyanate may exert chemopreventive effects against *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats.

Keywords: 4-methylthio-3-butenyl isothiocyanate, esophageal cancer, chemoprevention

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Matsushita K, Toyoda T, Inoue K, Morikawa T, Sone M, Ogawa K: Spontaneous infarcted adenoma of the mammary gland in a Wistar Hannover GALAS rat.
*J Toxicol Pathol.* 2017;30:57-62

Spontaneous massive infarction of mammary gland tumors has been reported to occur infrequently in humans. A subcutaneous mass (18 x 17 x 10 mm) was observed in the right axilla extending to the chest region of a 110-week-old female Wistar Hannover GALAS rat. Histopathologically, a well-circumscribed mass with lobular structures was present in the subcutis. Most of the mass was occupied by extensive coagulative necrosis of neoplastic cells with relatively uniform acinar and ductal structures. Although each necrotic acinar structure was separated by reticular fibers, periacinar stromal collagen fibers were not abundant. Considering the site of occurrence and histological features, the necrotic tissue was diagnosed as adenoma of the mammary gland. The necrotic region lacked hemorrhage and obvious inflammatory cell infiltration, indicating the necrosis was caused by infarction. Although multiple necrosis and focal infarction are occasionally observed in large-sized tumors in rodents, especially in adenocarcinomas, the present case was characteristic, with the massive infarction involving most parts of the tumor despite the relatively small size and low atypia of neoplastic cells. This is a rare case of spontaneous infarcted adenoma of the mammary gland in rats histologically resembling human cases.

Keywords: infarcted adenoma, mammary gland, Wistar Hannover GALAS rat

Taketa Y, Inoue K, Takahashi M, Sakamoto Y, Watanabe G*, Taya K*, Yoshida M: Effects of sulpiride and ethylene glycol monomethyl ether on endometrial carcinogenicity in Donryu rats.
*J Appl Toxicol.* 2016;36:769-76

Sulpiride and ethylene glycol monomethyl ether (EGME) are known ovarian toxicants that stimulate prolactin (PRL) secretion, resulting in hypertrophy of the corpora lutea and increased progesterone (P4) production. The purpose of the present study was to investigate how the PRL stimulatory agents affected uterine carcinogenesis and to clarify the effects of PRL on endometrial adenocarcinoma progression in rats. Ten-week-old female Donryu rats were treated once with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (20 mg/kg), followed by treatment with sulpiride (200 ppm) or EGME (1250 ppm) from 11 weeks of age to 12 months of age. Sulpiride treatment inhibited the incidence of uterine adenocarcinoma and precancerous lesions of atypical endometrial hyperplasia, whereas EGME had no effect on uterine carcinogenesis. Sulpiride markedly prevented the onset of persistent estrus throughout the study period, and EGME delayed and inhibited the onset of persistent estrus. Moreover, sulpiride-treated

animals showed high PRL and P4 serum levels without changes in the levels of estradiol-17$\beta$, low uterine weights and histological luteal cell hypertrophy. EGME did not affect serum PRL and P4 levels. These results suggest that the prolonged low estradiol-17$\beta$ to P4 ratio accompanied by persistent estrous cycle abnormalities secondary to the luteal stimulatory effects of PRL may explain the inhibitory effects of sulpiride on uterine carcinogenesis in rats.

Keywords: endometrial adenocarcinoma, ethylene glycol monomethyl ether, sulpiride

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Hibi D, Yokoo Y, Suzuki Y, Ishii Y, Jin M, Kijima A, Nohmi T, Nishikawa A, Umemura T: Lack of genotoxic mechanisms in early-stage furan-induced hepatocellular tumorigenesis in *gpt* delta rats.
*J Appl Toxicol*. 2017;37:142-9

Furan has been used as an intermediate in the chemical-manufacturing industry and has been shown to contaminate various foods. Although furan induces hepatocellular tumors in rodents, equivocal results from *in vitro* and *in vivo* mutagenicity tests have caused controversy regarding the involvement of genotoxic mechanisms in furan-induced carcinogenesis. In the present study, to elucidate the possible mechanisms underlying furan-induced hepatocarcinogenesis, a comprehensive medium-term analysis was conducted using *gpt* delta rats treated with furan at carcinogenic doses for 13 weeks. In the liver, the frequencies of *gpt* and Spi$^-$ mutants derived mainly from point and deletion mutations, respectively, were not changed, and there were no furan-specific gpt mutations in furan-treated rats. In contrast, the number and area of glutathione S-transferase placental form (GST-P)-positive foci were significantly increased in the high-dose group. Also, the ratio of PCNA-positive hepatocytes was significantly elevated in the same group, as supported by significant increases in cyclin d1 and cyclin e1 mRNA levels. Thus, it is highly probable that cell proliferation, but not genotoxic mechanisms, contribute to the development of GST-P foci in furan-treated rats. Based on the close relationship between GST-P and neoplastic hepatocytes, these data allowed us to hypothesize that cell proliferation following signal transduction other than the mitogen-activated protein kinase (MAPK)/ERK pathway may play a crucial role in early-stage furan-induced hepatocarcinogenesis.

Keywords: *gpt* delta rat, furan, hepatocarcinogenesis

Takeshima H*, Niwa T*, Toyoda T, Wakabayashi M*, Yamashita S*, Ushijima T*: The degree of methylation burden is determined by the exposure period to carcinogenic factors.
*Cancer Sci*. 2017;108:316-21

Aberrant DNA methylation accumulated in normal tissues, namely methylation burden, is associated with risk of carcinogenesis. The levels of methylation burden are known to be influenced by multiple factors, such as genetic factors and strengths of carcinogenic factors. However, the impact of the degree of exposure to a carcinogenic factor is still unclear. Here, using a Mongolian gerbil model of *Helicobacter pylori* (*H. pylori*)-induced gastritis, we aimed to clarify the impact of the degree of exposure on methylation burden in normal gastric tissues. DNA methylation levels of four CpG islands, HE6, SA9, SB5, and SD2, increased by *H. pylori* infection, depending upon the infection period. After eradication of *H. pylori*, DNA methylation levels decreased, but tended to be higher in gastric mucosae with a longer infection period. DNA molecules with dense methylation, but not those with sparse methylation, increased depending upon the infection period. DNA methylation levels of one of the four CpG islands, SA9, tended to be higher in gastric mucosae of gerbils infected with *H. pylori*, even 50 weeks after eradication than in those of non-infected gerbils. These results showed for the first time that the levels of methylation burden in normal tissues are influenced by the degree of exposure to a carcinogenic factor.

Keywords: *Helicobacter pylori*, methylation, gastric cancer

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Takahashi M, Ichimura R, Inoue K, Morikawa T, Watanabe G*, Yoshida M: The impact of neonatal exposure to 17alpha-ethynylestradiol on the development of kisspeptin neurons in female rats.
*Reprod Toxicol*. 2016;60:33-8

Neonatal exposure to 17alpha-ethynylestradiol

（EE）at relatively low doses leads to delayed effects characterized by the early onset of age-related anovulation. Kisspeptin neurons in the anteroventral periventricular nucleus（AVPV), located at the anterior hypothalamus, are proposed to play key roles in appearance of these delayed effects after maturation. To understand the initial changes, we investigated Kiss1 mRNA expression in the anterior and posterior hypothalamus before weaning in female rats that received neonatal exposure to EE at various doses（0.002-2,000 μg/kg). The level of Kiss1 mRNA in the anterior hypothalamus was decreased from 0.002 μg/kg which did not induce delayed effects. In the posterior hypothalamus, Kiss1 mRNA expression did not differ among the groups except 2,000 μg/kg group. These results suggest that neonatal exposure to EE affects the development of kisspeptin neurons and kisspeptin neurons in the AVPV are highly susceptible to neonatal EE treatment.

Keywords: delayed effect, 17alpha-ethynylestradiol, kiss1

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Kuwata K, Inoue K, Ichimura R, Takahashi M, Kodama Y, Shibutani M*, Yoshida M: Involvement of mouse constitutive androstane receptor in acifluorfen-induced liver injury and subsequent tumor development.

