Yoshida H, Shibata H, Izutsu K, Goda Y: Comparison of dissolution similarity assessment methods for products with large variations: f2 statistics and model-independent multivariate confidence region procedure for dissolution profiles of multiple oral products.

Biol Pharm Bull, 2017;40:722-5

The current Japanese Ministry of Health Labour and Welfare (MHLW)'s Guideline for Bioequivalence Studies of Generic Products uses averaged dissolution rates for the assessment of dissolution similarity between test and reference formulations. This study clarifies how the application of model-independent multivariate confidence region procedure (Method B), described in the European Medical Agency and U.S. Food and Drug Administration guidelines, affects similarity outcomes obtained empirically from dissolution profiles with large variations in individual dissolution rates. Sixty-one datasets of dissolution profiles for immediate release, oral generic, and corresponding innovator products that showed large variation in individual dissolution rates in generic products were assessed on their similarity by using the f2 statistics defined in the MHLW guidelines (MHLW f2 method) and two different Method B procedures, including a bootstrap method applied with f2 statistics (BS method) and a multivariate analysis method using the Mahalanobis distance (MV method). The MHLW f2 and BS methods provided similar dissolution similarities between reference and generic products. Although a small difference in the similarity assessment may be due to the decrease in the lower confidence interval for expected f2 values derived from the large variation in individual dissolution rates, the MV method provided results different from those obtained through MHLW f2 and BS methods. Analysis of actual dissolution data for products with large individual variations would provide valuable information towards an enhanced understanding of these methods and their possible incorporation in the MHLW guidelines.

Keywords: bootstrap analysis, dissolution similarity, multivariate ANOVA

Yoshida H, Kuwana A, Shibata H, Izutsu K, Goda Y: Comparison of Aerodynamic Particle Size Distribution Between a Next Generation Impactor and a Cascade Impactor at a Range of Flow Rates.

AAPS PharmSciTech. 2017;18:646-53

Wide variation in respiratory flow rates between patients emphasizes the importance of evaluating the aerodynamic particle size distribution (APSD) of dry powder inhaler (DPI) using a multi-stage impactor at different flow rates. US Pharmacopeia recently listed modified configurations of the Andersen cascade impactor (ACI) and new sets of cut-off diameter specifications for the operation at flow rates of 60 and 90 L/min. The purpose of this study was to clarify the effect of these changes on the APSD of DPI products at varied flow rates. We obtained APSD profiles of four DPIs and device combinations, Relenza®-Diskhaler® (GlaxoSmithKline Co.), Seebri®-Breezhaler® (Novartis Pharma Co.), Pulmicort®-Turbuhaler® (Astrazeneca Co.), and Spiriva®-Handihaler® (Nippon Boehringer Ingelheim Co.) using Next Generation Impactors (NGIs) and ACIs at flow rates from 28.3 to 90 L/min to evaluate the difference in the use of previous and new sets of cut-off diameter specifications. Processing the data using the new specifications for ACI apparently reduced large differences in APSD obtained by NGI and ACI with the previous specifications at low and high flow rates in all the DPIs. Selecting the appropriate configuration of ACI corresponding to the flow rate provided comparable APSD profiles of Pulmicort®-Turbuhaler® to those using NGIs at varied flow rates. The results confirmed the relevance of the current US Pharmacopeia specifications for ACI analysis in obtaining APSD profiles of DPI products at wide flow rates.

Keywords: Next Generation Impactor, Andersen cascade impactor, aerodynamic particle size distribution

Bera TK[*1], Abe Y, Ise T[*2], Oberle A[*3], Gallardo D[*1], Liu XF[*1], Nagata S[*2], Binder M[*3], Pastan I[*1]: Recombinant immunotoxins targeting B-cell maturation antigen are cytotoxic to myeloma cell lines and myeloma cells from patients.

Leukemia. 2018;32:569-72

B cell maturation antigen (BCMA), a member of the tumor necrosis factor receptor superfamily, is exclusively and highly expressed on normal

and malignant plasma cells. Therefore, BCMA is a promising antigen for targeted therapy to treat multiple myeloma. Recombinant immunotoxins are chimeric proteins in which an Fv or Fab is fused with Pseudomonas exotoxin A (PE). To develop immunotoxin targeting BCMA expressing cells, we have generated a panel of monoclonal antibodies against BCMA. Two of them, BM24 and BM306, were used to make recombinant immunotoxins targeting BCMA, by genetically attaching their Fabs to a 24 kDa form of PE. The Fab immunotoxins specifically killed BCMA expressing cell lines and malignant plasma cells isolated from multiple myeloma patients. The immunotoxins are very potent and act rapidly, a 10 minute treatment of H929 cells was sufficient to kill almost all cells. Immunotoxin alone produced partial regressions of H929 tumors in mice and when combined with Abraxane produced complete and prolonged regressions.

Keywords: immunotoxin, BCMA, multiple myeloma

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Sasaki T.*1, Sakamoto T., Otsuka M.*2: Detection of impurities in organic crystals by high-accuracy terahertz absorption spectroscopy.
Anal.Chem. 2018;90:1677-82

Quantitative detection of impurities in organic crystals was demonstrated by accurately measuring absorption frequencies using a continuous wave gallium phosphide terahertz spectrometer. THz spectra of l-asparagine monohydrate doped with l-aspartic acid at 0.05 to 12.5 wt % were obtained at 10 K. The three lowest frequency absorption peaks were baseline-resolved, allowing them to be examined independently. Using a least-squares curve fitting technique, impurities were detected at levels as low as 500 ppm. The sensitivity and detection limits of the technique depended strongly on the nature of both the host and the impurities. The projected limit of detection using the current system, given optimal materials, was estimated to be 51.7 ppm. In addition to quantitative assessments, impurities may also be identified by comparing frequency shifts of multiple absorptions.

Keywords: terahertz spectroscopy, qualitative analysis, organic impurity

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Hattori Y.*1, Seko Y.*1, Peerapattana J.*2, Otsuka K.*3 Sakamoto T., Otsuka M.*1: Rapid identification of oral dosage forms of counterfeit pharmaceuticals by discrimination using near-infrared spectroscopy.
Bio-Medical Materials and Engineering, 2018;29:1-14

Since it can take an enormous amount of time and cost to discriminate counterfeit medicines by using conventional methods, counterfeit medicines has been spread in the world markets. The purpose of this study was to develop a rapid and simple analytical method to discriminate counterfeit drugs using near infrared (NIR) spectroscopy. Seven types of brand name tablet and generic tablets containing atorvastatin calcium sesquihydrate (AT) preparations were used as simulated counterfeit medicines. NIR spectra of 35 AT tablet products were measured using a diffuse reflection method. The NIR spectral data were analyzed by principal component analysis (PCA). The PCA results suggested that the model had sufficient accuracy to discriminate the 7 types for AT tablets. The NIR spectral data were also analyzed using a soft independent modeling of class analogy (SIMCA) method. Predicting the classification of the AT tablet samples was performed based on all the validated AT tablet data using the SIMCA model, and the probability of classification of 7 types was 100%. The discrimination power spectrum of the SIMCA model indicated significant patterns based on diluents. The PCA and SIMCA classification of the AT tablets were depended on the major excipient combinations.

Keywords: counterfeit drugs, atorvastatin calcium sesquihydrate, near infrared spectroscopy

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Onuki Y*1, Funatani C*2, Yamamoto Y*3, Fukami T*4, Koide T, Hayashi Y*1, Takayama K*2: Stability of Mixed Preparations Consisting of Commercial

Moisturizing Creams with a Steroid Ointment Base Investigated by Magnetic Resonance Imaging.

Chem. Pharm. Bull., 2017;65:487-91

A moisturizing cream mixed with a steroid ointment is frequently prescribed to patients suffering from atopic dermatitis. However, there is a concern that the mixing operation causes destabilization. The present study was performed to investigate the stability of such preparations closely using magnetic resonance imaging(MRI). As sample preparations, five commercial moisturizing creams that are popular in Japan were mixed with an ointment base, a white petrolatum, at a volume ratio of 1 : 1. The mixed preparations were stored at 60° C to accelerate the destabilization processes. Subsequently, the phase separations induced by the storage test were monitored using MRI. Using advanced MR technologies including spin–spin relaxation time (T2) mapping and MR spectroscopy, we successfully characterized the phase-separation behavior of the test samples. For most samples, phase separations developed by the bleeding of liquid oil components. From a sample consisting of an oil-in-water-type cream, Urepearl Cream 10%, a distinct phase-separation mode was observed, which was initiated by the aqueous component separating from the bottom part of the sample. The resultant phase separation was the most distinct among the test samples. To investigate the phase separation quantitatively and objectively, we conducted a histogram analysis on the acquired T2 maps. The water-in-oil type creams were found to be much more stable after mixing with ointment base than those of oil-in-water type creams. This finding strongly supported the validity of the mixing operation traditionally conducted in pharmacies.

Keywords: mixed external preparation, magnetic resonance imaging, emulsion stability

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Yamamoto Y*1, Onuki Y*2, Fukami T*3, Metori K*4, Suzuki T*4, Koide T: Comparison of pharmaceutical properties among clobetasol propionate cream formulations: Considerations from near infrared spectra.

Vib. Spectrosc. 2017;93:17-22

The purpose of this study is to verify the usefulness of near infrared (NIR) spectroscopy for evaluation of pharmaceutical properties of cream formulations. The present study examined in detail the pharmaceutical properties of clobetasol propionate (CLB) cream formulations using NIR spectroscopy. The characteristic NIR spectrum of Dermovate1 (DRM), the original CLB cream formulation displayed a shoulder around 4800 cm^{-1} as well as absorption around 5200 cm^{-1}. A high concentration of propylene glycol (PG) in DRM was thought to be the main contributing factor to this characteristic aspect of the spectrum. Only a peak with absorption around 5200 cm^{-1} was obtained from the generic CLB creams, except for Myalone1 (MYA), suggesting a lower concentration of PG in those generic formulations compared to the original formulation. The spectrum obtained from the analysis of MYA was significantly different from the other formulations. The fact that it contained macrogol and a high PG concentration were likely the major factors that accounted for the spectral differences. High concentrations (25–30%) of PG were found in DRM and MYA by gas chromatography-mass spectrometry. Thus, even cream formulations with the same active pharmaceutical ingredients can have significantly different pharmaceutical properties. And it is suggested usefulness of NIR spectroscopy for evaluation of pharmaceutical property of cream formulation.

Keywords: steroidal formulation, cream, near infrared spectroscopy

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Inoue M*1, Hisada H*1, Koide T, Carriere J*2, Heyler R*2, Fukami T*1: Real-time formation monitoring of cocrystals with a different stoichiometry using probe-type low-frequency Raman spectroscopy.

Ind. Eng. Chem. Res. 2017;56:12693-7

In the cocrystallization process, real-time monitoring is eff ective for obtaining cocrystal products with consistent quality. Low-frequency Raman spectra refl ect the lattice vibrations derived from crystalline diff erences; therefore, it is expected to be useful for the monitoring of pharmaceutical cocrystals that are diffi cult to distinguish by Raman spectroscopy in the fi ngerprint region. In this work, we attempted to monitor the formation of cocrystals with 1:1 and 2:1 cocrystals consisting of carbamazepine and 4-aminobenzoic acid using probe-type low-frequency Raman spectroscopy. Real-time measurements were performed during stirring of a composition known to form 1:1 and 2:1 cocrystals by the reaction crystallization method, and the spectra derived from the cocrystals were confi rmed after 5 min. To monitor the transition of the cocrystals toward a stoichiometry of 2:1 from 1:1 and toward a stoichiometry of 1:1 from 2:1, specifi ed amounts of raw materials were added to the cocrystals suspended in ethanol. The cocrystals with diff erent stoichiometries were transformed after 3 h.

Keywords: Raman spectroscopy, low-frequency, cocrystal

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加藤くみ子，原矢佑樹：脂質膜微小胞の特性解析手法の開発.

BUNSEKI KAGAKU 2018;67:1-9

医薬品に利用される脂質膜微小胞の物理的化学的特性を解析するための手法を開発した．モノリス型キャピラリーカラムを用いたキャピラリー液体クロマトグラフィーシステムや原子間力顕微鏡法を活用する方法とその意義について概説した.

Keywords: lipid membrane vesicle, monolithic capillary column, atomic force microscopy

Takechi-Haraya Y, Goda Y, Sakai-Kato K: Imaging and size measurement of nanoparticles in aqueous medium by use of atomic force microscopy.

Analytical and Bioanalytical Chemistry. 2018;410:1525-31

Size control of nanoparticles in nanotechnology-based drug products is crucial for their successful development, since the in vivo pharmacokinetics of nanoparticles are size-dependent. In this study, we evaluated the use of atomic force microscopy (AFM) for imaging and size measurement of nanoparticles in aqueous medium. The height sizes of rigid polystyrene nanoparticles and soft liposomes were measured by AFM and were compared with the hydrodynamic sizes measured by dynamic light scattering (DLS). The lipid compositions of the studied liposomes were similar to those of commercial products. AFM proved to be a viable method for obtaining images of both polystyrene nanoparticles and liposomes in aqueous medium. For the polystyrene nanoparticles, the average height size observed by AFM was similar to the average number-weighted diameter obtained by DLS, indicating the usefulness of AFM for measuring the sizes of nanoparticles in aqueous medium. For the liposomes, the height sizes obtained by AFM differed depending upon the procedures of immobilizing the liposomes onto a solid substrate. In addition, the resultant average height sizes of the liposomes were smaller than those obtained by DLS. This knowledge will help the correct use of AFM as a powerful tool for imaging and size measurement of nanotechnology-based drug products for clinical use.

Keywords: atomic force microscopy, size measurement, liposome

Sakai-Kato K, Sakurai M, Takechi-Haraya Y, Nanjo K, Goda Y: Involvement of scavenger receptor class B type 1 and low-density lipoprotein receptor in the internalization of liposomes into HepG2 cells.

Biochimica et Biophysica Acta-Biomembranes. 2017;1859:2253-58

In this study, HepG2 cells, an in vitro model system for human hepatocytes, were used to evaluate the interaction of lipoprotein receptors with liposomes carrying fluorescently labeled cholesterol and their subsequent intracellular uptake. In these experiments, two lipoprotein receptors, scavenger receptor class B type 1 (SR-B1) and low-density lipoprotein receptor (LDLR), accounted for approximately 20% and 10%, respectively, of the intracellular uptake of the labeled liposomes. These findings indicate that additional mechanisms contributed to liposomal internalization. Liposomes modified with both apolipoproteins A-I and E were internalized in HepG2 cells in FBS-depleted

culture medium at the same levels as unmodified liposomes in FBS-containing culture medium, which indicates that apolipoproteins A-I and E were the major serum components involved in liposomal binding to SR-B1 or LDLR (or both). These results increase our understanding of the disposition of liposomes, processes that can directly affect the efficacy and safety of drug products.