*Toxicol Sci*. 2016;151:271-85

Acifluorfen（ACI), a protoporphyrinogen oxidase（PROTOX）inhibitor herbicide, promotes the accumulation of protoporphyrin IX（PPIX）and induces tumors in the rodent liver. Porphyria is a risk factor for liver tumors in humans; however, the specific mechanisms through which ACI induces hepatocarcinogenesis in rodents are unclear. Here, we investigated the mode of action of ACI-induced hepatocarcinogenesis, focusing on constitutive androstane receptor（CAR, NR1I3), which is essential for the development of rodent liver tumors in response to certain cytochrome P450（CYP）2B inducers. Dietary treatment with 2500 ppm ACI for up to 13 weeks increased Cyp2b10 expression in the livers of wild-type（WT）mice, but not in CAR-knockout（CARKO）mice. Microscopically, ACI treatment induced cytotoxic changes, including hepatocellular necrosis and inflammation, and caused regenerative changes accompanied by prolonged increases in the numbers of proliferating cell nuclear antigen（PCNA）-positive hepatocytes in WT mice. In contrast, these cytotoxic and regenerative changes in hepatocytes were significantly attenuated, but still observed, in CARKO mice. ACI treatment also increased liver PPIX levels similarly in both genotypes; however, no morphological evidence of porphyrin deposition was found in hepatocytes from either genotype. Treatment with 2500 ppm ACI for 26 weeks after initiation with diethylnitrosamine increased the incidence and multiplicities of altered foci and adenomas in hepatocytes from WT mice; these effects were significantly reduced in CARKO mice. These results indicated that prolonged cytotoxicity in the liver was a key factor for ACI-induced hepatocarcinogenesis, and that CAR played an important role in ACI-induced liver injury and tumor development in mice

Keywords: acifluorfen, constitutive androstane receptor, hepatocarcinogenesis

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Yokoo Y, Kijima A, Ishii Y, Takasu S, Tsuchiya T, Umemura T: Effects of *Nrf2* silencing on oxidative stress-associated intestinal carcinogenesis in mice.

*Cancer Med*. 2016;5:1228-38

To assess the risk of colorectal cancer in humans with inactivation of NRF2, *Nrf2*-proficient（$Nrf2^{+/+}$）and -deficient（$Nrf2^{-/-}$）mice were exposed to potassium bromate（$KBrO_3$）at concentrations of 750 or 1500 ppm for 52 weeks. Neoplastic proliferative lesions were observed in the small intestine and exhibited accumulations of β-catenin and cyclin D1. The lesions had characteristics similar to those in experimental models of human hereditary colorectal cancer. An additional 13-week study was performed to examine the role of *Nrf2* in the effects of oxidative stress. Significant increase in combined incidences of preneoplastic and neoplastic lesions in $Nrf2^{-/-}$ mice administered high-dose $KBrO_3$. In the short-term study, although 8-hydroxydeoxyguanosine（8-OHdG）levels in the epithelial DNA of $Nrf2^{-/-}$ mice at the high dose were significantly lower than those of the corresponding $Nrf2^{+/+}$ mice, the difference was very small. mRNA levels of *Nrf2*-regulated genes

were increased in *Nrf2*$^{+/+}$ mice. Overexpression of cyclooxygenase 2 (COX2) and increased numbers of proliferating cell nuclear antigen (PCNA)-positive cells in the jejunal crypts were observed in *Nrf2*$^{-/-}$ mice administered high-dose $KBrO_3$. Overall, these data suggested that individuals having single-nucleotide polymorphisms in *NRF2* may have a risk of colorectal cancer to some extent.

Keywords: cyclooxygenase-2, potassium bromate, NRF2

Ichimura R, Takahashi M, Morikawa T, Inoue K, Kuwata K, Usuda K[*1], Yokosuka M[*2], Watanabe G[*1], Yoshida M: Neonatal exposure to SERMs disrupts neuroendocrine development and postnatal reproductive function through alteration of hypothalamic kisspeptin neurons in female rats.

*Neurotoxicology*. 2016;56:64-75

Selective estrogen receptor modulators (SERMs) are a class of therapeutic chemicals which present tissue-specific estrogen receptor modulating activity. Neonatal exposure to SERMs has been reported to adversely affect central nervous system development, however, mechanism and involvement of hypothalamic kisspeptin neuron in this impairment remains undetermined. To clarify this uncertainty, neonates from female Donryu rats were subcutaneously injected with raloxifene (RLX) at 0.1, 1, and 10 mg/kg or tamoxifen (TMX) at 10 mg/kg on postnatal day 0, and then hypothalamic Kiss1 mRNA expression and gonadotropin levels were investigated during young adulthood and estrous cycling was monitored until middle age. Treatment with RLX or TMX at 10 mg/kg significantly depressed luteinizing hormone surge levels and Kiss1 mRNA expression in the anteroventral periventricular nucleus (AVPV), the control center of estrous cyclicity. The 10 mg/kg TMX group also showed decreased levels of follicle-stimulating hormone and Kiss1 mRNA expression in the arcuate nucleus (ARC). Early cessation of normal estrous cycling was observed in the 10 mg/kg RLX group, while the estrous cycle in the 10 mg/kg TMX group had ceased by the start of the analysis. The same dose of tamoxifen or raloxifene had either weak-estrogenic or anti-estrogenic activity on the uterus, respectively; however, treatment in adulthood with both SERMs did not affect Kiss1 mRNA expression in either the AVPV or ARC in the present study. These results indicate that neonatal exposure to SERMs could disrupt neuroendocrine development and postnatal reproductive function through the alteration of kisspeptin neurons.

Keywords: kisspeptin, selective estrogen receptor modulator, neonatal exposure

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Takahashi M, Ichimura R, Inoue K, Morikawa T, Kuwata K, Watanabe G[*1], Yoshida M[*2]: The role of estrogen receptor subtypes for induction of delayed effects on the estrous cycle and female reproductive organs in rats.

*Reprod Biol*. 2017;17:111-9

It has been reported that neonatal exposure to estrogens at relatively low doses can induce early onset anovulation as a delayed effect in female rats. Dysfunction of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) was proposed to be a trigger for this effect. To determine the roles of estrogen receptor (ER) subtypes in the induction of delayed effects, we conducted a series of experiments using Donryu rats to examine whether neonatal injection of an ER$\alpha$ agonist (PPT), an ER$\beta$ agonist (DPN) or an ER$\alpha$ antagonist (ICI) could induce delayed effects. Also, involvement of the kisspeptin neurons in the AVPV for induction of delayed effect by PPT and DPN was investigated. We observed that neonatal exposure to PPT, DPN and ICI induced the early onset of abnormal estrous cyclicity after sexual maturation, suggesting that the compounds capable of inducing delayed effects are not limited to ER$\alpha$ agonists. On the other hand, the data suggested the possibility that DPN and ICI functioned partially as ER$\alpha$ agonists in the neonatal brain. Regardless of the agents used, there is a possibility that dysfunction of kisspeptin neurons in the AVPV might contribute to induction of early onset anovulation.

Keywords: delayed effect, estrous cycle, kisspeptin

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Zhang H*, Nagaoka K*, Usuda K*, Nozawa K*,

Taya K*, Yoshida M, Watanabe G*: Estrogenic compounds impair primordial follicle formation by inhibiting the expression of proapoptotic Hrk in neonatal rat ovary.

*Biol Reprod*. 2016;95:78

Exposure to endocrine-disrupting chemicals (EDCs) during fetal and neonatal periods can have toxic effects that are irreversible and last a lifetime. However, the mechanism underlying this phenomenon is still unknown. Here, we show the effect of 17alpha-ethynyl estradiol (EE) on the development of the primordial follicle during early ovarian development in female mice. Microarray analysis revealed the downregulation of Hrk, an activator of apoptosis, in neonatal ovaries exposed to EE. Real-time PCR analysis also showed a decrease of Hrk mRNA expression in ovaries treated with EE both in vitro and in neonatal mice. An immunostaining assay showed that Hrk protein and cleaved caspase 3 colocalize in the oocytes at Postnatal Day 1 (PND1). The EE-exposed ovaries had a reduced number of oocytes positive for TUNEL staining compared to control ovaries at PND1. Abnormal follicle formation of EE-exposed ovaries was observed at PND7 and PND21. A TUNEL staining assay revealed that Hrk depletion reduced the number of apoptotic oocytes. In addition, downregulation of Hrk mRNA expression was observed in ovaries treated with other estrogenic chemicals. We propose a model in which EE inhibits oocyte apoptosis in the neonatal ovary by suppressing the expression of Hrk, thereby disrupted follicle formation and ovary function.

Keywords: 17alpha-ethynylestradiol, endocrine-disrupting chemicals, ovary

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Sekimoto M*, Sumi H*, Hosaka T*, Umemura T, Nishikawa A, Degawa M*: Aryl hydrocarbon receptor activation and CYP1A induction by cooked food-derived carcinogenic heterocyclic amines in human HepG2 cell lines.

*Food Chem Toxicol*. 2016;97:256-64

The ability of nine cooked food-derived heterocyclic aromatic amines (HCAs), such as 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methylpyrido[12-a:3',2'-*d*]imidazole (Glu-P-1), 2-amino-pyrido[12-a:3',2'-*d*]imidazole hydrochloride (Glu-P-2), 2-amino-9H-pyrido[2,3-*b*]indole (A$\alpha$C), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeA$\alpha$C), 2-amino-3-methylimidazo[4,5-*f*]quinolone (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenyl-1H-imidazo[4,5-*b*]pyridine (PhIP), to activate human aryl hydrocarbon receptor (hAhR) was examined using a HepG2-A10 cell line, which has previously established from human hepatocarcinoma-derived HepG2 cells for use in hAhR-based luciferase reporter gene assays. Trp-P-1, Trp-P-2, A$\alpha$C, MeA$\alpha$C, IQ and MeIQx showed a definite ability to induce not only luciferase (hAhR activation) in HepG2-A10 cells but also cytochrome P450 (CYP) 1A1/1A2 mRNAs in HepG2 cells, while such the ability of Glu-P-1, Glu-P-2, and PhIP was very low. In addition, all the HCAs examined, especially MeA$\alpha$C and MeIQx, had a definite capacity for inhibiting the activity of ethoxyresorfin O-deethylase (CYP1As, especially CYP1A1). The present findings demonstrate that all the HCAs examined have the ability to activate hAhR and its target genes, and further confirm that these HCAs become good substrates for human CYP1A subfamily enzyme (s).

Keywords: aryl hydrocarbon receptor, carcinogenic heterocyclic amine, CYP1A

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Kakehashi A*, Yoshida M, Tago Y*, Ishii N*, Okuno T*, Gi M*, Wanibuchi H*: *Pueraria mirifica* exerts estrogenic effects in the mammary gland and uterus and promotes mammary carcinogenesis in Donryu rats.

*Toxins*. 2016;8:E275

*Pueraria mirifica* (PM), a plant whose dried and powdered tuberous roots are now widely used in rejuvenating preparations to promote youthfulness in both men and women, may have major estrogenic influence. In this study, we investigated modifying effects of PM at various doses on mammary and endometrial carcinogenesis in female Donryu rats. Firstly, PM administered to ovariectomized animals at doses of 0.03%, 0.3%, and 3% in a phytoestrogen-low diet for 2 weeks caused significant increase in uterus weight. Secondly, a 4 week PM application to non-operated rats at a dose of 3% after 7,12-dimethylbenz[a]

anthracene（DMBA）initiation resulted in significant elevation of cell proliferation in the mammary glands. In a third experiment, postpubertal administration of 0.3%（200 mg/kg bw/day）PM to 5-week-old non-operated animals for 36 weeks following initiation of mammary and endometrial carcinogenesis with DMBA and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine（ENNG）, respectively, resulted in significant increase of mammary adenocarcinoma incidence. A significant increase of endometrial atypical hyperplasia multiplicity was also observed. Furthermore, PM at doses of 0.3%, and more pronouncedly, at 1% induced dilatation, hemorrhage and inflammation of the uterine wall. In conclusion, postpubertal long-term PM administration to Donryu rats exerts estrogenic effects in the mammary gland and uterus, and at a dose of 200 mg/kg bw/day was found to promote mammary carcinogenesis initiated by DMBA.

Keywords: *Pueraria mirifica*, estrogenic activity, mammary gland

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Kanemaru Y, Suzuki T, Sassa A, Matsumoto K*[1], Adachi N*[2], Honma M, Numazawa S*[3], Nohmi T: DNA polymerase kappa protects human cells against MMC-induced genotoxicity through error-free translesion DNA synthesis.

*Genes Environ.* 2017;39:6

Background: Interactions between genes and environment are critical factors for causing cancer in humans. The genotoxicity of environmental chemicals can be enhanced via the modulation of susceptible genes in host human cells. DNA polymerase kappa（Pol κ）is a specialized DNA polymerase that plays an important role in DNA damage tolerance through translesion DNA synthesis. To better understand the protective roles of Pol κ, we previously engineered two human cell lines either deficient in expression of Pol κ（KO）or expressing catalytically dead Pol κ（CD）in Nalm-6-MSH+ cells and examined cytotoxic sensitivity against various genotoxins. In this study, we set up several genotoxicity assays with cell lines possessing altered Pol κ activities and investigated the protective roles of Pol κ in terms of genotoxicity induced by mitomycin C（MMC）, a therapeutic agent that induces bulky DNA adducts and crosslinks in DNA.

Results: We introduced a frameshift mutation in one allele of the thymidine kinase（TK）gene of the KO, CD, and wild-type Pol κ cells（WT）, thereby establishing cell lines for the *TK* gene mutation assay, namely TK+/- cells. In addition, we formulated experimental conditions to conduct chromosome aberration（CA）and sister chromatid exchange（SCE）assays with cells. By using the WT TK+/- and KO TK+/- cells, we assayed genotoxicity of MMC. In the *TK* gene mutation assay, the cytotoxic and mutagenic sensitivities of KO TK+/- cells were higher than those of WT TK+/- cells. MMC induced loss of heterozygosity（LOH）, base pair substitutions at CpG sites and tandem mutations at GpG sites in both cell lines. However, the frequencies of LOH and base substitutions at CpG sites were significantly higher in KO TK+/- cells than in WT TK+/- cells. MMC also induced CA and SCE in both cell lines. The KO TK+/- cells displayed higher sensitivity than that displayed by WT TK+/- cells in the SCE assay.

Conclusions: These results suggest that Pol κ is a modulating factor for the genotoxicity of MMC and also that the established cell lines are useful for evaluating the genotoxicity of chemicals from multiple endpoints in different genetic backgrounds of Pol κ.

Keywords: DNA polymerase κ, genotoxicity assay, Mitomycin C

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Horibata K, Ukai A, Ogata A*, Nakae D*, Ando H*, Kubo Y*, Nagasawa A*, Yuzawa K*, Honma M: Absence of *in vivo* mutagenicity of multi-walled carbon nanotubes in single intratracheal instillation study using F344 *gpt* delta rats.

*Genes Environ.* 2017;39:4

It is known that fibrous particles of micrometer length, such as carbon nanotubes, which have same dimensions as asbestos, are carcinogenic. Carcinogenicity of nanomaterials is strongly related to inflammatory reactions; however, the genotoxicity mechanism（s）is unclear. Indeed, inconsistent results on genotoxicity of multi-walled carbon nanotubes（MWCNTs）have been shown in several reports.

Therefore, we analyzed the *in vivo* genotoxicity induced by an intratracheal instillation of straight MWCNTs in rats using a different test system-the *Pig-a* gene mutation assay-that can reflect the genotoxicity occurring in the bone marrow. Since lungs were directly exposed to MWCNTs upon intratracheal instillation, we also performed the *gpt* assay using the lungs. We detected no significant differences in *Pig-a* mutant frequencies (MFs) between the MWCNT-treated and control rats. Additionally, we detected no significant differences in *gpt* MFs in the lung between the MWCNT-treated and control rats. Our findings indicated that a single intratracheal instillation of MWCNTs was non-mutagenic to both the bone marrow and lung of rats.

Keywords: carbon nanotube, asbesto, *in vivo* genotoxicity

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Kimoto T*[1], Horibata K, Miura D*[1], Chikura S*[1], Okada Y*[1], Ukai A, Itoh S*[2], Nakayama S*[2], Sanada H*[3], Koyama N*[3], Muto S*[4], Uno Y*[4], Yamamoto M*[5], Suzuki Y*[6], Fukuda T*[6], Goto K*[6], Wada K*[7], Kyoya T*[8], Shigano M*[9], Takasawa H*[9], Hamada S*[9], Adachi H*[10], Uematsu Y*[10], Tsutsumi E*[11], Hori H*[11], Kikuzuki R*[12], Ogiwara Y*[12], Yoshida I*[13], Maeda A*[14], Narumi K*[15], Fujiishi Y*[15], Morita T, Yamada M, Honma M: The PIGRET assay, a method for measuring *Pig-a* gene mutation in reticulocytes, is reliable as a short-term *in vivo* genotoxicity test: Summary of the MMS/JEMS-collaborative study across 16 laboratories using 24 chemicals.
*Mutat Res*. 2016;811:3-15

The *in vivo* mutation assay using the X-linked phosphatidylinositol glycan class A gene (*Pig-a* in rodents, *PIG-A* in humans) is a promising tool for evaluating the mutagenicity of chemicals. Approaches for measuring *Pig-a* mutant cells have focused on peripheral red blood cells (RBCs) and reticulocytes (RETs) from rodents. The recently developed PIGRET assay is capable of screening $>1\times10^6$ RETs for *Pig-a* mutants by concentrating RETs in whole blood prior to flow cytometric analysis. Additionally, due to the characteristics of erythropoiesis, the PIGRET assay can potentially detect increases in *Pig-a* mutant frequency (MF) sooner after exposure compared with a *Pig-a* assay targeting total RBCs (RBC *Pig-a* assay). In order to test the merits and limitations of the PIGRET assay as a short-term genotoxicity test, an interlaboratory trial involving 16 laboratories was organized by the Mammalian Mutagenicity Study Group of the Japanese Environmental Mutagenicity Society (MMS/JEMS). First, the technical proficiency of the laboratories and transferability of the assay were confirmed by performing both the PIGRET and RBC *Pig-a* assays on rats treated with single doses of *N*-nitroso-*N*-ethylurea. Next, the collaborating laboratories used the PIGRET and RBC *Pig-a* assays to assess the mutagenicity of a total of 24 chemicals in rats, using a single treatment design and mutant analysis at 1, 2, and 4 weeks after the treatment. Thirteen chemicals produced positive responses in the PIGRET assay; three of these chemicals were not detected in the RBC *Pig-a* assay. Twelve chemicals induced an increase in RET *Pig-a* MF beginning 1 week after dosing, while only 3 chemicals positive for RBC *Pig-a* MF produced positive responses 1 week after dosing. Based on these results, we conclude that the PIGRET assay is useful as a short-term test for *in vivo* mutation using a single-dose protocol.