Keywords: liposome, lipoprotein receptor, apolipoprotein

Takechi-Haraya Y, Goda Y, Sakai-Kato K: Control of liposomal penetration into three-dimensional multicellular tumor spheroids by modulating liposomal membrane rigidity.
Molecular Pharmaceutics. 2017;14:2158-65

Effective penetration of drug-carrying nanoparticles into solid tumors is a major challenge in cancer therapy. Exploration of the physicochemical properties of nanoparticles that affect penetration efficiency is required to achieve maximum therapeutic effects. Here, we used confocal laser scanning microscopy to evaluate the efficiencies of penetration of fluorescently labeled liposomes into three-dimensional spheroids composed of HeLa cells. The prepared liposomes were composed of phosphatidylcholines and varying contents of cholesterol and/or a polyethylene glycol-modified phospholipid. We demonstrated that the efficiency of penetration into spheroids increased with the bending modulus (i.e., membrane rigidity) of the liposome, as determined by atomic force microscopy (correlation coefficient, 0.84). To clarify the mechanism by which membrane rigidity contributes to the penetration behavior of liposomes, we also analyzed the cellular uptake using monolayer cells. We showed that penetration efficiency was explained partially by cellular uptake efficiency, but that other factors such as liposome diffusion efficiency in the intercellular space of tumor spheroids contributed. Our results quantitatively demonstrate that the bending modulus of the liposomal membrane is a major determinant of liposomal penetration into three-dimensional spheroids. The present study will contribute to the understanding and control of tumor penetration of liposomal formulations.

Keywords: liposome, membrane rigidity, spheroid penetration

Takechi-Haraya Y, Sakai-Kato K, Goda Y: Membrane Rigidity Determined by Atomic Force Microscopy Is a Parameter of the Permeability of Liposomal Membranes to the Hydrophilic Compound Calcein.
AAPS PharmSciTech 2017;18:1887-93

We determined the permeability coefficient of a model hydrophilic drug, calcein, encapsulated within saturated lipid-based nano-sized liposomes of various lipid profiles. We demonstrated that the addition of cholesterol to liposomes containing saturated lipids increased the permeability of the liposomal membrane to calcein via a decrease in the membrane bending modulus, as determined by means of atomic force microscopy. We found an inverse correlation between the membrane bending modulus of saturated lipid-based nano-sized liposomes and the permeability coefficient of encapsulated calcein, demonstrating that bending modulus, as determined by means of atomic force microscopy, is a quantitative parameter describing the permeability of liposomal membranes to calcein.

Keywords: atomic force microscopy, liposomal membrane permeability, membrane rigidity

Krayukhina E[*1,2], Noda M[*1,2], Ishii K[*3], Maruno T[*1,2], Wakabayashi H[*1], Tada M, Suzuki T, Ishii-Watabe A, Kato M[*4], Uchiyama S[*1,2,3]: Analytical ultracentrifugation with fluorescence detection system reveals differences in complex formation between recombinant human TNF and different biological TNF antagonists in various environments.
mAbs. 2017;9(4):664-79.

A number of studies have attempted to elucidate the binding mechanism between tumor necrosis factor (TNF) and clinically relevant antagonists. None of these studies, however, have been conducted as close as possible to physiologic conditions, and so the relationship between the size distribution of TNF-antagonist complexes and the antagonists' biological activity or adverse effects remains elusive. Here, we characterized the binding stoichiometry and sizes of soluble TNF-antagonist complexes for adalimumab, infliximab, and etanercept that were formed in human serum and in phosphate-buffered saline (PBS). Fluorescence-detected sedimentation velocity analytical ultracentrifugation analyses revealed that adalimumab and infliximab formed a range of complexes with

TNF, with the major complexes consisting of 3 molcules of the respective antagonist and one or 2 molecules of TNF. Considerably greater amounts of high-molecular-weight complexes were detected for infliximab in human serum. The emergence of peaks with higher sedimentation coefficients than the adalimumab monomer as a function of added human serum albumin (HSA) concentration in PBS suggested weak reversible interactions between HSA and immunoglobulins. Etanerept exclusively formed 1:1 complexes with TNF in PBS, and a small amount of complexes with higher stoichiometry was detected in human serum. Consistent with these biophysical characterizations, a reporter assay showed that adalimumab and infliximab, but not etanercept, exerted FcγRIIa- and FcγRIIIa-mediated cell signaling in the presence of TNF and that infliximab exhibited higher potency than adalimumab. This study shows that assessing distribution profiles in serum will contribute to a more comprehensive understanding of the in vivo behavior of therapeutic proteins.

Keywords: TNF, monoclonal antibody, immune complex

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日向昌司，多田 稔，橋井則貴，石井明子：宿主細胞由来タンパク質の試験法に関する研究.
医薬品医療機器レギュラトリーサイエンス 2017;6:432-6.

Host cell proteins (HCP) is a kind of process-related impurities existing in biopharmaceuticals. A major safety concern of HCP is that HCP is not only induce anti-HCP antibody but also acting as adjuvant to enhance an anti-drug antibody development. To evaluate the amount of residual HCP, it is important to establish the purity test of the drug substance or the intermediates. Sandwich immunoassay (e.g. enzyme-linked immunosorbent assay; ELISA), a highly sensitive and specific quantitation assay, has been frequently used for the test of residual HCP. Since HCP is a mixture of various proteins, there are many points to consider in the establishment of a quantitative assay for HCP using sandwich immunoassay. In spite of the fact that there are many issues should be considered to establish appropriate HCP assay, the guideline of the assay has not been published. Recently US Pharmacopoeia and European Pharmacopoeia has published new chapters providing guidance on HCP assay, respectively. In order to prepare the draft of Japanese Pharmacopoeia general information for HCP assay, we assess the key points of the development of the HCP assay in reference to the Pharmacopoeias.

Keywords: host cell protein, biopharmaceutical, process-related impurity

Shiga Y*1, Murata D*1, Sugimoto A*1, Oshima Y*1, Tada M, Ishii-Watabe A, Imai K*2, Tomii K*2, Takeuchi T*3, Kagaya S*4, Sato A*1: Hinge-Deficient IgG1 Fc Fusion: Application to Human Lactoferrin.
Mol Pharm. 2017;14(9):3025-35.

Fusion of therapeutic proteins with the antibody Fc domain is a strategy widely applied to increase protein half-life in plasma. In our previous study, we generated a recombinant human lactoferrin (hLF) -immunoglobulin G1 Fc fusion protein (hLF-hinge-CH2-CH3) with improved stability, biological activity, and pharmacokinetics (Shiga, Y. et al. Eur J Pharm Sci., 2015, 67, 136-143) . However, the Fc domain in fusion proteins can potentially induce antibody-dependent and complement-dependent cytotoxicity and serious side effects. To overcome these drawbacks, we engineered an hLF-Fc fusion protein (hLF-CH2-CH3) without the Fc hinge region which is essential for engaging Fc receptors on immune cells and inducing complement-mediated cell lysis. The hLF-CH2-CH3 protein was stably expressed in Chinese hamster ovary (CHO) DG44 cells and compared for in vitro activities, thermal stability, pharmacokinetics, and attenuation of Fc-mediated immune effector functions with the conventional hinge-containing Fc fusion protein. Both hLF-hinge-CH2-CH3 and hLF-CH2-CH3 exhibited iron-binding activity, superior uptake by Caco-2 cells, similar thermal stability, and longer plasma half-life compared to recombinant hLF. However, in contrast to conventional hLF-hinge-CH2-CH3, hinge-deficient hLF-CH2-CH3 did not elicit Fc-mediated effector response potentially damaging for the target cells. Our findings demonstrate that conjugation of hinge-deficient Fc to therapeutic proteins is a promising strategy for

improving their pharmacokinetic properties without enhancing effector functions. Cell-expressed hinge-deficient hLF-CH2-CH3 is a potential drug candidate with improved plasma half-life for parenteral administration.

Keywords: lactoferrin, Fc-fusion protein, effector function

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Kobayashi T, Kamada I*1, Komura J*2, Toyoshima S*1, Ishii-Watabe A: Comparative study of the number of report and time-to-onset of the reported adverse event between the biosimilars and the originator of filgrastim.
Pharmacoepidemiol Drug Saf. 2017;26(8):917-24.

The objective of this study is to specify the most reported adverse events as preferred terms (PTs) and to compare the reported adverse events about some properties including the number of report and time-to-onset (TTO) distribution of the originator of filgrastim Neupogen® and its biosimilars in Europe, using VigiBase®. We identified the biosimilar which was reported as the suspected drug in more than 100 individual case safety reports (ICSRs) in Europe. Then we specified the top ranking 10 PTs in the cases reported with Neupogen® or each biosimilar as the suspected drug. We also compared the TTO of the most reported PTs using the data about the onset date of the PT and the start date of filgrastim. We used Kolmogorov-Smirnov method to detect significant difference. The total ICSR numbers with Neupogen® and 3 biosimilars, Zarzio®, Nivestim®, and Tevagrastim® were 1,301, 295, 156, and 127, respectively, in Europe. The most reported PTs with Neupogen® were bone pain, pyrexia, and dyspnoea. The TTO of bone pain and pyrexia with Zarzio® (N: 22 and 16, median: 1 and 0.5 days) were significantly shorter than those with Neupogen® ($P < 0.01$, N: 72 and 33, median: 3.5 and 3 days), respectively. The most reported PTs with biosimilars were drug ineffective and neutropenia. The difference in the TTO was identified between originator filgrastim Neupogen and its biosimilar regarding some PTs, which may suggest the difference in their safety profile.

Keywords: filgrastim, biosimilars, time-to-onset

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蛭田葉子，橋井則貴，久保田浩樹，鈴木琢雄，佐藤恭子，石井明子：日本薬局方ヘパリンナトリウム各条のナトリウム定性試験及び定量試験に関する研究.
医薬品医療機器レギュラトリーサイエンス 2017;11:780-87.

A qualitative test and an assay for sodium have been adopted in Heparin Sodium monographs of the United States Pharmacopeia (USP) and European Pharmacopoeia (EP), respectively. Although calcium assays have been adopted in Heparin Calcium monograph of Japanese Pharmacopoeia (JP) XVII, a sodium test has not yet been adopted in JP Heparin Sodium monograph. Therefore, a test for sodium is required to ensure the quality of pharmaceutical heparin sodium products that are distributed in Japan and to enhance international harmonization. Our study demonstrates the applicability of the JP General Test, Qualitative Test (sodium salt) <1.09>, and atomic Absorption Spectrophotometry <2.23> to the test for sodium of JP Heparin Sodium monograph.

Keywords: heparin sodium, qualititve test, JP Qualitative Test (sodium salt) <1.09>

Takigawa M*1, Iida M*1, Nagase S*1, Suzuki H*2, Watari A*1, Tada M, Okada Y*1, Doi T*1, Fukasawa M*3, Yagi K*1, Kunisawa J*2, Kondoh M*1 : Creation of a Claudin-2 Binder and Its Tight Junction-Modulating Activity in a Human Intestinal Model.
J Pharmacol Exp Ther. 2017;363(3):444-451.

Disruption of the gastrointestinal epithelial barrier is a hallmark of chronic inflammatory bowel diseases (IBDs). The transmembrane protein claudin 2 (CLDN2) is a component of epithelial tight junctions (TJs). In the intestines of patients with IBDs, the expression of the pore-forming TJ protein CLDN2 is upregulated. Although CLDN2 is involved in these leaky barriers, whether it can be a target to enhance TJ integrity is unknown because a CLDN2-specific inhibitor has not been developed. Here, we used DNA immunization to generate a monoclonal antibody (mAb) that recognized an extracellular loop

of CLDN2. Treatment of epithelial cell monolayers with the mAb increased barrier integrity. In addition, the anti-CLDN2 mAb attenuated the decrease in TJ integrity induced by the proinflammatory cytokine tumor necrosis factor-α（TNF-α), and cotreatment of cells with anti-TNF-α mAb and anti-CLDN2 mAb showed additive attenuating effects. These findings indicate that CLDN2 may be a target for enhancing TJ integrity, and CLDN2 binder may be an enhancer of mucosal barrier integrity and a potential therapeutic option for IBDs.

Keywords: claudin-2, monoclonal antibody, tight junction

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Shibata H, Nishimura K, Miyama C, Tada M, Suzuki T, Saito Y, Ishii-Watabe A : Comparison of different immunoassay methods to detect human anti-drug antibody using the WHO erythropoietin antibody reference panel for analytes.
J Immunol Methods. 2018;452:73-77.

Development of an appropriate assay to detect anti-drug antibody（ADA）is important for assessing immunogenicity to therapeutic protein products. However, characterizing ADA assay methods is difficult because human ADA as a reference standard is not available in most cases. We compared the analytical performance of three ligand-binding assay methods for ADA, namely, surface plasmon resonance（SPR), electrochemiluminescence（ECL), and biolayer interferometry（BLI）methods, by using the anti-erythropoietin（EPO）monoclonal antibody reference panel developed by the World Health Organization（WHO）in 2015. Dose-dependent binding responses were observed for all nine anti-EPO antibodies in the anti-EPO panel by the SPR and BLI methods. In contrast, the ECL method did not clearly detect binding of low-affinity anti-EPO antibodies. Regarding IgG2 and IgM antibodies derived from the same clone, IgG2 exhibited a higher binding response in the SPR assay, whereas the IgM binding response was higher than that of IgG2 in the ECL assay. In the case of the BLI method, there was no consistent pattern observed in the binding responses of IgG2 or IgM. Results of the anti-EPO antibody reference panel, which contains a variety of monoclonal antibodies, indicated that the ability to detect ADAs differed among these assay methods. Therefore, with ligand-binding assays, differences in assay platforms can affect the sensitivity and other characteristics of assays to detect ADAs. These results show that understanding the analytical performance of ADA assays is important for an appropriate assessment of immunogenicity. Our study also indicated the benefits of using the established human ADA reference panel to assess the assay methods for ADA detection.