Keywords: *Pig-a* assay, reticulocyte, PIGRET assay

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Horibata K, Ukai A, Honma M: Evaluation of mutagenicity of acrylamide using RBC *Pig-a* and PIGRET assays by single peroral dose in rats.
*Mutat Re*s. 2016;811:54-9

The *Pig-a* gene mutation assay, a powerful tool for evaluating *in vivo* genotoxicity, is based on flow cytometric enumeration of red blood cells (RBCs), which are deficient in glycosylphosphatidylinositol anchored proteins caused by mutation (s) in the *Pig-a* gene. Various approaches for measuring cells with mutated *Pig-a* gene have been developed. The *Pig-a* assay targeting concentrated reticulocytes - the PIGRET assay - has the potential to detect genotoxicity in early stages of the study. To verify the potential and usefulness of the PIGRET assay for short-term testing, we conducted a joint research with the Mammalian Mutagenicity Study (MMS) Group of the Japanese Environmental Mutagen Society. As part of this study, we evaluated the genotoxicity of a single oral administration of acrylamide (AA) at 25, 50, 100, 137.5, and 175mg/kg using the PIGRET and *Pig-a* assays targeting RBCs (RBC *Pig-a* assay) at 7, 14, and 28 days after dosing. Toxic effects induced by AA, such as hind limb weak-paralysis, reduction of body weight gain, and reticulocytosis, were observed in AA-treated groups. However, we detected no significant increases in *Pig-a* mutant frequencies using either the PIGRET or RBC *Pig-a* assay. Therefore, we concluded that the genotoxicity of AA could not be detected by these assays under our experimental conditions.

Keywords: acrylamide, *Pig-a* assay, reticulocyte

Johnson GE[*1], Yamamoto M[*2], Suzuki Y[*3], Adachi H[*4], Kyoya T[*5], Takasawa H[*6], Horibata K, Tsutsumi E[*7], Wada K[*8], Kikuzuki R[*9], Yoshida I[*10], Kimoto T[*11], Maeda A[*12], Narumi K[*13]: Measuring reproducibility of dose response data for the *Pig-a* assay using covariate benchmark dose analysis.

*Mutat Res*. 2016;811:135-9

The reproducibility of the *in vivo Pig-a* gene mutation test system was assessed across 13 different Japanese laboratories. In each laboratory rats were exposed to the same dosing regimen of *N*-nitroso-*N*-ethylurea (ENU), and red blood cells (RBCs) and reticulocytes (RETs) were collected for mutant phenotypic analysis using flow cytometry. Mutant frequency dose response data were analysed using the PROAST benchmark dose (BMD) statistical package. Laboratory was used as a covariate during the analysis to allow all dose responses to be analysed at the same time, with conserved shape parameters. This approach has recently been shown to increase the precision of the BMD analysis, as well as providing a measure of equipotency. This measure of equipotency was used here to demonstrate a reasonable level of interlaboratory reproducibility. Increased reproducibility could have been achieved by increasing the number of cells scored, as this would reduce the number of zero values within the mutant frequency data. Overall, the interlaboratory trial was successful, and these findings support the transferability of the *in vivo Pig-a* gene mutation assay.

Keywords: *Pig-a* gene mutation assay, benchmark dose, interlaboratory reproducibility

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Lorge E[*1], Moore MM[*2], Clements J[*3], O'Donovan M[*4], Fellows MD[*5], Honma M, Kohara A[*6], Galloway S[*7], Armstrong MJ[*7], Thybaud V[*8], Gollapudi B[*9], Aardema MJ[*10], Tanir JY[*11]: Standardized cell sources and recommendations for good cell culture practices in genotoxicity testing.

*Mutat Res*. 2016;809:1-15

Good cell culture practice and characterization of the cell lines used are of critical importance in *in vitro* genotoxicity testing. The objective of this initiative was to make continuously available stocks of the characterized isolates of the most frequently used mammalian cell lines in genotoxicity testing anywhere in the world ('IVGT' cell lines). This project was organized under the auspices of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) Project Committee on the Relevance and Follow-up of Positive Results in In Vitro

Genetic Toxicity (IVGT) Testing. First, cell isolates were identified that are as close as possible to the isolate described in the initial publications reporting their use in genotoxicity testing. The depositors of these cell lines managed their characterization and their expansion for preparing continuously available stocks of these cells that are stored at the European Collection of Cell Cultures (ECACC, UK) and the Japanese Collection of Research Bioresources (JCRB, Japan). This publication describes how the four 'IVGT' cell lines, i.e. L5178Y TK+/- 3.7.2C, TK6, CHO-WBL and CHL/IU, were prepared for deposit at the ECACC and JCRB cell banks. Recommendations for handling these cell lines and monitoring their characteristics are also described. The growth characteristics of these cell lines (growth rates and cell cycles), their identity (karyotypes and genetic status) and ranges of background frequencies of select endpoints are also reported to help in the routine practice of genotoxicity testing using these cell lines.

Keywords: CHO-WBL, CHL/IU, TK6

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Horibata K, Ukai A, Ishikawa S*, Sugano A*, Honma M: Monitoring genotoxicity in patients receiving chemotherapy for cancer: application of the *PIG-A* assay.

*Mutat Res.* 2016;808:20-6

The recently introduced *Pig-a in vivo* gene mutation assay measures endogeneous mutations of *Pig-a* (human, *PIG-A*), an X-linked gene that is conserved across species from rodents to humans. Flow cytometric analysis enables the enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient erythrocytes, resulting from a mutation in *Pig-a*/*PIG-A*, in only a few microliters of peripheral blood. *Pig-a*/*PIG-A* mutations appear to function in a neutral manner, allowing evaluation of the accumulated genotoxic effects of repeated exposures. To date, most *Pig-a* studies have been conducted in rodents; only a few reports regarding human applications of the *PIG-A* assay have been published. We have conducted a *PIG-A* assay in the context of human genotoxicity monitoring. Peripheral blood was collected from healthy human donors and chemotherapy-treated cancer patients at Yamagata University Hospital. To investigate the *PIG-A* mutant frequency (MF) induced by chemotherapy, red blood cells were analyzed via flow cytometry following staining with allophycocyanin-conjugated anti-CD235ab (erythrocyte specific) and fluorescein isothiocyanate-conjugated anti-CD59 antibodies (GPI-anchored protein specific). Reticulocyte frequencies (%RET) were also analyzed using a phycoerythrin-conjugated anti-CD71 antibody to monitor bone marrow suppression and reticulocytosis. Two of 27 patients exhibited a significantly elevated frequency of *PIG-A* mutants. Although we observed either a reduced or an increased %RET in all patients, no association was observed between this factor and the *PIG-A* MF. Unfortunately, we could not analyze blood samples collected before treatment during therapeutic processes. Additionally, the sampling time point for some patients was too short to express the *PIG-A* mutant phenotypes. Therefore, the possibility of natively high *PIG-A* MFs prior to treatment must be considered. The human *PIG-A* assay shows promise as a human genotoxicity monitoring method.

Keywords: human *PIG-A* assay, human monitoring, chemotherapy

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Masumura K, Toyoda-Hokaiwado N, Ukai A, Gondo Y*, Honma M, Nohmi T: Dose-dependent *de novo* germline mutations detected by whole-exome sequencing in progeny of ENU-treated male *gpt* delta mice.

*Mutat Res.* 2016;810:30-9

Germline mutations are an important component of genetic toxicology; however, mutagenicity tests of germline cells are limited. Recent advances in sequencing technology can be used to detect mutations by direct sequencing of genomic DNA (gDNA).

We previously reported induced *de novo* mutations detected using whole-exome sequencing in the offspring of *N*-ethyl-*N*-nitrosourea (ENU)-treated mice in a single-dose experiment (85 mg/kg, i.p., weekly on two occasions). In this study, two lower doses (10 and 30 mg/kg) were added, and dose-response of inherited germline mutations was analyzed. Male *gpt* delta transgenic mice treated with ENU in three dose groups were mated with untreated females 10 weeks after the last treatment, and offspring were obtained. The ENU-treated male mice showed dose-dependent increases in *gpt* mutant frequencies in their sperm, testis, and liver. gDNA of one family (parents and four offspring) from each dose group was used for whole-exome sequencing, and unique *de novo* mutations in the offspring were detected. Frequencies of inherited mutations increased with dosage more than 25-fold in the highest dose group. The mutation spectrum of the inherited mutations showed characteristics of ENU-induced mutations, such as A:T base substitutions. No confirmed mutations were observed in the control group. Filtering using the alternate reads ratio resulted in the mutation frequencies and spectra similar to those obtained by the Sanger sequencing confirmation. These results suggest that direct sequencing analysis may be a useful tool to investigate inherited germline mutations induced by environmental mutagens.

Keywords: germline mutation, whole-exome sequencing, *gpt* delta mouse

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Suzuki T, Yasui M, Honma M: Mutator phenotype and DNA double-strand break repair in BLM helicase-deficient human cells.
*Mol Cell Biol*. 2016;36:2877-89

Bloom syndrome (BS), an autosomal recessive disorder of the BLM gene, predisposes sufferers to various cancers. To investigate the mutator phenotype and genetic consequences of DNA double-strand breaks (DSBs) in BS cells, we developed BLM helicase-deficient human cells by disrupting the BLM gene. Cells with a loss of heterozygosity (LOH) due to homologous recombination (HR) or nonhomologous end joining (NHEJ) can be restored with or without site-directed DSB induction. BLM cells exhibited a high frequency of spontaneous interallelic HR with crossover, but noncrossover events with long-tract gene conversions also occurred. Despite the highly interallelic HR events, BLM cells predominantly produced hemizygous LOH by spontaneous deletion. These phenotypes manifested during repair of DSBs. Both NHEJ and HR appropriately repaired DSBs in BLM cells, resulting in hemizygous and homozygous LOHs, respectively. However, the magnitude of the LOH was exacerbated in BLM cells, as evidenced by large deletions and long-tract gene conversions with crossover. BLM helicase suppresses the elongation of branch migration and crossover of double Holliday junctions (HJs) during HR repair, and a deficiency in this enzyme causes collapse, abnormal elongation, and/or preferable resolution to crossover of double HJs, resulting in a large-scale LOH. This mechanism underlies the predisposition for cancer in BS.

Keywords: Bloom syndrome, homologous recombination, nonhomologous end joining

Suzuki T, Grúz P, Honma M, Adachi N, Nohmi T: The role of DNA polymerase ζ in translesion synthesis across bulky DNA adducts and cross-links in human cells.
*Mutat Res*. 2016;791-792:35-41

Translesion DNA synthesis (TLS) is a cellular defense mechanism against genotoxins. Defects or mutations in specialized DNA polymerases (Pols) involved in TLS are believed to result in hypersensitivity to various genotoxic stresses. Here, DNA polymerase ζ (Pol ζ)-deficient (KO: knockout) and Pol ζ catalytically dead (CD) human cells were established and their sensitivity towards cytotoxic activities of various genotoxins was examined. The CD cells were engineered by altering the DNA sequence encoding two amino acids essential for the catalytic activity of Pol ζ, i.e., D2781 and D2783, to alanines. Both Pol ζ KO and CD cells displayed a prolonged cell cycle and higher incidence of micronuclei formation than the wild-type (WT) cells in the absence of exogenous genotoxic treatments, and the order of abnormality was CD>KO>WT cells. Both KO and CD cells exhibited higher sensitivity towards the killing effects of benzo[*a*]pyrene diol epoxide, mitomycin C, potassium bromate, *N*-methyl-*N*'-nitro-N-nitrosoguanidine, and ultraviolet C irradiation than WT cells, and there were no differences between the

sensitivities of KO and CD cells. Interestingly, neither KO nor CD cells were sensitive to the cytotoxic effects of hydrogen peroxide. Since KO and CD cells displayed similar sensitivities to the genotoxins, we employed only KO cells to further examine their sensitivity to other genotoxic agents. KO cells were more sensitive to the cytotoxicity of 4-nitroquinoline *N*-oxide, styrene oxide, cisplatin, methyl methanesulfonate, and ethyl methanesulfonate than WT cells. However, the KO cells displayed sensitivity camptothecin, etoposide, bleomycin, hydroxyurea, crotonealdehyde, and methylglyoxal in a manner similar to the WT cells. Our results suggest that Pol ζ plays an important role in the protection of human cells by carrying out TLS across bulky DNA adducts and cross-links, but has no or limited role in the protection against strand-breaks in DNA.

Keywords: translesion DNA synthesis, DNA polymerase ζ, bulky DNA adduct

Sassa A, Çağlayan M*, Rodriguez Y*, Beard WA*, Wilson SH*, Nohmi T, Honma M, Yasui M: Impact of Ribonucleotide Backbone on Translesion Synthesis and Repair of 7,8-Dihydro-8-oxoguanine.

*J Biol Chem*. 2016;291:24314-23

Numerous ribonucleotides are incorporated into the genome during DNA replication. Oxidized ribonucleotides can also be erroneously incorporated into DNA. Embedded ribonucleotides destabilize the structure of DNA and retard DNA synthesis by DNA polymerases (pols), leading to genomic instability. Mammalian cells possess translesion DNA synthesis (TLS) pols that bypass DNA damage. The mechanism of TLS and repair of oxidized ribonucleotides remains to be elucidated. To address this, we analyzed the miscoding properties of the ribonucleotides riboguanosine (rG) and 7,8-dihydro-8-oxo-riboguanosine (8-oxo-rG) during TLS catalyzed by the human TLS pols κ and η *in vitro* The primer extension reaction catalyzed by human replicative pol α was strongly blocked by 8-oxo-rG. pol κ inefficiently bypassed rG and 8-oxo-rG compared with dG and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG), whereas pol η easily bypassed the ribonucleotides. pol α exclusively inserted dAMP opposite 8-oxo-rG. Interestingly, pol κ preferentially inserted dCMP opposite 8-oxo-rG, whereas the insertion of dAMP was favored opposite 8-oxo-dG. In addition, pol η accurately bypassed 8-oxo-rG. Furthermore, we examined the activity of the base excision repair (BER) enzymes 8-oxoguanine DNA glycosylase (OGG1) and apurinic/apyrimidinic endonuclease 1 on the substrates, including rG and 8-oxo-rG. Both BER enzymes were completely inactive against 8-oxo-rG in DNA. However, OGG1 suppressed 8-oxo-rG excision by RNase H2, which is involved in the removal of ribonucleotides from DNA. These results suggest that the different sugar backbones between 8-oxo-rG and 8-oxo-dG alter the capacity of TLS and repair of 8-oxoguanine.

Keywords: ribonucleotide, oxidative damage, translesion DNA synthesis

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Sassa A, Kanemaru Y, Kamoshita N, Honma M, Yasui M: Mutagenic consequences of cytosine alterations site-specifically embedded in the human genome.

*Genes Environ*. 2016;38:17

Introduction: Cytosine residues in CpG dinucleotides often undergo various types of modification, such as methylation, deamination, and halogenation. These types of modifications can be pro-mutagenic and can contribute to the formation of mutational hotspots in cells. To analyze mutations induced by DNA modifications in the human genome, we recently developed a system for tracing DNA adducts in targeted mutagenesis (TATAM). In this system, a modified/damaged base is site-specifically introduced into intron 4 of thymidine kinase genes in human lymphoblastoid cells. To further the understanding of the mutagenesis of cytosine modification, we directly introduced different types of altered cytosine residues into the genome and investigated their genomic consequences using the TATAM system.

Findings: In the genome, the pairing of thymine and 5-bromouracil with guanine, resulting from the deamination of 5-methylcytosine and 5-bromocytosine, respectively, was highly pro-mutagenic compared with the pairing of uracil with guanine, resulting from the deamination of cytosine residues.

Conclusions: The deamination of 5-methylcytosine and 5-bromocytosine rather than that of normal cytosine dramatically enhances the mutagenic potential in the

human genome.
Keywords: deamination, gene mutation, genome

Cao Y*, Yang L*, Feng N*, Shi O*, Xi J*, You X*, Yin C*, Yang H*, Horibata K, Honma M, Qian B*, Weng W*, Luan Y*: A population study using the human erythrocyte *PIG-A* assay.
*Environ Mol Mutagen*. 2016;57:605-14

Erythrocyte-based *PIG-A* assay is sensitive and reliable in detecting exposure to mutagenetic agents in animal studies, but there are few data from human populations. In this study, we employed a method for detecting CD59 phenotypic variants, resulting from mutation in the *PIG-A* gene, in human red blood cells (RBCs), and determined the CD59-deficient RBC ($RBC^{CD59-}$) frequencies in 217 subjects from general population. The majority of subjects had a relatively low mutant frequencies (MFs) (average, $5.25 \pm 3.6 \times 10^{-6}$, median, $4.38 \times 10^{-6}$, for all subjects), but with males having a significantly greater MFs ($5.97 \pm 4.0 \times 10^{-6}$) than females ($4.19 \pm 2.5 \times 10^{-6}$). There was no correlation between MFs and age. In addition, MFs showed no difference between smoker and nonsmoker, and also no association with smoke duration in male subjects. However, there was a significant correlation between cigarette-pack-years which indicated that the MF was only slightly elevated with the increase of cigarette-pack-years. Moreover, intraindividual variations were investigated in three volunteer subjects over 300 days, and the MFs were relatively stable and repeatable. Furthermore, a pilot study by using white blood cell (WBC) assay based on labeling with FLAER was performed in volunteer subjects. The MFs of FLAER-deficient WBC (WBCFLAER-) and $RBC^{CD59-}$ were consistently elevated in two subjects. Our findings provide baseline data that will be helpful in designing further studies using the *PIG-A* assay to monitor the genotoxic effects of carcinogens in human populations.
Keywords: erythrocyte-based *PIG-A* assay, human populations, human *PIG-A* assay