Keywords: anti-drug antibody assay, anti-erythropoietin antibody reference panel, biolayer interferometry

Nakamori S*1,2, Takahashi J*1,2, Hyuga S*2, Tanaka-Kagawa T*3, Jinno H*4, Hyuga M, Hakamatsuka T, Odaguchi H*2, Goda Y, Hanawa T*2, Kobayashi Y*1,2: Ephedra Herb extract activates/desensitizes transient receptor potential vanilloid 1 and reduces capsaicin-induced pain.
J Nat Med. 2017;71(1):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 Ca^{2+} 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: capsaicin, Ephedra Herb, TRPV1

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Takemoto H*1,2, Takahashi J*1,2, Hyuga S*2, Odaguchi H*2, Uchiyama N, Maruyama T, Yamashita T*3, Hyuga M, Oshima N*4, Amakura Y*5, Hakamatsuka T, Goda Y, Hanawa T*2, and Kobayashi Y*1,2: Ephedrine Alkaloids-Free Ephedra Herb Extract, EFE, Has No Adverse Effects Such as Excitation, Insomnia, and Arrhythmias.

Biol. Pharm. Bull. 2018;41:247–253.

Ephedrine alkaloids-free Ephedra Herb extract (EFE) has been developed to eliminate the adverse effects caused by ephedrine alkaloid-induced sympathetic hyperactivation. Previously, we reported that EFE possesses analgesic, anti-influenza, and cancer metastatic inhibitory effects at comparable levels to that of Ephedra Herb extract (EHE). However, it has not yet been demonstrated that EFE is free from the known side effects of EHE, such as excitation, insomnia, and arrhythmias. In this study, the incidence of these adverse effects was compared between mice administered EHE and those administered EFE. Increased locomotor activity in an open-field test, reduced immobility times in a forced swim test, and reduced sleep times in a pentobarbital-induced sleep test were observed in EHE-treated mice, when compared to the corresponding values in vehicle-treated mice. In contrast, EFE had no obvious effects in these tests. In electrocardiograms, atrial fibrillation (i.e., irregular heart rhythm, absence of P waves, and appearance of f waves) was observed in the EHE-treated mice. It was suggested that this atrial fibrillation was induced by stimulation of adrenaline β 1 receptors, but not by hypokalemia. However, EFE did not affect cardiac electrophysiology. These results suggest that the abovementioned side effects are caused by ephedrine alkaloids in EHE, and that EFE is free from these adverse effects, such as excitation, insomnia, and arrhythmias. Thus, EFE is a promising new botanical drug with few adverse effects.

Keywords: Ephedra Herb, adverse effect, ephedrine alkaloid

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Egashira Y*1,2, Nagatoishi S*1,3, Kiyoshi M, Ishii-Watabe A, Tsumoto K*1,3: Characterization of Glycoengineered Anti-HER2 Monoclonal Antibodies Produced by Using a Silkworm-Baculovirus Expression System.

J Biochem. 2018;1-8.

Silkworm-baculovirus expression systems are efficient means for production of recombinant proteins that provide high expression levels and post-translational modifications. Here, we characterized the stability, glycosylation pattern, and antibody-dependent cell-mediated cytotoxicity activity of anti-HER2 monoclonal antibodies containing native or glycoengineered mammalian-like N-glycans that were produced by using a silkworm-baculovirus expression system. Compared with a monoclonal antibody produced by using a Chinese hamster ovary cell expression system, the glycoengineered monoclonal antibody had comparable thermal stability and a higher antibody-dependent cell-mediated cytotoxicity activity. These results suggest that silkworm-baculovirus expression systems are potentially useful as next-generation expression systems for the cost-effective production of therapeutic antibodies.

Keywords: baculovirus, monoclonal antibody, *N*-glycosylation

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Kiyoshi M, Caaveiro JMM[*1,2], Tada M, Tamura H[*1], Tanaka T[*3], Terao Y[*3], Morante K[*1], Harazono A, Hashii N, Shibata H, Kuroda D[*1], Nagatoishi S[*1], Oe S[*3], Ide T[*3], Tsumoto K[*1,4,5], Ishii-Watabe A.: Assessing the Heterogeneity of the Fc-Glycan of a Therapeutic Antibody Using an engineered FcγReceptor IIIa-Immobilized Column.

Sci Rep. 2018;8(1):3955.

The N-glycan moiety of IgG-Fc has a significant impact on multifaceted properties of antibodies such as in their effector function, structure, and stability. Numerous studies have been devoted to understanding its biological effect since the exact composition of the Fc N-glycan modulates the magnitude of effector functions such as the antibody-dependent cell mediated cytotoxicity (ADCC), and the complement-dependent cytotoxicity (CDC). To date, systematic analyses of the properties and influence of glycan variants have been of great interest. Understanding the principles on how N-glycosylation modulates those properties is important for the molecular design, manufacturing, process optimization, and quality control of therapeutic antibodies. In this study, we have separated a model therapeutic antibody into three fractions according to the composition of the N-glycan by using a novel FcγRIIIa chromatography column. Notably, Fc galactosylation was a major factor influencing the affinity of IgG-Fc to the FcγRIIIa immobilized on the column. Each antibody fraction was employed for structural, biological, and physicochemical analysis, illustrating the mechanism by which galactose modulates the affinity to FcγRIIIa. In addition, we discuss the benefits of the FcγRIIIa chromatography column to assess the heterogeneity of the N-glycan.

Keywords: antibody isolation, purification, antibody therapy

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Gotoh Y[*1], Niimi S, Matsuura T[*2], Ishizuka Y[*3]: Improvement of hydroxyapatite-coated nonwoven fabrics by coating with silk fibroin for use as a scaffold for culture of human hepatocellular carcinoma-derived FLC-5 cells.

J Insect Biotechnol Sericology. 2017;86:29-33.

We modified hydroxyapatite (HA)-coated nonwoven polyethylene/polypropylene fabrics by coating with silk fibroin (SF) to improve them as a three-dimensional substrate for culturing human hepatocellular carcinoma-derived FLC-5 cells. After 25 days of culture, FLC-5 cells cultured on nonwoven fabrics coated with HA and HA plus SF (HA-SF) partially formed multicellular aggregates, whereas the cells cultured on tissue culture plates formed monolayers. FLC-5 cells cultured on nonwoven fabrics and tissue culture plates for 25 days were subjected to quantitative assay for cell number and albumin secretion. The lowest cell number and the largest amount of albumin were found for nonwoven fabrics coated with HA-SF. Normalizing albumin values to cell number data demonstrated that albumin secretory function per cell on 2 kinds of nonwoven fabrics was remarkably higher than that on tissue plates. Moreover, albumin secretory function per cell on nonwoven fabrics coated with HA-SF was twice as high as that on HA-coated nonwoven fabrics. These preliminary results suggest that modification of HA-coated nonwoven fabrics by SF coating induced functional improvement of FLC-5 cells.

Keywords: hepatic tissue engineering, nonwoven fabrics coating, silk fibroin coating

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袴塚高志：英訳版日本薬局方各条における生薬関連用語の整理及び生薬各条英訳の抜本的見直し.

医薬品医療機器レギュラトリーサイエンス, 2017;48:788-793

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Keywords：日本薬局方，生薬，和英対訳表

Maruyama T, Ezaki M[*1], Shiba M[*2], Yamaji H[*2], Yoshitomi T, Kawano N[*1], Zhu S[*3], Cheng X[*4], Yokokura T[*5], Yamamoto Y[*6], Fuchino H[*1], Sun H[*4], Komatsu K[*2], Kawahara N[*1]: Botanical origin and chemical constituents of the commercial SAPOSHNIKOVIAE RADIX and its related crude drugs available in Shaanxi and around regions.

J Nat Med. 2018;72:267-273

Saposhnikoviae radix (SR) is described in the Japanese Pharmacopoeia as a crude drug derived from the root of *Saposhnikovia divaricata* Schischkin (Umbelliferae). According to Flora of China, the root of *Peucedanum ledebourielloides* K. F. Fu is used as a regional substitute for SR. Therefore, we surveyed the botanical origin of the drug used in China, especially Shaanxi and the surrounding regions, through nucleotide sequence analysis of the internal transcribed spacer region of rDNA. As a result, several samples from Shaanxi（陝西）and Shanxi（山西）provinces were identified as *Peucedanum ledebourielloides*. To prevent this substitute from being distributed as genuine SR, we developed a thin-layer chromatography analysis condition to enable a specific compound of this species to be easily detected. The specific compound was identified as xanthalin, based on 1D- and 2D-NMR and high-resolution mass spectrometry data. The established TLC conditions were as follows-extraction solvent, *n*-hexane; applied volume, 5 μL; chromatographic support, silica gel; developing solvent, *n*-hexane:ethyl acetate:acetic acid (20:10:1); developing length, 7 cm; detection, UV (365 nm); R_f value, 0.4 (blue fluorescence; xanthalin).

Keywords: Saposhnikoviae radix, Shaanxi province, *Peucedanum ledebourielloides*

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Yoshitomi T, Oshima N[*1], Goto Y, Nakamori S[*2], Wakana D[*3], Anjiki N[*4], Sugimura K[*4], Kawano N[*4], Fuchino H[*4], Iida O[*4], Kagawa S[*5], Jinno H[*6], Kawahara N[*4], Kobayashi Y[*2], Maruyama T: Construction of prediction models for the transient receptor potential vanilloid subtype 1 (TRPV1)-stimulating activity of ginger and processed ginger Based on LC-HRMS data and PLS regression analyses.

J Agric Food Chem. 2017;65:3581-3588

To construct a model formula to evaluate the thermogenetic effect of ginger (*Zingiber officinale* Roscoe) from the ingredient information, we established transient receptor potential vanilloid subtype 1 (TRPV1)-stimulating activity prediction models by using a partial least-squares projections to latent structures (PLS) regression analysis in which the ingredient data from liquid chromatography–high-resolution mass spectrometry (LC-HRMS) and the stimulating activity values for TRPV1 receptor were used as explanatory and objective variables, respectively. By optimizing the peak extraction condition of the LC-HRMS data and the data preprocessing parameters of the PLS regression analysis, we succeeded in the construction of a TRPV1-stimulating activity prediction model with high precision ability. We then searched for the components responsible for the TRPV1-stimulating activity by analyzing the loading plot and s-plot of the model, and we identified [6]-gingerol (**1**) and hexahydrocurcumin

(**3**) as TRPV1-stimulating activity components.
Keywords: Ginger, TRPV1, PLS

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Uchikura T*, Tanaka H*, Sugiwaki H*, Yoshimura M*, Sato-Masumoto N, Tsujimoto T, Uchiyama N, Hakamatsuka T, Amakura Y*: Preliminary quality evaluation and characterization of phenolic constituents in Cynanchi Wilfordii Radix.
Molecules 2018;23:656

A new phenolic compound, 2-*O*-β-laminaribiosyl-4-hydroxyacetophenone (1), was isolated from Cynanchi Wilfordii Radix (CWR, the root of *Cynanchum wilfordii* Hemsley), along with 10 known aromatic compounds, including cynandione A (2), bungeisides-C (7) and –D (8), p-hydroxyacetophenone (9), 2',5'-dihydroxyacetophenone (10), and 2',4'-dihydroxyacetophenone (11). The structure of the new compound (1) was elucidated using spectroscopic methods and chemical methods. The structure of cynandione A (2), including a linkage mode of the biphenyl parts that remained uncertain, was unambiguously confirmed using the 2D ^{13}C–^{13}C incredible natural abundance double quantum transfer experiment (INADEQUATE) spectrum. Additionally, health issues related to the use of Cynanchi Auriculati Radix (CAR, the root of *Cynanchum auriculatum* Royle exWight) instead of CWR have emerged. Therefore, constituents present in methanolic extracts of commercially available CWRs and CARs were examined using UV-sensitive high-performance liquid chromatography (HPLC), resulting in common detection of three major peaks ascribed to cynandione A (2), *p*-hydroxyacetophenone (9), and 2',4'-dihydroxyacetophenone (11). Thus, to distinguish between these ingredients, a thin-layer chromatography (TLC) method, combined with only UV irradiation detection, focusing on wilfosides C1N (12) and K1N (13) as marker compounds characteristic of CAR, was performed. Furthermore, we propose this method as a simple and convenient strategy for the preliminary distinction of CWR and CAR to ensure the quality and safety of their crude drugs.
Keywords: *Cynanchum wilfordii,* phenolic glycoside, 2-*O*-β-laminaribiosyl-4-hydroxyacetophenone

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内山奈穂子，政田さやか，細江潤子，袴塚高志，合田幸広：定量NMRを利用した機能性関与成分の定量に用いる市販試薬の絶対純度.
食品化学学会誌 2017;24:125-130

The new system of Foods with Function Claims (FFCs) have been launched in April 2015. FFCs are foods submitted to the Secretary-General of the Consumer Affairs Agency as products whose labels bear function claims based on scientific evidence on food safety and effectiveness, under the responsibility of the manufacturers. More than 1,000 FFCs have been submitted as of November 2017. In a registered report to the Secretary-General, the manufacturers are required to describe in detail the analytical method for the quantitative determination of functional substances (FSs) to ensure the quality control of FFCs. Usually, the quantification of FSs are performed by HPLC analysis using commercial available laboratory grade agents as reference standards. Up to now, the purities of commercial available laboratory grade agents are also determined by an HPLC. Lately, a quantitative NMR (qNMR) have been developed as the absolute quantification method to determine the purities of organic compounds, including pure natural compounds, which are traceable to the International System of Units (SI). In this study, we determined the absolute purities of two commercial available laboratory grade agents, glabridin and cyanidin 3-*O*-glucoside, used for the quantification of FSs by ^{1}H-qNMR analysis. Glabridin is an ingredient of licorice, cyanidin 3-*O*-glucoside is one of the anthocyanins and an ingredient of bilberry and black currant etc. The absolute purities of glabridin and cyanidin 3-*O*-glucoside were 98.02% and 87.22%, respectively. It was revealed that their absolute purities determined by ^{1}H-qNMR analysis were lower than their purities estimated from area percentage of main peak under HPLC analysis (>99.7% and ≧99%, respectively).

The lower absolute purities of the two natural compounds by ^{1}H-qNMR analysis might be caused by contamination of impurities including water and solvents which are difficult to detect by HPLC. Therefore, the ^{1}H-qNMR analysis would be a reliable method for the accurate purity determination of the commercial reagents used as reference.

Keywords：定量NMR，グラブリジン，シアニジン-3-*O*-グルコシド

Malyshevskaya O*, Aritake K*, Kaushik MK*, Uchiyama N, Cherasse Y*, Kikura-Hanajiri R, Urade Y*: Natural（Δ^{9}-THC）and synthetic（JWH-018）cannabinoids induce seizures by acting through the cannabinoid CB1 receptor.

Scientific Reports 2017;7:10516

Natural cannabinoids and their synthetic substitutes are the most widely used recreational drugs. Numerous clinical cases describe acute toxic symptoms and neurological consequences following inhalation of the mixture of synthetic cannabinoids known as “Spice.” Here we report that an intraperitoneal administration of the natural cannabinoid Δ^{9}-tetrahydrocannabinol, one of the main constituent of marijuana, or the synthetic cannabinoid JWH-018 triggered electrographic seizures in mice, recorded by electroencephalography and videography. Pretreatment of mice with AM-251, a cannabinoid receptor 1-selective antagonist, completely prevented these cannabinoid-induced seizures. These data imply that abuse of cannabinoids can be dangerous and represents an emerging public health threat. Additionally, our data strongly suggest that AM-251 could be used as a crucial abortive therapy for cannabinoid-induced seizures or similar life-threatening conditions.

Keywords: cannabinoids, seizure, cannabinoid CB1 receptor

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内山奈穂子，鎌倉浩之，政田さやか，辻本恭，細江潤子，徳本廣子，丸山卓郎，合田幸広，袴塚高志：C型肝炎治療薬の偽造品に関する成分分析.

薬学雑誌 2017;137:1265-1276

In January 2017, counterfeits of the hepatitis C drug 'HARVONI® Combination Tablets' (HARVONI®) were found at a pharmacy chain through unlicensed suppliers in Japan. A total of five lots of counterfeit HARVONI® (samples 1–5) bottles were found, and the ingredients of the bottles were all in tablet form. Among them, two differently shaped tablets were present in two of the bottles (categorized as samples 2A, 2B, 4A, and 4B). We analyzed the total of seven samples by high-resolution LC-MS, GC-MS and NMR. In samples 2A, 3 and 4B, sofosbuvir, the active component of another hepatitis C drug, SOVARDI® Tablets 400 mg (SOVARDI®), was detected. In sample 4A, sofosbuvir and ledipasvir, the active components of HARVONI®, were found. A direct comparison of the four samples and genuine products showed that three samples (2A, 3, 4B) are apparently SOVARDI® and that sample 2A is HARVONI®. In samples 1 and 5, several vitamins but none of the active compounds usually found in HARVONI® (i.e., sofosbuvir and ledipasvir) were detected. Our additional investigation indicates that these two samples are likely to be a commercial vitamin supplement distributed in Japan. Sample 2B, looked entirely different from HARVONI® and contained several herbal constitutents (such as ephedrine and glycyrrhizin) that are used in Japanese Kampo formulations. A further analysis indicated that sample 2B is likely to be a Kampo extract tablet of Shoseiryuto which is distributed in Japan. Considering this case, it is important to be vigilant to prevent a recurrence of distribution of counterfeit drugs.

Keywords: counterfeit drug, hepatitis C drug, LC-MS

Oshima N*[1,6], Maruyama T, Yamashita T*[2], Uchiyama N, Amakura Y*[3], Hyuga S*[4], Hyuga M, Hakamatsuka T, Odaguchi H*[4], Hanawa T*[4], Goda Y: Two flavone *C*-glycosides as quality control markers for the manufacturing process of ephedrine alkaloids-free Ephedra Herb extract (EFE) as a crude drug preparation.

J Nat Med. 2018;72:73-79

As part of our continuing study of ephedrine alkaloids-free Ephedra Herb extract (EFE) in pursuit of its approval as a crude drug preparation, we identified two quantitative markers for the quality control of the manufacturing process of EFE and sought to establish cost-effective and simple methods for quantitative analyses. We analysed Ephedra Herb

extracts grown in different habitats and collection years by liquid chromatography/high-resolution mass spectrometry (LC/HRMS) and detected two notable peaks common to each extract. These peaks were identified as vicenin-2 (**1**) and isovitexin 2"-*O*-rhamnoside (**2**). Quantitative analyses using the isocratic condition of LC/MS showed that the content percentages of **1** and **2** in EFE were 0.140–0.146% and 0.350–0.411%, respectively. We concluded that **1** and **2** were adequate quality control markers for quantitative analysis of EFE. Furthermore, we quantitatively analysed apigenin (**3**), an aglycon common to **1** and **2**, and found that the conversion factors of **1** to **3** and **2** to **3** were 1.3 and 1.5, respectively. Therefore, we concluded that **3** was a secondary standard for quantifying the contents of **1** and **2** in EFE. A series of results obtained from this study will be valuable for the quality control of EFE.

Keywords: Ephedra Herb, ephedrine alkaloids-free Ephedra Herb extract, quality control markers

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Sato-Masumoto N, Uchikura T*1, Sugiwaki H*1, Yoshimura M*1, Masada S, Atsumi T*2, Watanabe M*3, Tanaka N*4, Uchiyama N, Amakura Y*1, Hakamatsuka T: Survey on the original plant species of crude drugs distributed as Cynanchi Wilfordii Radix and its related crude drugs in the Korean and Chinese markets.

Biol Pharm Bull. 2017;40:1693-1699

Cynanchi Wilfordii Radix (CWR) is used in Korea as a substitute for Polygoni Multiflori Radix (PMR), which is a crude drug traditionally used in East Asian countries. Recently, the use of Cynanchi Auriculati Radix (CAR) in place of PMR and CWR has emerged a major concern in the Korean market. In Japan, PMR is permitted to be distributed as a pharmaceutical regulated by the Japanese Pharmacopoeia 17th edition (JP17). Although CWR and CAR have not traditionally been used as medicines, CWR was recently introduced as a health food. The distribution of unfamiliar CWR-containing products could lead to the misuse of original species for PMR and CWR like in Korea. To prevent this situation, the original species of plant products distributed as PMR, CWR, and CAR in the Korean and Chinese markets were surveyed and identified by their genes and components. The results revealed that all two PMR in the Korean market were misapplied as CAR, and that CAR was incorrectly used in eight of thirteen products distributed as CWR in both markets. As PMR is strictly controlled by JP17, the risk of mistaking PMR for CWR and CAR would be low in Japan. In contrast, the less stringent regulation of health food products and the present situation of misidentification of CWR in the Korean and Chinese markets could lead to unexpected health hazards. To ensure the quality and safety of crude drugs, it is important to use the information about the genes and components of these crude drugs.

Keywords: *Cynanchum wilfordii, Cynanchum auriculatum, Polygonum multiflorum*

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Nose M*1, Tada M*1, Kojima R*1, Nagata K*1, Hisaka S*1, Masada S, Homma M*2, Hakamatsuka T: Comparison of glycyrrhizin content in 25 major kinds of Kampo extracts containing Glycyrrhizae Radix used clinically in Japan.

J Nat Med. 2017;71:711-722

To confirm the basis of the safety regulation, in this study we comprehensively determined the glycyrrhizin (GL) content of 25 major kinds of Kampo extracts compounding Glycyrrhizae Radix. We found that Schisandrae Fructus in Sho-seiryu-To decoction caused

a lowered pH condition and drastically decreased the extraction efficacy of GL from Glycyrrhizae Radix. Moreover, we were able to confirm that the extraction efficiency of GL from Glycyrrhizae Radix is dependent on the pH value of the extraction solvent. Furthermore, the GL contents are well correlated with pseudoaldosteronism incidence data obtained from the Japanese Adverse Drug Event Report database on the 25 kinds of Kampo extracts. This suggests that the GL content is a better index to consider to avoid the adverse effects of Glycyrrhizae Radix-containing Kampo formulas.

Keywords: Glycyrrhizin, Kampo extracts, pH

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Masada S, Uchiyama N, Hakamatsuka T: An analysis of isoflavones in “Foods with Functional Claims” containing *Puerariae thomsonii* flower extract.
日本食品化学学会誌 2018;25:39-44

We evaluated the amount of isoflavones in 8 FFCs of which functional components are *Puerariae thomsonii* flower-derived isoflavones (as tectorigenin derivatives). These FFCs contained tectorigenin derivatives (sum of tectorigenin 7-*O*-(6-*O*-xylosylglucoside), tectoridin, and tectorigenin), corresponding to 106 ~ 117% of the labeled values within a small margin of error. This result indicated that the tested FFCs have as high quality as herbal medicinal products for the chemical component level. Additionally, the aglycone content (tectorigenin equivalents) in 8 FFCs ranged from 25 to 31 mg/day. Since tectorigenin derivatives is thought to have strong estrogenic effects even though their binding affinities for estrogen receptors are weak, further investigation and discussion would be needed for ensuring the efficacy and safety of FFCs containing *Puerariae thomsonii* flower-derived isoflavones.

Keywords: *Puerariae thomsonii* flower extract, tectorigenin, foods with functional claims

Kawahara G*, Maeda H*, Kikura-Hanajiri R, Yoshida K*, Hayashi YK*: The psychoactive drug 25B-NBOMe recapitulates rhabdomyolysis in zebrafish larvae.
Forensic Toxicology 2017;35:369-375

N-Benzyl-substituted 2C class phenethylamines (NBOMes) are psychoactive designer drugs, with strong hallucinogenic and stimulant effects, even at low doses. The designer drug, 2-(4-bromo-2, 5-dimethoxyphenyl)-*N*-(2-methoxybenzyl) ethanamine (25B-NBOMe) is considered to be one of the most potent agonists of the serotonin-2A (5-HT_{2A}) receptor. Recently, we reported the first lethal case of 25B-NBOMe intoxication with severe rhabdomyolysis, concluded by clinical, pathological and toxicological analyses. There are currently no good animal models that closely recapitulate serotonin receptor-dependent rhabdomyolysis. In the present study, we created animal models of rhabdomyolysis using zebrafish larvae to study the pathomechanism of rhabdomyolysis, and demonstrated that 25B-NBOMe can simulate lethal rhabdomyolysis in this animal. Treatment of the larvae with 25B-NBOMe decreased their survival rate, locomotion, altered birefringence of the skeletal muscle and immunostainings for dystroglycan (a myoseptal protein) and myosin heavy chain (a myofibril protein), which were consistent with rhabdomyolysis. This 25B-NBOMe-induced rhabdomyolysis was inhibited by the 5-HT_{2A} receptor antagonists ritanserin and aripirazole, but not by the 5-HT_{1A} + 5-HT_{1B} receptor antagonist propranolol and the 5-HT_3 receptor antagonist granisetron, indicating 5-HT_{2A}-dependent rhabdomyolysis. The 25B-NBOMe-treated zebrafish is, therefore, a highly useful model of rhabdomyolysis for studying the pathomechanism of rhabdomyolysis as well as for therapeutic drug screening.

Keywords: 25B-NBOMe intoxication, 5-HT_{2A} receptor, zebrafish larvae

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Hashimoto T*1,2, Hanajiri R, Yasuda N*1, Nakamura Y*1, Mizuno N*1, Honda S*1, Hayakawa S*1,3, Nishiwaki Y*1,4, Kimura S*1: Single-crystal structure analysis of designer drugs circulating in the Japanese drug market by the synchrotron radiation X-ray diffraction.
Powder Diffraction 2017;32:112-117

Over the past 20 years, many designer drugs derived from controlled substances have been widely distributed as easily available psychoactive substances and have become a serious problem in

Japan. In order to determine the absolute structures of four new designer drugs derived from medicines (methylphenidate and phenmetrazine) X-ray single-crystal structure analyses were performed using the BL26B1 beamline of synchrotron radiation facility SPring-8. The results show that the molecular configuration of these designer drugs (having two asymmetric carbons), which were distributed in the illegal drug market had threo-forms as found for methylphenidate and phenmetrazine.

Keywords: synchrotron XRD, designer drugs

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Maeda H*, Kikura-Hanajiri R, Kawamura M*, Nagashima E*, Yoshida K*: AB-CHMINACA induced sudden death from non-cardiogenic pulmonary edema.

Clinical Toxicology 2018;56:143-145

Despite widespread use of diverse synthetic cannabinoid (sCB) compounds, the pathophysiology associated with intoxication with many sCB compounds, including AB-CHMINACA, is poorly understood, as is their metabolism and distribution into blood and organs. A young man died shortly after ingesting an herb product containing sCB compounds. Toxicological analyses of blood samples revealed high levels of AB-CHMINACA (7.61 ± 0.59 ng/mL) and its metabolites (M2, 56.73 ± 4.16 ng/mL; M4, 2.29 ± 0.14 ng/mL) and trace amounts of 5-fluoro-AMB, FUB-PB-22, and AB-FUBINACA. The autopsy revealed severe pulmonary edema, and histology showed air bubbles in the alveolar effusion, suggesting rapid progression of edema. Low blood levels of *N*-terminal pro-brain natriuretic peptide excluded cardiogenic pulmonary edema. Histological examination revealed diffuse neuronal (brain) and myocardial (sub-endocardial) hyper-eosinophilia, indicating hypoxic encephalopathy and systemic hypoxemia, respectively. The findings show that AB-CHMINACA induced rapid progression of pulmonary edema resulting in hypoxic encephalopathy and systemic hypoxemia, possibly through severe seizures. The high blood ratio of the M2 metabolite to the parent compound, AB-CHMINACA, demonstrates rapid metabolism. This highlights the usefulness of quantification of M2 in diagnosing AB-CHMINACA intoxication.

Keywords: synthetic cannabinoid, pulmonary edema, sudden death

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Kitajima M*1, Yanagisawa T*1, Tsukahara M*1, Yamaguchi Y*1, Kogure N*1, Kikura-Hanajiri R, Goda Y, Iida O*2, Sugimura Y*2, Kawahara N*2, Takayama H*1: Biphenyl ether and biphenyl quinolizidine lactone alkaloids from *Heimia salicifolia*.

Tetrahedron 2018;74:441-452

Three new biphenyl ether quinolizidine lactone alkaloids and 13 new biphenyl quinolizidine lactone alkaloids were isolated from Heimia salicifolia (Lythraceae) together with seven known alkaloids. Their structures were determined by spectroscopic analyses and chemical conversions.