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Sugiyama K, Furusawa H, Shimizu M*, Grúz P, Honma M: Epigenetic mutagen as histone modulator can be detected by yeast flocculation.
*Mutagenesis*. 2016;31:687-93

We have previously reported that flocculation of a yeast co-transformed with the human DNA methyltransferase 1 (*DNMT1*) and *DNMT3B* genes was inhibited by DNMT inhibitors. It is well known that epigenetic mutagens can disturb nucleosome positioning via DNA methylation and/or histone modification. In this study we first examined the effects of trichostatin A (TSA), a histone deacetylase inhibitor, on the flocculation level of yeast. TSA dose-dependently promoted the flocculation exhibited by the yeast transformed with the DNMT genes or empty vectors. Furthermore, TSA induced the expression of the flocculin-encoding gene *FLO1* The anthracene-derived alizarin, a natural madder root dye, has a potential for carcinogenesis promotion; however, the mode of action has not been elucidated. It is considered that epigenetic changes can promote cancer. Alizarin but not anthracene enhanced the flocculation level of the yeast. Similar to TSA, alizarin also upregulated *FLO1* mRNA. Surprisingly, western blotting indicated that alizarin, but not anthracene, reduced the level of histone H3 in yeast, and alizarin-treated cells frequently displayed abnormally shaped nuclei. These findings suggest that alizarin uniquely influences nucleosome structure. Taken together with our previous findings, this study suggests that the DNMT gene-transformed yeast strains are a useful tool for screening various classes of epigenetic mutagens.
Keywords: epigenetic mutagen, yeast, *FLO1*

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Manganelli S*[1], Benfenati E*[1], Manganaro A*[1], Kulkarni S*[2], Barton-Maclaren TS*[2], Honma M: New quantitative structure-activity relationship models improve predictability of Ames mutagenicity for aromatic azo compounds.
*Toxicol Sci*. 2016;153:316-26

Existing Quantitative Structure-Activity Relationship (QSAR) models have limited predictive capabilities for aromatic azo compounds. In this study, 2 new models were built to predict Ames mutagenicity of this class of compounds. The first one made use of descriptors based on simplified molecular input-line entry system (SMILES), calculated with the CORAL software. The second model was based on the k-nearest neighbors algorithm. The statistical quality of the predictions

from single models was satisfactory. The performance further improved when the predictions from these models were combined. The prediction results from other QSAR models for mutagenicity were also evaluated. Most of the existing models were found to be good at finding toxic compounds but resulted in many false positive predictions. The 2 new models specific for this class of compounds avoid this problem thanks to a larger set of related compounds as training set and improved algorithms.

Keywords: QSAR, aromatic azo compounds, Ames mutagenicity

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Suzuki T, Grúz P, Honma M, Adachi N, Nohmi T: Sensitivity of human cells expressing low-fidelity or weak-catalytic-activity variants of DNA polymerase ζ to genotoxic stresses.

*DNA Repair* (*Amst*). 2016;45:34-43

Translesion DNA polymerases (TLS pols) play critical roles in defense mechanisms against genotoxic agents. The defects or mutations of TLS pols are predicted to result in hypersensitivity of cells to environmental mutagens. In this study, human cells expressing DNA polymerase ζ (Pol ζ) variants with low fidelity or weak catalytic activity have been established with Nalm-6-MSH+ cells and their sensitivity to mutagenicity and cytotoxicity of benzo[a] pyrene diol epoxide (BPDE) and ultraviolet-C light (UV-C) was examined. The low-fidelity mutants were engineered by knocking-in DNA sequences that direct changes of leucine 2618 to either phenylalanine (L2618F) or methionine (L2618M) of Pol ζ. The weak-catalytic-activity mutants were generated by knocking-in DNA sequences that direct changes of either tyrosine 2779 to phenylalanine (Y2779F) or aspartate 2781 to asparagine (D2781N). In addition, a +1 frameshift mutation, i.e., CCC to CCCC, was introduced in the coding region of the TK1 gene to measure the mutant frequencies. Doubling time and spontaneous TK mutant frequencies of the established cell lines were similar to those of the wild-type cells. The low-fidelity mutants displayed, however, higher sensitivity to the mutagenicity of BPDE and UV-C than the wild-type cells although their cytotoxic sensitivity was not changed. In contrast, the weak-catalytic-activity mutants were more sensitive to the cytotoxicity of BPDE and UV-C than the wild-type cells, and displayed much higher sensitivity to the clastogenicity of BPDE than the wild-type cells in an in vitro micronucleus assay. These results indicate that human Pol ζ is involved in TLS across DNA lesions induced by BPDE and UV-C and also that the TLS plays important roles in induction of mutations, clastogenicity and in cellular survival of the damaged human cells. Similarities and differences in in vivo roles of yeast and human Pol ζ in genome integrity are discussed.

Keywords: translesion DNA polymerases, benzo[*a*] pyrene diol epoxide, DNA polymerase ζ

Petkov PI*1, Schultz TW*2, Donner EM*3, Honma M, Morita T, Hamada S*4, Wakata A*5, Mishima M*6, Maniwa J*7, Todorov M*1, Kaloyanova E*1, Kotov S*1, Mekenyan OG*1: Integrated Approach to Testing and Assessment for Predicting Rodent Genotoxic Carcinogenesity.

*J Appl Toxicol*. 2016;36:1536-50

We investigated the performance of an integrated approach to testing and assessment (IATA), designed to cover different genotoxic mechanisms causing cancer and to replicate measured carcinogenicity data included in a new consolidated database. Genotoxic carcinogenicity was predicted based on positive results from at least two genotoxicity tests: one *in vitro* and one *in vivo* (which were associated with mutagenicity categories according to the Globally Harmonized System classification). Substances belonging to double positives mutagenicity categories were assigned to be genotoxic carcinogens. In turn, substances that were positive only in a single mutagenicity test were assigned to be mutagens. Chemicals not classified by the selected genotoxicity endpoints were assigned to be negative genotoxic carcinogens and subsequently evaluated for their capability to elicit non-genotoxic carcinogenicity. However, non-genotoxic carcinogenicity mechanisms were not currently included in the developed IATA. The IATA is docked to the OECD Toolbox and uses measured data for different genotoxicity endpoints when available. Alternatively, the system automatically provides

predictions by SAR genotoxicity models using the OASIS Tissue Metabolism Simulator platform. When the developed IATA was tested against the consolidated database, its performance was found to be high, with sensitivity of 74% and specificity of 83%, when measured carcinogenicity data were used along with predictions falling within the models' applicability domains. Performance of the IATA would be slightly changed to a sensitivity of 80% and specificity of 72% when the evaluation by non-genotoxic carcinogenicity mechanisms was taken into account.

Keywords: integrated approach to testing and assessment, genotoxic carcinogenicity, non-genotoxic carcinogenicity

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Benfenati E[*1], Belli M[*1], Borges T[*2], Casimiro E[*3], Cester J[*4], Fernandez A[*4], Gini G[*5], Honma M, Kinzl M[*6], Knauf R[*7], Manganaro A[*8], Mombelli E[*9], Petoumenou MI[*1], Paparella M[*6], Paris P[*10], Raitano G[*1]: Results of a round-robin exercise on read-across. *SAR QSAR Environ Res*. 2016;27:371-84

A round-robin exercise was conducted within the CALEIDOS LIFE project. The participants were invited to assess the hazard posed by a substance, applying *in silico* methods and read-across approaches. The exercise was based on three endpoints: mutagenicity, bioconcentration factor and fish acute toxicity. Nine chemicals were assigned for each endpoint and the participants were invited to complete a specific questionnaire communicating their conclusions. The interesting aspect of this exercise is the justification behind the answers more than the final prediction in itself. Which tools were used? How did the approach selected affect the final answer?