Keywords: alkaloid, quinolizidine, *Heimia salicifolia*

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Minakata K*, Hasegawa K*, Yamagishi I*, Nozawa H*, Kikura-Hanajiri R, Suzuki M*, Kitamoto T*, Suzuki O*, Watanabe K* : Sensitive quantification of 5F-PB-22 and its three metabolites 5F-PB-22 3-carboxyindole, PB-22 *N*-5-hydroxypentyl and PB-22 *N*-pentanoic acid in authentic urine specimens obtained from four individuals by liquid chromatography-tandem mass spectrometry.

Forensic Toxicol. 2018;36:151–159

Urine is the most suitable specimen to collect from individuals because of noninvasiveness and relatively large volumes obtainable. In authentic urine specimens, however, synthetic cannabinoids having the structures of quinolinyl ester indoles, such as 5F-PB-22, PB-22 and BB-22, in unchanged forms as well as their metabolites, have not been quantified yet. Therefore, the aim of this study was to establish a sensitive analytical method for the quantification of 5F-PB-22 and its three metabolites 5F-PB-22 3-carboxyindole, PB-22 *N*-5-hydroxypentyl and PB-22 *N*-pentanoic acid in authentic urine samples in four cases. These compounds were extracted from

β-glucuronide-hydrolyzed and unhydrolyzed urine via liquid-liquid extraction. The identification and quantification were performed using the QTRAP type of a liquid chromatography-tandem mass spectrometer. The limits of detection were 3-30 pg/mL and their summed quantitation range was 10-10,000 pg/mL. The devised method was applied to quantify these compounds in authentic urine specimens obtained from four individuals. The levels of 5F-PB-22 were 5.1, 13.6, 94.7 and 470 pg/mL; those of 5F-PB-22 3-carboxyindole were 8.25, 3.39, 23.2 and 880 ng/mL; and those of PB-22 *N*-pentanoic acid were 12.0, 57.4, 959 and 2090 pg/mL, respectively, in four unhydrolyzed urine samples, and the levels of PB-22 *N*-5-hydroxypentyl could be quantified as 29.9 and 131 pg/mL in two unhydrolyzed urine samples. The 5F-PB-22 and its metabolites PB-22 *N*-5-hydroxypentyl have been detected from authentic human urine samples for the first time. Also, this is the first report dealing with the quantification of the three metabolites in human urine samples.

Keywords: 5F-PB-22, in vivo metabolites in urine, QTRAP mass spectrometry

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Kuroda T, Yasuda S, Nakashima H, Takada N, Matsuyama S, Kusakawa S, Umezawa A*1, Matsuyama A*2, Kawamata S*3, Sato Y: Identification of a Gene Encoding Slow Skeletal Muscle Troponin T as a Novel Marker for Immortalization of Retinal Pigment Epithelial Cells.
Scientific Reports. 2017;7:8163-8174

Human pluripotent stem cells (hPSCs) are leading candidate raw materials for cell-based therapeutic products (CTPs). In the development of hPSC-derived CTPs, it is imperative to ensure that they do not form tumors after transplantation for safety reasons. Because cellular immortalization is a landmark of malignant transformation and a common feature of cancer cells, we aimed to develop an *in vitro* assay for detecting immortalized cells in CTPs. We employed retinal pigment epithelial (RPE) cells as a model of hPSC-derived products and identified a gene encoding slow skeletal muscle troponin T (*TNNT1*) as a novel marker of immortalized RPE cells by comprehensive microarray analysis. *TNNT1* mRNA was commonly upregulated in immortalized RPE cells and human induced pluripotent stem cells (hiPSCs), which have self-renewal ability. Additionally, we demonstrated that *TNNT1* mRNA expression is higher in several cancer tissues than in normal tissues. Furthermore, stable expression of *TNNT1* in ARPE-19 cells affected actin filament organization and enhanced their migration ability. Finally, we established a simple and rapid qRT-PCR assay targeting *TNNT1* transcripts that detected as low as 3% of ARPE-19 cells contained in normal primary RPE cells. Purified hiPSC-derived RPE cells showed *TNNT1* expression levels below the detection limit determined with primary RPE cells. Our qRT-PCR method is expected to greatly contribute to process validation and quality control of CTPs.

Keywords: hiPSCs, retinal pigment epithelial, immortalization

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Ohtsuki S*, Takahashi Y*, Inoue T, Takakura Y*, Nishikawa M*: Reconstruction of Toll-like receptor 9-mediated responses in HEK-Blue hTLR9 cells by transfection of human macrophage scavenger receptor 1 gene.
Scientific Reports, 2017:7,13661.

We used human Toll-like receptor 9 (hTLR9)-expressing HEK-Blue hTLR9 cells, which release secreted embryonic alkaline phosphatase (SEAP) upon response to CpG DNA, to evaluate the immunological properties of nucleic acid drug candidates. Our preliminary studies showed that phosphodiester CpG DNA hardly induced any SEAP secretion in HEK-Blue hTLR9 cells. In the current study, therefore, we developed HEK-Blue hTLR9 cells transduced with human macrophage scavenger receptor-1 (hMSR1), a cell-surface DNA receptor, and determined whether HEK-Blue hTLR9/hMSR1 cells respond to phosphorothioate (PS) CpG DNA and phosphodiester (PO) CpG DNA. We selected PS CpG2006, a single-stranded PO CpG DNA (ssCpG),

and a tetrapod-like structured DNA (tetrapodna) containing ssCpG (tetraCpG) as model TLR9 ligands. Alexa Fluor 488-labeled ligands were used for flow cytometry. Unlike the mock-transfected HEK-Blue hTLR9 cells, the HEK-Blue hTLR9/hMSR1 cells efficiently took up all three CpG DNAs. SEAP release was almost proportional to the uptake. Treatment of HEK-Blue hTLR9/hMSR1 cells with an anti-hMSR1 antibody significantly reduced the uptake of ssCpG and tetraCpG. Collectively, reconstruction of TLR9-mediated responses to CpG DNA in HEK-Blue hTLR9 cells can be used to evaluate the toxicity of nucleic acid drug candidates with diverse physicochemical properties.

Keywords: hMSR1, hTLR9, CpG DNA

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Shibata N, Nagai K*, Morita Y*, Ujikawa O*, Ohoka N, Hattori T, Koyama R*, Sano O*, Imaeda Y*, Nara H*, Cho N*, Naito M: Development of Protein Degradation Inducers of Androgen Receptor by Conjugation of Androgen Receptor Ligands and Inhibitor of Apoptosis Protein Ligands.

J Med Chem 2018;61:543-75.

Targeted protein degradation using small molecules is a novel strategy for drug development. We have developed hybrid molecules named specific and nongenetic inhibitor of apoptosis protein [IAP] -dependent protein erasers (SNIPERs) that recruit IAP ubiquitin ligases to degrade target proteins. Here, we show novel SNIPERs capable of inducing proteasomal degradation of the androgen receptor (AR). Through derivatization of the SNIPER(AR) molecule at the AR ligand and IAP ligand and linker, we developed 42a (SNIPER(AR)-51), which shows effective protein knockdown activity against AR. Consistent with the degradation of the AR protein, 42a inhibits AR-mediated gene expression and proliferation of androgen-dependent prostate cancer cells. In addition, 42a efficiently induces caspase activation and apoptosis in prostate cancer cells, which was not observed in the cells treated with AR antagonists. These results suggest that SNIPER(AR)s could be leads for an anticancer drug against prostate cancers that exhibit AR-dependent proliferation.

Keywords: androgen receptor, cIAP1, ubiquitin-proteasome system

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Shimasaki K*[1], Watanabe-Takahashi M*[1], Umeda M*[2], Funamoto S*[1], Saito Y*[1], Noguchi N*[1], Kumagai K*[3], Hanada K*[3], Tsukahara F*[4], Maru Y*[4], Shibata N, Naito M, Nishikawa K*[1]: Pleckstrin homology domain of p210 BCR-ABL interacts with cardiolipin to regulate its mitochondrial translocation and subsequent mitophagy.

Genes Cells. 2018;23:22-34.

Chronic myeloid leukemia (CML) is caused by the chimeric protein p210 BCR-ABL encoded by a gene on the Philadelphia chromosome. Although the kinase domain of p210 BCR-ABL is an active driver of CML, the pathological role of its pleckstrin homology (PH) domain remains unclear. Here, we carried out phospholipid vesicle-binding assays to show that cardiolipin (CL), a characteristic mitochondrial phospholipid, is a unique ligand of the PH domain. Arg726, a basic amino acid in the ligand-binding region, was crucial for ligand recognition. A subset of wild-type p210 BCR-ABL that was transiently expressed in HEK293 cells was dramatically translocated from the cytosol to mitochondria in response to carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment, which induces mitochondrial depolarization and subsequent externalization of CL to the organelle's outer membrane, whereas an R726A mutant of the protein was not translocated. Furthermore, only wild-type p210 BCR-ABL, but not the R726A mutant, suppressed CCCP-induced mitophagy and subsequently enhanced reactive oxygen species production. Thus, p210 BCR-ABL can change its intracellular localization via interactions between the PH domain and CL to cope with mitochondrial damage. This suggests that p210 BCR-ABL could have beneficial effects for cancer proliferation, providing new insight into the PH domain′s contribution to CML pathogenesis.

Keywords: BCR-ABL, pleckstrin homology domain, cardiolipin

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Shimokawa K*, Shibata N, Sameshima T*, Miyamoto N*, Ujikawa O*, Nara H*, Ohoka N, Hattori T, Cho N*, Naito M: Targeting the Allosteric Site of Oncoprotein BCR-ABL as an Alternative Strategy for Effective Target Protein Degradation.
ACS Med. Chem. Lett. 2017;8:1042-7.

Protein degradation technology based on hybrid small molecules is an emerging drug modality that has significant potential in drug discovery and as a unique method of post-translational protein knockdown in the field of chemical biology. Here, we report the first example of a novel and potent protein degradation inducer that binds to an allosteric site of the oncogenic BCR-ABL protein. BCR-ABL allosteric ligands were incorporated into the SNIPER (Specific and Nongenetic inhibitor of apoptosis protein [IAP]-dependent Protein Erasers) platform, and a series of *in vitro* biological assays of binding affinity, target protein modulation, signal transduction, and growth inhibition were carried out. One of the designed compounds, 6 (SNIPER(ABL)-062), showed desirable binding affinities against ABL1, cIAP1/2, and XIAP and consequently caused potent BCR-ABL degradation.
Keywords: BCR-ABL, allosteric site, ubiquitin-proteasome system

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Shibata N, Miyamoto N*, Nagai K*, Shimokawa K*, Sameshima T*, Ohoka N, Hattori T, Imaeda Y*, Nara H*, Cho N*, Naito M: Development of protein degradation inducers of oncogenic BCR-ABL protein by conjugation of ABL kinase inhibitors and IAP ligands.
Cancer Sci. 2017;108:1657-66.

Chromosomal translocation occurs in some cancer cells, which results in the expression of aberrant oncogenic fusion proteins that include BCR-ABL in chronic myelogenous leukemia (CML). Inhibitors of ABL tyrosine kinase, such as imatinib and dasatinib, exhibit remarkable therapeutic effects, although emergence of drug resistance hampers the therapy during long-term treatment. An alternative approach to treat CML is to downregulate the BCR-ABL protein. We have devised a protein knockdown system by hybrid molecules named Specific and Non-genetic inhibitor of apoptosis protein [IAP]-dependent Protein Erasers (SNIPER), which is designed to induce IAP-mediated ubiquitylation and proteasomal degradation of target proteins, and a couple of SNIPER(ABL) against BCR-ABL protein have been developed recently. In this study, we tested various combinations of ABL inhibitors and IAP ligands, and the linker was optimized for protein knockdown activity of SNIPER (ABL). The resulting SNIPER(ABL)-39, in which dasatinib is conjugated to an IAP ligand LCL161 derivative by polyethylene glycol (PEG) ×3 linker, shows a potent activity to degrade the BCR-ABL protein. Mechanistic analysis suggested that both cellular inhibitor of apoptosis protein 1 (cIAP1) and X-linked inhibitor of apoptosis protein (XIAP) play a role in the degradation of BCR-ABL protein. Consistent with the degradation of BCR-ABL protein, the SNIPER (ABL)-39 inhibited the phosphorylation of signal transducer and activator of transcription 5 (STAT5) and Crk like proto-oncogene (CrkL), and suppressed the growth of BCR-ABL-positive CML cells. These results suggest that SNIPER(ABL)-39 could be a candidate for a degradation-based novel anti-cancer drug against BCR-ABL-positive CML.
Keywords: BCR-ABL, dasatinib, ubiquitin-proteasome system

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Hattori T, Okitsu K, Yamzaki N, Ohoka N, Shibata N, Misawa T, Kurihara M, Demizu Y, Naito M: Simple and efficient knockdown of His-tagged proteins by ternary molecules consisting of a His-tag ligand, a ubiquitin ligase ligand, and a cell-penetrating peptide.
Bioorg Med Chem Lett. 2017;27:4478-81.

We designed and synthesized hybrid molecules for a protein knockdown method based on the recognition of a His-tag fused to a protein of interest (POI). The synthesized target protein degradation inducers contained three functional moieties: a His-tag ligand (nickel nitrilotriacetic acid [Ni-NTA]), an E3 ligand (bestatin [BS] or MV1), and a carrier peptide (Tat or nonaarginine [R9]). The designed hybrid molecules, BS-Tat-Ni-NTA, MV1-Tat-Ni-NTA, BS-R9-Ni-NTA, and MV1-R9-Ni-NTA, efficiently degraded His-tagged cellular retinoic acid binding protein 2 via the ubiquitin–proteasome system (UPS). This system will become a useful tool for research into selective protein

degradation inducers that act via the UPS.
Keywords: His-tag, Ni-NTA, ubiquitin–proteasome system

Inoue Y*, Kawachi S*, Ohkubo T*, Nagasaka M*, Ito S*, Fukuura K*, Itoh Y*, Ohoka N, Morishita D*, Hayashi H*: The CDK inhibitor p21 is a novel target gene of ATF4 and contributes to cell survival under ER stress.
FEBS Lett. 2017;591:3682-91.

Activating transcription factor 4 (ATF4) is well known for its role in the endoplasmic reticulum (ER) stress response. ATF4 also transcriptionally induces multiple effectors that determine cell fate depending on cellular context. In addition, ATF4 can communicate both pro-apoptotic and pro-survival signals. How ATF4 mediates its prosurvival roles, however, requires further investigation. Here, we report that the CDK inhibitor p21 is a novel target gene of ATF4. We identified two ATF4-responsive elements, one of which directly binds ATF4, within the first intron of the p21 gene. Importantly, overexpression of p21 enhances cell survival following ER stress induction, while p21 knockdown increases cell death. These results suggest that p21 induction plays a vital role in the cellular response to ER stress and indicate that p21 is a prosurvival effector of ATF4.
Keywords: ATF4, ER stress, p21

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Ohoka N, Misawa T, Kurihara M, Demizu Y, Naito M: Development of a peptide-based inducer of protein degradation targeting NOTCH1.
Bioorg Med Chem Lett. 2017;27:4985-8.

We previously developed a protein knockdown system by small-molecule hybrid compounds named SNIPERs (Specific and Nongenetic IAP-dependent Protein Erasers). Here we report a peptide-based protein knockdown system for inducing degradation of a transcriptional factor NOTCH1. The molecules designed were composed of two biologically active scaffolds: a peptide that binds to the surface of the target protein NOTCH1 and a small-molecule MV1 that binds to the E3 ubiquitin ligase inhibitor of apoptosis protein (IAP), which are expected to cross-link these proteins in cells. Hybrid molecules specifically induced the degradation of the NOTCH1 protein by the proteasome. This system could be a useful method to develop various degradation inducers against a large number of proteins to which small-molecule ligands have not been found.
Keywords: NOTCH1, proteasome, ubiquitin

Ohoka N, Morita Y*1, Nagai K*1, Shimokawa K*1, Ujikawa O*1, Fujimori I*1, Ito M*1, Hayase Y*1, Okuhira K*2, Shibata N, Hattori T, Sameshima T*1, Sano O*1, Koyama R*1, Imaeda Y*1, Nara H*1, Cho N*1, Naito M: Derivatization of inhibitor of apoptosis protein (IAP) ligands yields improved inducers of estrogen receptor α degradation.
J Biol Chem. 2018;293:6776-90.

Aberrant expression of proteins often underlies many diseases, including cancer. A recently developed approach in drug development is small molecule-mediated, selective degradation of dysregulated proteins. We have devised a protein-knockdown system that utilizes chimeric molecules termed specific and nongenetic IAP-dependent protein erasers (SNIPERs) to induce ubiquitylation and proteasomal degradation of various target proteins. SNIPER(ER)-87 consists of an inhibitor of apoptosis protein (IAP) ligand LCL161 derivative that is conjugated to the estrogen receptor α (ERα) ligand 4-hydroxytamoxifen by a PEG linker, and we have previously reported that this SNIPER efficiently degrades the ERα protein. Here, we report that derivatization of the IAP ligand module yields SNIPER(ER)s with superior protein-knockdown activity. These improved SNIPER(ER)s exhibited higher binding affinities to IAPs and induced more potent degradation of ERα than does SNIPER(ER)-87. Further, they induced simultaneous degradation of cellular inhibitor of apoptosis protein 1 (cIAP1) and delayed degradation of X-linked IAP (XIAP). Notably, these reengineered SNIPER(ER)s efficiently induced apoptosis in MCF-7 human breast cancer cells that require IAPs for continued cellular survival. We found that one of these molecules, SNIPER(ER)-110, inhibits the growth of MCF-7 tumor xenografts in mice more potently than the previously characterized SNIPER(ER)-87. Mechanistic analysis revealed that our novel SNIPER(ER)s preferentially recruit XIAP, rather than cIAP1, to degrade ERα. Our results suggest that derivatized IAP ligands could facilitate

further development of SNIPERs with potent protein-knockdown and cytocidal activities against cancer cells requiring IAPs for survival.
Keywords: ERalpha, protein knockdown, proteasome

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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 2017;105:1514-24.

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., Nomura, Y., Fukui, C., Kawakami, T., Ikeda, T.*1, Mukai, T.*2, Yuba, T.*2, Inamura, K.*2, Yamaoka, H.*2, Miyazaki, K.*3, Okazaki, H.*1, Haishima, Y.: Pilot study on novel blood containers with alternative plasticizers for red cell concentrate storage.
PLOS ONE 2017;12:e0185737.

A concern for the safety of Di (2-ethylhexyl) phthalate (DEHP) on human health has led to the development of alternative plasticizers. We showed that two types of non-DEHP blood containers: polyvinyl chloride (PVC) blood bags containing di-isononyl-cyclohexane-1,2-dicarboxylate (DINCH) and di (2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTH), or 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester (DL9TH) and DOTH, demonstrated the same quality of red cell concentrates storing as the DEHP blood containers. Since DOTH, DINCH, and DL9TH were reported to be safe, DOTH/DINCH and DOTH/DL9TH blood containers are promising candidate substitutes for DEHP blood containers.
Keywords: di (2-ethylhexyl) phthalate, alternative plasticizer, blood container

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Nomura, Y., Hasegawa, C., Morishita, Y., Haishima, Y.: A biological study establishing the endotoxin limit for in vitro proliferation of human mesenchymal stem cells.
Regenerative Therapy 2017;7:45-51.

Human multipotent mesenchymal stem cells (hMSCs) have applications in tissue engineering, cell-based therapy, and medical devices. Although endotoxin testing is a requirement for evaluating the quality and safety of transplanted MSCs, there have been no reports on the dose response to endotoxins to establish limits for in vitro MSC culture systems. The present study aimed to accurately quantify the risk of endotoxin contamination in cell culture systems in order to establish the acceptable endotoxin limit for hMSC proliferation.
Keywords: endotoxin limit, regenerative medicine product, proliferative capacity

Nomura, Y., Yamazaki, K.*1, Amano, R.*2, Takada, K.*2, Nagata, T.*3, Kobayashi, N.*4, Tanaka, Y.*5, Fukunaga, J.*5, Katahira, M.*3, Kozu, T.*5, Nakamura, Y.*6,7, Haishima, Y., Torigoe, H.*1, Sakamoto, T.*2: Conjugation of two RNA aptamers improves binding affinity to AML1 Runt domain.
Journal of Biochemistry 2017;162:431-436.

The newly designed aptamer Apt14 was generated by the conjugation of two RNA aptamers (Apt1 and Apt4) obtained by SELEX against AML1 Runt domain, resulting in improvement in its binding performance. The residues of AML1 Runt domain in contact with Apt14 were predicted in silico and confirmed by mutation and NMR analyses. Conjugation of two aptamers that bind to different sites of the target protein is a facile and robust strategy to develop an aptamer with higher performance.
Keywords: aptamer, design, structure

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Takada, K.*1, Amano, R.*1, Nomura, Y., Tanaka, Y.*2, Sugiyama, S.*3, Nagata, T.*4, Katahira, M.*4, Nakamura, Y.*5,6, Kozu, T.*7, Sakamoto, T.*1: Characterisation of an aptamer against Runt domain of AML1 (RUNX1) by NMR and mutational analyses.

FEBS Open Bio 2018;8:264–270.

The aptamers have been reported that can bind to a wide range of target molecules with high affinity and specificity. Previously, we reported an RNA aptamer that shows high affinity to the Runt domain (RD) of the AML1 protein, a transcription factor with roles in haematopoiesis and immune function. In this study, we identified the secondary structure by nuclear magnetic resonance spectroscopy and performed a mutational study to reveal the residue critical for binding to the RD. It was suggested that the large contact area was formed by a DNA-mimicking motif and a multibranched loop, which confers the high affinity and specificity of binding.

Keywords: AML1, mutation, aptamer

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Miyajima A, Sakemi-Hoshikawa K, Usami M, Mitsunaga K*1, Irie T, Ohno Y*2, Sunouchi M: Thyrotoxic rubber antioxidants, 2-mercaptobenzimidazole and its methyl derivatives, cause both inhibition and induction of drug-metabolizing activity in rat liver microsomes after repeated oral administration. *Biochemical and Biophysical Research Communications*. 2017;492:116–20.

We examined the effects of thyrotoxic rubber antioxidants, 2-mercaptobenzimidazole (MBI, 0.3 mmol/kg/day) and its methyl derivatives, methyl-MBIs [4-methyl-MBI (4-MeMBI, 0.6 mmol/kg/day), 5-methyl-MBI (5-MeMBI, 0.6 mmol/kg/day), and 4 (or 5)-methyl-MBI (4(5)-MeMBI, 0.6 or 1.2 mmol/kg/day)], on the drug-metabolizing activity in male rat liver microsomes by 8-day repeated oral administration. The weight of liver and thyroid were increased by all the test chemicals; MBI was most potent, and there was no additive or synergistic effect between 4-MeMBI and 5-MeMBI. MBI decreased the cytochrome P450 (CYP) content, NADPH-cytochrome P450 reductase (POR) activity, 7-ethoxycoumarin O-deethylation (ECOD) activity, and flavin-containing monooxygenase (FMO) activity, but increased the 7-pentoxyresorufin O-dependentylation (PROD) activity, suggesting inhibition of the drug-metabolizing activity on the whole but induce some activities such as the CYP2B activity. On the contrary, all the methyl-MBIs increased the CYP content, CYB5 content, ECOD activity, 7-ethoxyresorufin O-deethylation (EROD) activity, and PROD activity, indicating that they are mostly inducible of the CYP activity. However, the methyl-MBIs decreased the FMO activity, and 5-MeMBI and 4(5)-MeMBI appeared inhibitory for CYPs 2C11 and 2C13. Between 4-MeMBI and 5-MeMBI, there was no additive or synergistic effect on the drug-metabolizing activity, but was counteraction. It was concluded that MBI and methyl-MBIs had both inhibitory and inducible effects on the drug-metabolizing activity in rat liver microsomes at thyrotoxic doses. The effects of 4(5)-MeMBI indicated that the increased liver weight alone can be a hepatotoxic sign but not an adaptive no-adverse response in toxicity studies. The present results were related to the toxicokinetic profiles of MBI and 4(5)-MeMBI in the repeated toxicity studies.

Keywords: benzimidazole, cytochrome P450, drug-metabolizing activity

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De Jong WH*1, Hoffmann S*2, Lee M*3, Kandárová H*3, Pellevoisin C*5, Haishima Y, Rollins B*6, Zdawczyk A*7, Willoughby J*8, Bachelor M*9, Schatz T*10, Skoog S*11, Parker S*12, Sawyer A*13, Pescio

P[*14], Fant K[*15], Kim KM[*16], Kwon JS[*16], Gehrke H[*17], Hofman-Hüther H[*18], Meloni M[*18], Julius C[*19], Briotet D[*20], Letasiova S[*4], Kato R, Miyajima A, De La Fonteyne LJJ[*21], Videau C[*5], Tornier C[*5], Turley AP[*3], Christiano N[*22], Rollins TS[*3], Coleman KP[*23]: Round Robin study to evaluate the Reconstructed Human Epidermis（RhE）model as an in vitro skin irritation test for detection of irritant activity in medical device extracts.

Toxicol In Vitro, 2018 doi: 10.1016/j.tiv.2018.01.001.

Assessment of skin irritation is an essential component of the safety evaluation of medical devices. OECD Test Guideline 439 describes the use of reconstructed human epidermis(RhE) as an in vitro test system for classification of skin irritation by neat chemicals. An international round robin study was conducted to evaluate the RhE method for determination of skin irritant potential of medical device extracts. Four irritant polymers and three non-irritant controls were obtained or developed that had demonstrated their suitability to act as positive or negative test samples. The RhE tissues (EpiDerm™ and SkinEthic™ RHE) were dosed with 100 µL aliquots of either saline or sesame oil extract. Incubation times were 18h (EpiDerm™) and 24h (SkinEthic™ RHE). Cell viability reduction > 50% was indicative of skin irritation. Both the EpiDerm™ and SkinEthic™ RHE tissues were able to correctly identify virtually all of the irritant polymer samples either in the saline, sesame oil or both solvent extracts. Our results indicate that RhE tissue models can detect the presence of strong skin irritants at low levels in dilute medical device polymer extracts. Therefore, these models may be suitable replacements for the rabbit skin irritation test to support the biological evaluation of medical devices.

Keywords: medical devices, irritation, alternative testing

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迫田秀行，岡本吉弘，菅野伸彦*：マイクロスラリージェットエロージョン法を用いた人工関節超高分子量ポリエチレンコンポーネントの劣化評価.

臨床バイオメカニクス 2017;38:223-228.

Lipids such as squalene（SQ）are absorbed in ultra-high molecular weight polyethylene（UHMWPE）components of joint prostheses during use *in vivo*. The degradation of UHMWPE induced by the absorbed lipids *in vivo* is suspected since the lipid-induced degradation of UHWWPE has been reported by *in-vitro* studies using SQ as a model lipid. However, direct evidence of the lipid-induced degradation of UHMWPE *in vivo* has not been reported.

In this study, the micro slurry-jet erosion（MSE）method, which can evaluate the strength of a material near the surface with a high resolution in the direction of the depth, was applied to *in-vitro* materials simulating lipid- and radical-induced degradation and nine retrieved UHMWPE components from hip joint prostheses.

Reductions in the strength of *in-vitro* materials due to simulated lipid- and radical-induced degradation

were successfully evaluated using the MSE method. The strength of specimens simulating lipid-induced degradation was the lowest at the surfaces, while that of specimens simulating radical-induced degradation was the lowest below the surfaces. The strength at the surfaces (0-50 μm) in the retrieved specimens was lower than that below the surfaces (100-150 μm) in all cases. Due to the similarity in the degradation profile between retrieved specimens and the *in-vitro* material with simulated lipid-induced degradation, it was considered that the reduction in strength in the retrieved specimens was due to lipid-induced degradation *in vivo*.

Since simulated lipid-induced degradation has been reported to increase the wear rate of UHMWPE *in vitro,* it was suggested that UHMWPE has a higher wear rate *in vivo* due to lipid-induced degradation than those reported by *in-vitro* wear simulator studies.

Keywords: joint prosthesis, UHMWPE, lipids, degradation, strength

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Relationship between deamidation intensity and allergenicity of acid hydrolysed wheat proteins preparations: from France to Japan

Clinical and Translational Allergy, 2017;7:18

Introduction: Hydrolyzed wheat proteins (HWP) were used as ingredients in food and cosmetics. From the 2000's severe food allergy to HWP has been reported in individuals elsewhere tolerant to native wheat proteins. Denery et al. demonstrated that deamidation of wheat proteins, a consequence of acid hydrolysis, generate essential neo-epitopes in these particular allergy to wheat. More recently in Japan, an acid-HWP preparation (a-HWP), named GluPearl 19S, elicited severe skin reactions and food allergy in more than 1800 individuals and was likely to contain deamidated gluten proteins. Level of deamidation depends on treatment intensity; a-HWP preparations with either low or high level of deamidation can be found as ingredient. This study aimed at exploring the impact of deamidation level of wheat proteins on the degranulation of basophils sensitized with IgE from patient allergic to a-HWP.