Keywords: read-across, bioconcentration factor, mutagenicity

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Masumura K, Toyoda-Hokaiwado N, Ukai A, Gondo Y*, Honma M, Nohmi T: Estimation of the frequency of inherited germline mutations by whole exome sequencing in ethyl nitrosourea-treated and untreated *gpt* delta mice.
*Genes Environ*. 2016;38:10

Introduction: Germline mutations are heritable and may cause health disadvantages in the next generation. To investigate trans-generational mutations, we treated male *gpt* delta mice with *N*-ethyl-*N*-nitrosourea (ENU) (85 mg/kg intraperitoneally, weekly on two occasions). The mice were mated with untreated female mice and offspring were obtained. Whole exome sequencing analyses were performed to identify de novo mutations in the offspring. At 20 weeks after the treatment, the *gpt* mutant frequencies in the sperm of ENU-treated mice were 21-fold higher than those in the untreated control. Liver DNA was extracted from six mice, including the father, mother, and four offspring from each family of the ENU-treated or untreated mice. In total, 12 DNA samples were subjected to whole exome sequencing analyses. We identified *de novo* mutations in the offspring by comparing single nucleotide variations in the parents and offspring. In the ENU-treated group, we detected 148 mutation candidates in four offspring and 123 (82%) were confirmed as true mutations by Sanger sequencing. In the control group, we detected 12 candidate mutations, of which, three (25%) were confirmed. The frequency of inherited mutations in the offspring from the ENU-treated family was $184 \times 10^{-8}$ per base, which was 17-fold higher than that in the control family. The *de novo* mutation spectrum in the next generation exhibited characteristic ENU-induced somatic mutations, such

as base substitutions at A:T bp. These results suggest that direct sequencing analyses can be a useful tool for investigating inherited germline mutations and that the germ cells could be a good endpoint for evaluating germline mutations, which are transmitted to offspring as inherited mutations.

Keywords: germline mutation, whole exome sequencing, *gpt* delta mouse

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Ahlberg E*[1], Amberg A*[2], Beilke LD*[3], Bower D*[4], Cross KP*[4], Custer L*[5], Ford KA*[6], Van Gompel J*[7], Harvey J*[8], Honma M, Jolly R*[9], Joossens E*[10], Kemper RA*[11], Kenyon M*[12], Kruhlak N*[13], Kuhnke L*[14], Leavitt P*[5], Naven R*[12], Neilan C*[15], Quigley DP*[4], Shuey D*[15], Spirkl HP*[2], Stavitskaya L*[13], Teasdale A*[16], White A*[8], Wichard J*[14], Zwickl C*[9], Myatt GJ*[17]: Extending (Q) SARs to incorporate proprietary knowledge for regulatory purposes: A case study using aromatic amine mutagenicity.

*Regul Toxicol Pharmacol*. 2016;13:1-12

Statistical-based and expert rule-based models built using public domain mutagenicity knowledge and data are routinely used for computational (Q) SAR assessments of pharmaceutical impurities in line with the approach recommended in the ICH M7 guideline. Knowledge from proprietary corporate mutagenicity databases could be used to increase the predictive performance for selected chemical classes as well as expand the applicability domain of these (Q) SAR models. This paper outlines a mechanism for sharing knowledge without the release of proprietary data. Primary aromatic amine mutagenicity was selected as a case study because this chemical class is often encountered in pharmaceutical impurity analysis and mutagenicity of aromatic amines is currently difficult to predict. As part of this analysis, a series of aromatic amine substructures were defined and the number of mutagenic and non-mutagenic examples for each chemical substructure calculated across a series of public and proprietary mutagenicity databases. This information was pooled across all sources to identify structural classes that activate or deactivate aromatic amine mutagenicity. This structure activity knowledge, in combination with newly released primary aromatic amine data, was incorporated into Leadscope's expert rule-based and statistical-based (Q) SAR models where increased predictive performance was demonstrated.

Keywords: (Q) SAR, ICH M7, pharmaceutical impurities

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Amberg A*[1], Beilke L*[2], Bercu J*[3], Bower D*[4], Brigo A*[5], Cross KP*[4], Custer L*[6], Dobo K*[7], Dowdy E*[3], Ford KA*[8], Glowienke S*[9], Van Gompel J*[10], Harvey J*[11], Hasselgren C*[4], Honma M, Jolly R*[12], Kemper R*[13], Kenyon M*[7], Kruhlak N*[14], Leavitt P*[6], Miller S*[4], Muster W*[5], Nicolette J*[15], Plaper A*[16], Powley M*[14], Quigley DP*[4], Reddy MV*[17], Spirkl HP*[1], Stavitskaya L*[14], Teasdale A*[18], Weiner S*[19], Welch DS*[15], White A*[11], Wichard J*[20], Myatt GJ*[4]: Principles and procedures for implementation of ICH M7 recommended (Q) SAR analyses.

*Regul Toxicol Pharmacol*. 2016;77:13-24

The ICH M7 guideline describes a consistent approach to identify, categorize, and control DNA reactive, mutagenic, impurities in pharmaceutical products to limit the potential carcinogenic risk related to such impurities. This paper outlines a series of principles and procedures to consider when generating (Q) SAR assessments aligned with the ICH M7 guideline to be included in a regulatory submission. In the absence of adequate experimental data, the results from two complementary (Q) SAR methodologies may be combined to support an initial hazard

classification. This may be followed by an assessment of additional information that serves as the basis for an expert review to support or refute the predictions. This paper elucidates scenarios where additional expert knowledge may be beneficial, what such an expert review may contain, and how the results and accompanying considerations may be documented. Furthermore, the use of these principles and procedures to yield a consistent and robust (Q) SAR-based argument to support impurity qualification for regulatory purposes is described in this manuscript.
Keywords: ICH M7, (Q) SAR, mutagenic impurities

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Sugiyama K, Muroi M*1, Kinoshita M, Hamada O, Minai Y*2, Konishi Y, Kamata Y*3, Tanamoto K*1: NF-κB activation via MyD88-dependent Toll-like receptor signaling is inhibited by trichothecene mycotoxin deoxynivalenol.
*J Toxicol Sci*, 2016;41:273-9

Macrophages induce the innate immunity by recognizing pathogens through Toll-like receptors (TLRs), which sense pathogen-associated molecular patterns. Myeloid differentiation factor 88 (MyD88), which is an essential adaptor molecule for most TLRs, mediates the induction of inflammatory cytokines through nuclear factor κB (NF-κB). Trichothecene mycotoxin deoxynivalenol (DON) shows immunotoxic effects by interrupting inflammatory mediators produced by activated macrophages. The present study investigates the effect of DON on NF-κB in activated macrophages through MyD88-dependent pathways. DON inhibited NF-κB-dependent reporter activity induced by MyD88-dependent TLR agonists. In addition, lipopolysaccharide-induced phosphorylation of interleukin-1 receptor-associated kinase 1 and inhibitor κBα were attenuated by DON. Furthermore, DON downregulated the expression level of MyD88. These results suggest that DON inhibits NF-κB activation in macrophages stimulated with TLR ligands via MyD88-dependent TLR signals. Therefore exposure to DON may lead to the inhibition of MyD88-dependent pathway of TLR signaling.
Keywords: Toll-like receptor, MyD88, deoxynivalenol

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Tanabe S, Kawabata T*1, Aoyagi K*2, Yokozaki H*3, Sasaki H*2: Gene expression and pathway analysis of *CTNNB1* in cancer and stem cells.
*World J Stem Cells*. 2016;8:384-95

AIM: To investigate β-catenin (CTNNB1) signaling in cancer and stem cells, the gene expression and pathway were analyzed using bioinformatics. METHODS: The expression of the catenin β1 (CTNNB1) gene, which codes for β-catenin, wasanalyzed in mesenchymal stem cells (MSCs) and gastric cancer (GC) cells. Beta-catenin signaling and the mutation of related proteins were also analyzed using the cBioPortal for Cancer Genomics and HOMology modeling of Complex Structure (HOMCOS) databases. RESULTS: The expression of the CTNNB1 gene was up-regulated in GC cells compared to MSCs. The expression of EPH receptor A8 (EPHA8), synovial sarcoma translocation chromosome 18 (SS18), interactor of little elongation complex ELL subunit 1 (ICE1), patched 1 (PTCH1), mutS homolog 3 (MSH3) and caspase recruitment domain family member 11 (CARD11) were also shown to be altered in GC cells in the cBioPortal for Cancer Genomics analysis. 3D complex structures were reported for E-cadherin 1 (CDH1), lymphoid enhancer binding factor 1

(LEF1), transcription factor 7 like 2 (TCF7L2) and adenomatous polyposis coli protein (APC) with β-catenin. CONCLUSION: The results indicate that the epithelial-mesenchymal transition (EMT)-related gene CTNNB1 plays an important role in the regulation of stem cell pluripotentcy and cancer signaling.

Keywords: β-catenin, epithelial-mesenchymal transition, mesenchymal stem cell

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Yamamoto N*1, Kato Y*2, Sato A*2, Hiramatsu N*1, Yamashita H*1, Ohkuma M*1, Miyachi E*1, Horiguchi M*1, Hirano K*1, Kojima H: Establishment of a new immortalized human corneal epithelial cell line (iHCE-NY1) for use in evaluating eye irritancy by *in vitro* test methods.

*In Vitro Cell Dev Biol Anim*. 2016 Aug;52(7):742-8

*In vitro* test methods that use human corneal epithelial cells to evaluate the eye irritation potency of chemical substances do not use human corneal epithelium because it has been difficult to maintain more than four passages. In this study, we make a new cell line comprising immortalized human corneal epithelial cells (iHCE-NY1). The $IC_{50}$ of iHCE-NY1 cells is slightly higher than that of Statens Seruminstitut Rabbit Cornea (SIRC) cells, which are currently used in some *in vitro* test methods. CDKN1A in iHCE-NY1 cells was used as a marker of gene expression to indicate cell cycle activity. This enabled us to evaluate cell recovery characteristics at concentrations lower than the $IC_{50}$ of cytotoxic tests.