Methods: Impact of the deamidation level of gliadins and a-HWPs upon IgE reactivity of 8 a-HWP allergic patients was determined by ELISA. Impact of deamidation on basophil degranulation was also explored with humanized Rat Basophil Leukemia cells passively sensitized with IgE from patients and subjected to crosslinking with a set of deamidated samples. Finally IgE Repertoire specific to deamidated wheat protein was then explored by inhibition with INRA-DG1, a mouse monoclonal antibody specific for deamidated gliadins.

Results: Intensity of binding of patient IgE onto a-HWP and the degranulation potency were correlated with level of deamidation. Pre-incubation of deamidated gluten with INRA-DG1 mAb inhibited half of its degranulation capacity with patient IgE. These results suggested that the patient IgE repertoire specific for deamidated gluten proteins is likely to be limited to a very few specificities. GluPearl 19S, involved in the Japanese cases, was determined as highly deamidated. It was the most recognized sample among the 5 deamidated glutens tested in this study.

Conclusion: Although differences exist between French and Japanese cases (such as the tolerance of native wheat proteins), this result suggested that Japanese and French cases suffered from the same unconventional allergy to wheat.

Keywords: hydrolysed wheat proteins, allergenicity, deamidation

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A review of toxicity studies of carbon nanotubes.

Journal of Occupational Health, 2017;59:394-407.

Objective: We reviewed studies on pulmonary, reproductive, and developmental toxicity caused by carbon nanotubes (CNTs). In paricular, we analyzed how CNT exposure affects the several processes of pulmonary toxicity, including inflammation, injury, fibrosis, and pulmonary tumors. Methods: In pulmonary toxicity, there are various processes, including inflammation, injury, fibrosis, respiratory tumor in the lungs, and biopersistence of CNTs and genotoxicity as tumor-related factors, to develop the respiratory tumor. We evaluated the evidence for the

carcinogenicity of CNTs in each process. In the fields of reproductive and developmental toxicity, studies of CNTs have been conducted mainly with mice. We summarized the findings of reproductive and developmental toxicity studies of CNTs. Results: In animal studies, exposure to CNTs induced sustained inflammation, fibrosis, lung cancer following long-term inhalation, and gene damage in the lung. CNTs also showed high biopersistence in animal studies. Fetal malformations after intravenous and intraperitoneal injections and intratracheal instillation, fetal loss after intravenous injection, behavioral changes in offsprings after intraperitoneal injection, and a delay in the delivery of the first litter after intratracheal instillation were reported in mice-administered multi-walled carbon nanotubes (MWCNTs). Single-walled carbon nanotubes (SWCNTs) appeared to be embryolethal and teratogenic in mice when given by intravenous injection; moreover, the tubes induced death and growth retardation in chicken embryos. Conclusion: CNTs are considered to have carcinogenicity and can cause lung tumors. However, the carcinogenicity of CNTs may attenuate if the fiber length is shorter. The available data provide initial information on the potential reproductive and developmental toxicity of CNTs.

Keywords: carbon nanotube, pulmonary toxicity, reproductive toxicity

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Kawakami T, Isama K*1, Kagawa-Tanaka T*2, Jinno H*3: Analysis of glycols, glycol ethers, and other volatile organic compounds present in household water-based hand pump sprays.

J Environ Sci Health A Tox Hazard Subst Environ Eng 2017;52:1204-10.

The aim of this investigation is to clarify the types and concentrations of VOCs present in various commercial household water-based hand pump spray products used in Japan, and to estimate their average concentrations in indoor air when the spray product is used. We selected glycol and glycol ethers as the main target compounds, as these chemicals were detected at high frequencies and concentrations in a national survey of Japanese indoor air pollution. The extraction of these chemicals using graphite carbon cartridges was examined, with good recoveries and reproducibilities being obtained. Eighteen chemicals were analyzed in 54 commercial products and 8 chemicals were detected. More specifically, dipropylene glycol (DPG) was present in 44 samples (1.1×10^{1}-1.8×10^{4} μg/mL); propylene glycol (PG) was present in 22 samples (1.5×10^{1}-2.9×10^{4} μg/mL); diethylene glycol monoethyl ether (DGMEE) was found in 15 samples (trace amount-1.9×10^{3} μg/mL); diethylene glycol (DEG) was present in 9 samples (1.0×10^{1}-2.4×10^{3} μg/mL); 1,3-butandiol (13BG) was found in 5 samples (trace amount-7.4×10^{3} μg/mL); 2-ethyl-1-hexanol (2E1H) was detected in 5 samples (3.2×10^{-1}-4.4×10^{1} μg/mL); diethylene glycol monobutyl ether (DGMBE) was present in 4 samples (2.1×10^{1}-7.1×10^{1} μg/mL); and 3-methoxy-3-methylbutanol (MMB) was found in 2 samples (2.4×10^{1}-4.7×10^{2} μg/mL). In addition, the average concentrations of these chemicals in indoor air were estimated using their maximum concentrations observed in the spray product. The estimated average concentrations of the chemicals in indoor air were determined to range between 1.0×10^{-2} and 1.0 mg/m^3, with the exception of 2E1H and DGMBE. Furthermore, the estimated average concentrations of PG, 13BG, and DGMEE in indoor air were comparable to or higher than those reported in a national survey of Japanese indoor air pollution. It therefore appeared that household water-based hand pump sprays may contribute to the presence of these chemicals in indoor air. In contrast, estimated average concentrations of 2E1H in indoor air were low, its concentrations observed in a national survey of Japanese indoor air pollution are likely due to the use of plasticizers and paints.

Keywords: graphite carbon cartridge, glycol and glycol ether, household water-based hand pump spray

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液体クロマトグラフィータンデム質量分析による水道水中の臭素酸分析条件の検討と妥当性評価.

水環境学会誌 2017;40:223-33.

水道水中の臭素酸イオン（BrO_3^-）を既存の告示法よりも高精度かつ迅速・簡便に分析するために，LC/MS/MSによる測定方法を検討し，臭素酸イオンを高感度に検出でき，さらに水道水中に含まれる他の陰イオンを良好に分離可能な測定条件を確立した．さらに，本研究で確立した測定条件が全国の水道水に適用できるかどうかを検証するために，水道事業体等の23機関において水道水に臭素酸イオンを基準値（0.01 mg/L）およびその1/10（0.001 mg/L）となるように添加した試料を調製し，各機関で最適化した様々な測定条件で試験を行った．その結果，いずれの機関においても厚生労働省が示している「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ことから，本分析法は水道水中の臭素酸イオンを基準値の1/10（0.001 mg L-1）まで高精度に分析可能であると評価した.

Keywords: bromate, drinking water, liquid chromatography

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A chimeric IgE that mimics IgE from patients allergic to acid-hydrolyzed wheat proteins is a novel tool for *in vitro* allergenicity assessment of functionalized glutens

PLOS ONE, 2017;12:e0187415

BACKGROUND: Acid-hydrolyzed wheat proteins (acid-HWPs) have been shown to provoke severe allergic reactions in Europe and Japan that are distinct from classical wheat allergies. Acid-HWPs were shown to contain neo-epitopes induced by the deamidation of gluten proteins. However, products with variable rates of deamidation can be found.

OBJECTIVES: In this work, we studied the effect of the extent of wheat proteins deamidation on its allergenicity. A recombinant chimeric IgE was produced and compared to patients' IgE for its capacity to assess the IgE-mediated triggering potential of acid-HWPs.

METHODS: Sera from acid-HWP allergic patients were analyzed via ELISA and a functional basophil assay for their IgE reactivity to wheat proteins with different deamidation levels. A chimeric mouse/human IgE (chIgE-DG1) specific for the main neo-epitope, QPEEPFPE, involved in allergy to acid-HWPs was characterized with respect to its functionality and its reactivity compared to that of patients' IgE.

RESULTS: Acid-HWPs with medium (30%) and high (50-60%) deamidation levels displayed a markedly stronger IgE binding and capacity to activate basophils than those of samples with weak (15%) deamidation levels. The monoclonal chIgE-DG1 allowed basophil degranulation in the presence of deamidated wheat proteins. ChIgE-DG1 was found to mimic patients' IgE reactivity and displayed the same ability to rank acid-HWP products in a degranulation assay.

CONCLUSION: Increasing the deamidation level of products from 15% to 60% resulted in an approximately 2-fold increase in their antigenicity and a 100-fold increase in their eliciting potential. The chimeric ChIgE-DG1 may be a useful tool to evaluate functionalized glutens for their allergenic potential.

By mimicking patient sera reactivity, chIgE-DG1 also provided data on the patients' IgE repertoire and on the functionality of certain repeated epitopes in gluten proteins.

Keywords: hydrolysed wheat proteins, allergenicity, deamidation

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竹原友貴*，庄田裕紀子*，河上強志：医療用弾性ストッキングに含まれる2-*n*-octyl-4-isothiazolin-3-one（OIT）による接触皮膚炎の1例.

J Environ Dermatol Cutan Allergol 2017;11:326-32.

65歳男性．下肢静脈瘤に対して弾性ストッキングを5年間着用し，下腿に境界明瞭な紅斑を生じた．弾性ストッキングによるパッチテストが陽性を呈し，成分分析と成分パッチテストの結果，2-*n*-octyl-4-isothiazolin-3-one（OIT）による接触皮膚炎と診断した．OITは，イソチアゾリノン系防腐剤であり，海外では塗料，接着剤，木製製品，皮革の防腐剤として使用され，職業性接触皮膚炎の発生が散見されるが，わが国での報告は少ない．医療用弾性ストッキングは下肢の静脈血，リンパ液の還流促進を目的とした医療機器であるが，今回，OITは防臭目的で使用されていた．弾性ストッキングは長期間，肌に直接触れ，かつ，着用者にはうっ滞性皮膚炎の合併率が高いことから，感作されやすい．イソチアゾリノン系防腐剤の弾性ストッキングや衣類への使用には注意が必要と考え報告する．

Keywords: contact dermatitis, 2-*n*-octyl-4-isothiazolin-3-one, medical compression stocking

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味村真弓*1，中島晴信，河上強志，伊佐間和郎*2：繊維製品に含まれるトリス(1-アジリジニル)ホスフィンオキシド(略称：APO)の分析法の改定に向けた検討.

大阪健康安全基盤研年報 2017;1:93-100.

「有害物質を含有する家庭用品の規制に関する法律」により，繊維製品への使用が禁止されている防炎加工剤のトリス(1-アジリジニル)ホスフィンオキシド(APO)の公定法を改定するために，APO-d_{12}をサロゲート物質として用いた GC/MS 分析法の検討を行った．SIM モードにおける定量・定性イオンを，APO では m/z 131，m/z 90，APO-d_{12}では m/z 139，m/z 95 とした．設定した分析条件で作成した検量線は，0.25～10 μg/mL の間で相関係数 0.998 以上の良好な直線性が得られた．検出限界は S/N=3として 0.075 μg/mL であった．また，フロリジルカラムを用いた試料の精製法を検討し，APO 及びサロゲート物質共に良好な回収率が得られる前処理法を確立した．次に，素材の異なる複数の繊維製品を用いて添加回収試験を行った．綿製品では良好な回収率が得られたが，化繊やその混紡製品では回収率が非常に低いものがあった．そこで，低回収率を示した製品のメタノール抽出液に標準品を添加し添加回収試験を行ったところ，APO 及びサロゲート物質共に低回収率となり，製品からメタノール中に抽出された夾雑物質が影響していることが推測された．今回作成した分析法は，サロゲート物質の使用により回収率が補正され，製品中の正確な APO 残留量を知ることができる．構築した方法は公定法の改良法として十分適用できる可能性があると考えられた．この方法で，市販の防炎加工製品 8 製品を分析調査したが，いずれの製品からも APO は検出されなかった.

Keywords: tris（1-aziridinyl）phosphine oxide, textile product, GC-MS

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Miyazaki H*1, Yamashita K*1, Uchino T, Takezawa T*2,Kojima H :Development of a novel *in vitro* skin sensitization test method using a collagen vitrigel membrane chamber

AATEX 2017;22:141–154.

Several *in vitro* cell-based methods for predicting the skin sensitizing potential have been reported; however, such methods are limited by the poor water solubility of many organic molecules. Therefore, we developed a novel test method using a collagen vitrigel membrane（CVM）chamber, termed the Vitrigel Skin Sensitization Test（Vitrigel-SST）. We first determined the optimal concentration of dimethyl sulfoxide（DMSO）for test chemical solutions subjected to the Vitrigel-SST. When medium containing 10% DMSO was subjected to the Vitrigel-SST using THP-1 cells, cytotoxicity was not observed until the 3 h time point. Thus, we decided to use 10% DMSO for Vitrigel-SST. Test chemicals were dispersed or dissolved in medium containing 10% DMSO. THP-1 cells were exposed to the test solutions for 30 min through the CVM, and the test solutions were replaced to fresh medium. After

48 h incubation, interleukin 8 (IL-8) productions was measured. Twenty-fore test chemicals were evaluated to demonstrate the capacity of the Vitrigel-SST to predict chemical-induced skin sensitization. Cells exposed fifteen chemicals, including five chemicals with poor water solubility, increased maximum IL-8 production ($IL\text{-}8_{MAX}$) more than 2-fold ($\geqq 2$) that of cells exposed to the control treatment. $IL\text{-}8_{MAX}$ of five non-skin sensitizers was less than 2. The results 20 of 24 chemicals were matched with that of LLNA when the $IL\text{-}8_{MAX}$ of 2 was adoped as cut off value. These results demonstrate that the Vitrigel-SST is a promising new skin sensitization test and is suitable to evaluate the skin sensitization potential of chemicals with poor water solubility.