Keywords: corneal epithelium, eye irritation, *in vitro* model

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Marx U*1, Andersson TB*2,3, Bahinski A*4, Beilmann M*5, Beken S*6, Cassee FR*7,8, Cirit M*9, Daneshian M*10, Fitzpatrick S*11, Frey O*12, Gaertner C*13, Giese C*14, Griffith L*9, Hartung T*10,15, Heringa MB*7, Hoeng J*16, de Jong WH*7, Kojima H, Kuehnl J*17, Leist M*10, Luch A*18, Maschmeyer I*1, Sakharov D*19, Sips AJ*7, Steger-Hartmann T*20, Tagle DA*21, Tonevitsky A*22, Tralau T*18, Tsyb S*23, van de Stolpe A*24, Vandebriel R*7, Vulto P*25, Wang J*26, Wiest J*27, Rodenburg M*7, Roth A*28: Biology-inspired microphysiological system approaches to solve the prediction dilemma of substance testing.

*ALTEX*. 2016;33(3):272-321

The recent advent of microphysiological systems - microfluidic biomimetic devices that aspire to emulate the biology of human tissues, organs and circulation *in vitro* - is envisaged to enable a global paradigm shift in drug development. An extraordinary US governmental initiative and various dedicated research programs in Europe and Asia have led recently to the first cutting-edge achievements of human single-organ and multi-organ engineering based on microphysiological systems. The expectation is that test systems established on this basis would model various disease stages, and predict toxicity, immunogenicity, ADME profiles and treatment efficacy prior to clinical testing. Consequently, this technology could significantly affect the way drug substances are developed in the future. Furthermore, microphysiological system-based assays may revolutionize our current global programs of prioritization of hazard characterization for any new substances to be used, for example, in agriculture, food, ecosystems or cosmetics, thus, replacing laboratory animal models used currently. Thirty-six experts from academia, industry and regulatory bodies present here the results of an intensive workshop (held in June 2015, Berlin, Germany). They review the status quo of microphysiological systems available today against industry needs, and assess the broad variety of approaches with fit-for-purpose potential in the drug development cycle. Feasible technical solutions to reach the next levels of human biology *in vitro* are proposed. Furthermore, key organ-on-a-chip case studies, as well as various national and international programs are highlighted. Finally, a roadmap into the future is outlined, to allow for more predictive and regulatory-accepted substance testing on a global scale.

Keywords: microphysiological systems, organ-on-a-chip, predictive toxicology

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Morita T, Hamada S*1, Masumura K, Wakata A*2, Maniwa J*3, Takasawa H*1, Yasunaga K*1, Hashizume T*4, Honma M: Evaluation of the sensitivity and specificity of *in vivo* erythrocyte micronucleus and transgenic rodent gene mutation tests to detect rodent carcinogen.

*Mutation Research*. 2016;802:1-29

Sensitivity and/or specificity of the *in vivo* erythrocyte micronucleus (MN) and transgenic rodent mutation (TGR) tests to detect rodent carcinogens and non-carcinogens were investigated. The Carcinogenicityand Genotoxicity eXperience (CGX) dataset created by Kirkland et al. was used for the carcinogenicityand *in vitro* genotoxicity data, i.e., Ames and chromosome aberration (CA) tests. Broad literature surveys were conducted to gather *in vivo* MN or TGR test data to add to the CGX dataset. Genotoxicity data *in vitro* were also updated slightly. Data on 379 chemicals (293 carcinogens and 86 non-carcinogens) were available for the *in vivo* MN test; sensitivity, specificity or concordances were calculated as 41.0%, 60.5% or 45.4%, respectively. For the TGR test, data on 80 chemicals (76 carcinogens and 4 non-carcinogens) were available; sensitivity was calculated as 72.4%.

Keywords: genotoxicity *in vivo*, sensitivity, specificity

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Fujita Y*, Morita T, Matsumura S*, Kawamoto T*, Itoh Y*, Nishiyama N*, Honda H*: Comprehensive retrospective evaluation of existing *in vitro* chromosomal aberration test data by cytotoxicity index transformation.

*Mutation Research*. 2016;802:38-49

Using a mathematical approach to estimate new indices from the relative cell count, we constructed an evaluation flow that quantitatively estimates how often the previous test conclusions change when applying the updated cytotoxicity criteria. The new evaluation flow was applied to a retrospective evaluation of 285 chemicals in two databases. The effects of the employment of new cytotoxicity indices are investigated at a large scale. Using the new evaluation flow, 90 chemicals were estimated as positive, 39 were designated as estimated negative (13 probably negative and 26 possibly negative), and 140 were designated as negative.

Keywords: *in vitro* chromosomal aberration test, cytotoxicity index, retrospective evaluation

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Morita T, Uneyama C: Genotoxicity assessment of

4-methylimidazole: Regulatory perspectives.
*Genes and Environment*. 2016;38:20

Genotoxic assessments of 4-Methylimidazole (4-MI) in different regulatory bodies are presented and the risk evaluation of 4-MI is discussed based on new genotoxicity data.

Keywords: 4-Methylimidazole, carcinogenicity, risk assessment

Hirata-Koizumi M, Ise R[*1], Kato H[*1], Matsuyama T[*1], Nishimaki-Mogami T, Takahashi M, Ono A, Ema M[*2], Hirose A: Transcriptome analyses demonstrate that Peroxisome Proliferator-Activated Receptor α (PPARα) activity of an ultraviolet absorber, 2-(2'-hydroxy-3',5'-di-tert-butylphenyl) benzotriazole, as possible mechanism of their toxicity and the gender differences.
*J Toxicol Sci*. 2016;41:693-700

2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)benzotriazole (HDBB), the Benzotriazole UVstabilizer (BUVSs) known as UV-320, is widely used in plastic materials for protection against UV-irradiation. Previously, we reported that oral ingestion of HDBB induce hepatotoxicity including hepatocyte hypertrophy and necrosis in rats and, males was more susceptible compared with females in young rats while no sex-related difference was observed in preweaning rats. Phenotypes observed in our previous study imply involvement of peroxisome proliferator-activated receptor (PPAR) α in HDBB hepatotoxicity, however, direct evidence that HDBB can activate PPARα has not been provided and the mechanism which underlying the gender difference of HDBB hepatotoxicity was not clearly elucidated. Here, we conduct transcriptome analysis using microarray expression profiles in the livers of rats administered HDBB. PPARα agonist activity of HDBB was elucidated by comparison with gene expression data of typical PPARα agonist, i.e. clofibrate, WY-14643, gemfibrozil, and fenofibrate, from TG GATEs database. Moreover, we analyzed for PPARα mRNA expression in the liver of developing male and female rats. PPARα mRNA expression level was higher in males than in females on postnatal days (PNDs) 28 and 35, whereas no sex-related difference was found on PNDs 7 and 22. These results suggest that HDBB exerts its hepatotoxicity through the PPARα signal pathway and the sex-related difference in PPARα expression may contribute to the sex-related difference in susceptibility to hepatotoxicity.

Keywords: 2-(2'-hydroxy-3',5'-di-tert-butylphenyl) benzotriazole, peroxisome proliferator-activated receptor α, benzotriazole UV-stabilizer

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Matsumoto M, Todo H[*1], Akiyama T, Hirata-Koizumi M, Sugibayashi K[*1], Ikarashi Y, Ono A, Hirose A, Yokoyama K[*2]: Risk assessment of skin lightening cosmetics containing hydroquinone.
*Regul Toxicol Pharmacol*. 2016;81:128-35

Following reports on potential risks of hydroquinone (HQ), HQ for skin lightening has been banned or restricted in Europe and the US. In contrast, HQ is not listed as a prohibited or limited ingredient for cosmetic use in Japan, and many HQ cosmetics are sold without restriction. To assess the risk of systemic effects of HQ, we examined the rat skin permeation rates of four HQ (0.3%, 1.0%, 2.6%, and 3.3%) cosmetics. The permeation coefficients ranged from $1.2 \times 10^{-9}$ to $3.1 \times 10^{-7}$ cm/s, with the highest value superior than the HQ aqueous solution ($1.6 \times 10^{-7}$ cm/s). After dermal application of the HQ cosmetics to rats, HQ in plasma was detected only in the treatment by highest coefficient cosmetic. Absorbed HQ levels treated with this highest coefficient cosmetic in humans were estimated by numerical methods, and we calculated the margin of exposure (MOE) for the estimated dose (0.017 mg/kg-bw/day in proper use) to a benchmark dose for rat renal tubule adenomas. The MOE of 559 is judged to be in a range safe for the consumer. However, further consideration may be required for regulation of cosmetic ingredients.

Keywords: hydroquinone, dermal absorption, permeation coefficients

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