Keywords: skin, sensitizer, collagen, vitrigel interleukin-8

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Intensity of deamidation in the epitopes of acid-hydrolyzed wheat proteins is a key parameter for their allergenicity

Clinical and Translational Allergy, 2018;8:15

Background: Acid-hydrolyzed wheat proteins (a-HWP) were used as ingredients in food and cosmetics. From the 2000's, cases of severe food allergy to HWP have been reported in people tolerant to native wheat proteins. Denery *et al.* demonstrated that deamidation of wheat proteins, the main consequence of acid-hydrolysis, generates neo-epitopes responsible for this particular allergy to wheat. More recently in Japan, a soap containing a-HWP elicited severe skin reactions and food allergy in more than 2000 people. Gliadins and glutenins, the main components of wheat proteins, are characterized by homologous domains constituted of repeated sequences of 6–8 amino acids rich in glutamines. During acid-hydrolysis, the random process of deamidation results in heterogeneous deamidation in each repeated sequences. This work investigated the effect of the deamidation rates of the repeated sequences of a-HWP on their triggering potency.

Methods: Three batches of deamidated gliadins were produced by increasing the acid-hydrolysis duration. These 3 samples and 5 industrial HWP samples involved in European or Japanese cases of allergy were characterized for their content in native, weakly deamidated and highly deamidated repeated sequences by competitive ELISA. Their triggering potency was determined using a basophils assay with HWP-allergic patients' sera.

Results: Competitive ELISAs showed that native sequences were progressively converted to deamidated sequences when acid-hydrolysis duration increased. Among the deamidated sequences the content in highly deamidated sequences progressively increased with the treatment duration while the content in weakly deamidated sequence remained constant. Industrial HWPs appeared extremely heterogeneous and displayed various levels of native, weakly and highly deamidated sequences. The ability to activate basophils sensitized with HWP-allergic patients appeared related to the content in highly deamidated sequences.

Conclusions: Repeated domains of gliadins and glutenins in a-HWPs are a mix of native, weakly deamidated and highly deamidated sequences which proportions vary among the products released on the market. The content in highly deamidated sequences predominantly contributed to the triggering potency of a-HWP samples.

Keywords: hydrolysed wheat proteins, allergenicity, deamidation

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Saito-Shida S, Sakai T, Nemoto S, Akiyama H: Quantitative analysis of veterinary drugs in bovine muscle and milk by liquid chromatography quadrupole time-of-flight mass spectrometry.

Food Addit. Contam. Part A 2017;34:1153-1161

A simple and reliable multiresidue method for quantitative determination of veterinary drugs in bovine muscle and milk using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) was developed. Critical MS parameters such as capillary voltage, cone voltage, collision

energy, desolvation gas temperature, and extraction mass window were carefully optimised to obtain the best possible sensitivity. Analytical samples were prepared using extraction with acetonitrile and hexane in the presence of anhydrous sodium sulphate and acetic acid, followed by ODS cartridge clean-up. The developed method was validated for 82 veterinary drugs in bovine muscle and milk at spike levels of 0.01 and 0.1 mg kg^{-1}. With the exception of cefoperazone and phenoxymethylpenicillin, all of these compounds exhibited sufficient signal intensity at 0.01 μg ml^{-1} (equivalent to 0.01 mg kg^{-1}), indicating the high sensitivity of the developed method. For most targets, the determined accuracies were within 70–120%, with repeatability and reproducibility being below 20% at both levels. Except for sulfathiazole in bovine muscle, no interfering peaks at target compound retention times were detected in the blank extract, indicating that the developed method is highly selective. The absence of sulfathiazole in bovine muscle was confirmed by simultaneous acquisition at low and high collision energies to afford exact masses of molecular adduct and fragment ions. Satisfactory linearity was observed for all compounds, with matrix effects being negligible for most targets in bovine muscle and milk at both spike levels. Overall, the results suggest that the developed LC-QTOF-MS method is suitable for routine regulatory-purpose analysis of veterinary drugs in bovine muscle and milk.

Keywords: veterinary drugs, multiresidue method, LC-QTOF-MS

朝倉敬行*，北村真理子*，関亘*，飯田智成*，中里光男*，安田和男*，根本了：LC-MS/MSによる農産物および畜水産物中のジニコナゾールの分析法.

食品衛生学雑誌 2017;58(4):195-200

LC-MS/MSを用いた農産物および畜水産物中のジニコナゾールの分析法を開発した．農産物は，アセトンで抽出し，*n*-ヘキサンに転溶後，必要に応じてアセトニトリル/ヘキサン分配により脱脂し，フロリジルおよびグラファイトカーボンミニカラムによる精製を行い，LC-MSIMSにて測定した．また，畜水産物については，アセトン－n-ヘキサン(1:2,v/v)混液で抽出し，アセトニトリル/ヘキサン分配で脱脂し(はちみつを除く)，フロリジルミニカラムで精製した後，LC-MS/MSにて測定した．農産物および畜水産物計16食品に0.01 mg/kg添加して回収試験を行ったところ，真度88.3～108%，併行精度0.5～5.1%であった．本分析法における定量限界値は，0.01 mg/kgであった.

Keywords：ジニコナゾール，殺菌剤，LC-MS/MS

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小林麻紀*，酒井奈穂子*，上條恭子*，小池裕*，根本了，新藤哲也*：LC-MS/MSによる農産物中の塩酸ホルメタネート分析法.

食品衛生学雑誌 2017;58(5):221-228

農産物中の塩酸ホルメタネート分析法について検討を行った．塩酸ホルメタネートはアセトニトリル中で安定であったことから，試料からアセトニトリルで抽出し，エチレンジアミン-*N*-プロピルシリル化シリカゲル(PSA)およびグラファイトカーボン(GC)ミニカラムで精製し，LC-MS/MSで測定を行い，絶対検量線法で定量した．10品目の農産物(玄米，大豆，ほうれんそう，キャベッ，ばれいしょ，オレンジ，ライム，りんご，ネクタリン，緑茶)を対象に残留基準値濃度または一律基準値濃度(0.01 ppm)における添加回収試験を行った結果，真度(n=5)は92.3～103%，併行精度は1.3～5.4%，定量限界は0.01 mg/kgであった.

Keywords：塩酸ホルメタネート，農産物，LC-MS/MS

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塩野 弘二，志田（齊藤）静夏，今村 正隆，根本 了，穐山 浩：水素化物発生原子吸光法による農産物中のヒ素分析法.

日本食品化学学会誌 2017;24(3):114–118

The maximum residue limits (MRL) for arsenic in the eleven agricultural products as pesticide residues is set in Japan. A method for the determination of arsenic pesticide residues in the agricultural products using hydride-generation atomic absorption spectrometry was developed. The samples were digested by wet ashing method using nitric and sulfuric acid, pre-reduced by hydrochloric acid and potassium iodide to prepare test solution, prior to measurement by hydride generation atomic absorption spectrophotometer. The absorbance of test solution after pre-reduction was stable until 30 minutes. The calibration curve in the concentration of 1.0-10 ng/mL had good linearly. The proposed method was validated for 11 kinds of agricultural products at MRL. The results showed excellent recoveries (98.7-105.4%) and repeatability (2.1-4.7%). The study showed that the

developed method using hydride generation atomic absorption spectrometry is reliable and applicable for the determination of arsenic in the agricultural products as the regulation of pesticide residue.
Keywords: arsenic, atomic absorption spectrophotometer, hydride generation

Saito-Shida S, Hayashi T, Nemoto S, Akiyama H: Determination of total avilamycin residues as dichloroisoeverninic acid in porcine muscle, fat, and liver by LC-MS/MS.
Food chemistry 2018;249:84-90

A sensitive and reliable method for determining the total avilamycin residues was developed using LC–MS/MS. Avilamycin (consisting of avilamycin A and 15 other minor factors) and its metabolites in porcine muscle, fat, and liver were analysed as the marker residue dichloroisoeverninic acid (DIA), in accordance with the maximum residue limit (MRL) established by international organisations such as Codex Alimentarius Commission and other regulatory bodies. The analytes were extracted from samples with acetone, hydrolysed to DIA, partitioned into ethyl acetate, and cleaned up prior to the LC-MS/MS analysis. The method was validated at Codex MRL and 0.01 mg/kg. The results show excellent recoveries ranging from 100–108%, with the relative standard deviations <6%. Matrix effects were negligible for all types of samples, indicating effective sample clean-up. The absence of interfering peaks close to the retention time in blank samples demonstrates high selectivity. Overall, this method is reliable and suitable for regulatory-purpose analysis.
Keywords: avilamycin, dichloroisoeverninic acid, LC-MS/MS

Saito-Shida S, Hamasaka T*, Nemoto S, Akiyama H: Multiresidue determination of pesticides in tea by liquid chromatography-high-resolution mass spectrometry: Comparison between Orbitrap and time-of-flight mass analyzers.
Food Chemistry 2018;256:140–148

Liquid chromatography (LC)-Orbitrap mass spectrometry (MS) and LC-time-of-flight (TOF) MS operating in full scan mode at a mass resolution of 140000 (*m/z* 200) and 30000 (m/z 556), respectively, were compared for quantification of pesticide residues in tea. Both methods were validated for 146 pesticides at spike levels of 0.1 and 0.01 mg/kg and compared in terms of recovery, intra- and inter-day precisions, selectivity, linearity, and matrix effect. The results of both analyses were comparable, and recovery and intra- and inter-day precisions were within the acceptable ranges for most pesticides. LC-Orbitrap MS was slightly superior to LC-TOF MS in terms of sensitivity and selectivity due to its higher resolution. However, even using high-resolution LC-Orbitrap MS with a narrow mass window of ±3 ppm, interference by coeluting matrix components was observed, indicating that full scan data are insufficient for unequivocal identification, and additional information such as fragment ions is necessary.
Keywords: pesticide, liquid chromatography-Orbitrap mass spectrometry, liquid chromatography-time-of-flight mass spectrometry

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坂井隆敏，根本了，手島玲子，穐山浩：LC-MS/MSを用いた畜水産物中のニトロイミダゾール類および主要代謝物の分析法.
食品衛生学雑誌 2017;58(4):180-187

A sensitive and reliable method for the simultaneous determination of four nitroimidazoles (ipronidazole (IPZ), dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ)) and three metabolites (IPZ-OH, MNZ-OH and 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)) in livestock and fishery products was developed. The analytes were extracted from samples with acetone containing acetic acid. The crude extracts were defatted by liquid–liquid partition using acetonitrile and *n*-hexane followed by solid-phase extraction using a cartridge column packed with divinylbenzene–*N*-vinylpyrolidone copolymer bearing sulfo groups. The analytes in the eluate from the cartridge column were extracted with ethyl acetate after addition of ammonium sulfate. The solvent was removed from the extract, and the residue was dissolved in 0.1 vol% formic acid. The HPLC separation was performed on a C18 column with a gradient formed from water containing 0.1 vol% formic acid and acetonitrile containing 0.1 vol% formic acid. For detection of the analytes, tandem mass spectrometry with positive ion electrospray ionization was used. The recovery tests were performed on 10 livestock

and fishery products. The truenesse ranged from 74.6 to 111.1%, with repeatability of 0.5–8.3 RSD% for the entire procedure. The limit of quantification was 0.0001 mg/kg for IPZ, IPZ-OH, MNZ and MNZ-OH, and 0.0002 mg/mg for DMZ, RNZ and HMMNI.
Keywords: nitroimidazoles, LC-MS/MS, livestock and fishery products

Tsutsumi T, Takatsuki S, Teshima R, Matsuda R, Watanabe T, Akiyama H: Dioxin concentrations in dietary supplements containing animal oil on the Japanese market between 2007 and 2014.
Chemosphere 2018;191:514–519

We determined the concentrations of dioxins (polychlorinated dibenzo-*p*-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls) in 46 dietary supplement products, containing the oil of fish, marine mammals, or egg yolk, on the Japanese market between 2007 and 2014. Dioxins were detected in 43 of the 46 products tested at concentrations from 0.00015 to 67 pg TEQ/g. The highest concentration of dioxins was found in a shark liver oil product which varied insignificantly in five batches collected over a two-year period. The dioxin intakes from these five batches reached 2.3 to 2.8 pg TEQ/kg bw/day, or 58% to 70%, respectively, of the Japanese tolerable daily intake (TDI) of 4 pg TEQ/kg bw/day. However, the dioxin intakes from most of the other products tested were less than 5% of the TDI. Although rare, supplements based on animal oils may contain relatively high concentrations of dioxins, leading to a substantial increase in dioxin intakes.
Keywords: dioxin, supplement, animal oil

片岡洋平，渡邉敬浩，林恭子，小澤蘭*，滝澤和宏*，穐山浩：ミネラルウォーター類製品における六価クロム濃度の実態調査.
食品衛生学雑誌 2017;58(6):275-280

ミネラルウォーター類（MW）製品中の六価クロム分析法を構築し，その性能を評価し妥当性を確認した．さらに定量下限値を推定した本法を用いて，2016年に市場流通していたMW類150製品における六価クロム濃度の実態を調査した．実態調査に併せて分析した添加試料からは93～107％の範囲で回収率が得られ，妥当性確認した分析法の適用性が高いことが示された．調査した150製品のうち65製品から六価クロムが検出され，検出率は43％であった．また，検出された濃度の最小値は0.0001mg/L，最大値は0.0019mg/L，中央値は0.0003mg/Lであった．0.0001～0.0002mg/Lの範囲で六価クロムが検出される製品数が最も多かった．本研究において実施した実態調査では，食品衛生法により設定されている規格値（0.05mg/L）を超過する濃度で六価クロムが検出される製品は発見されなかった.
Keywords：六価クロム，イオンクロマトグラフィー，ミネラルウォーター

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Zaima K*1, Fukamachi A*1, Yagi R*1, Ito Y*2, Sugimoto N, Akiyama H, Shinomiya K*1, Harikai N*1: Kinetic study of the equilibration between carminic acid and its two isomers isolated from cochineal dye.
Chem Pharm Bull 2017;65:306-310.

Carminic acid (CA) is a major component of cochineal dye used in food additives, cosmetics, and pharmaceuticals. CA and its isomers, 2-*C*-α-glucofuranoside and 2-*C*-β-glucofuranoside of kermesic acid (DCIV and DCVII, respectively), were isolated from cochineal dye and the equilibrium constants (*K*) between CA, DCIV and DCVII were investigated. DCIV was partially converted to CA and DCVII, and DCVII was converted to CA and DCIV, whereas CA was very stable and only very slightly converted to DCIV and DCVII. Most of the DCIV and DCVII was converted to CA under aqueous conditions. The kinetic rate constants (*k*) for the degradation of DCIV within the first day of incubation at 24℃ was determined to be 0.901 d^{-1} and for the degradation of DCVII it was determined to be 1.102 d^{-1}. The *k* value for the formation of CA from the remaining DCIV was calculated to be 0.146 d^{-1} and for the formation of CA from the produced DCVII it was found to be 0.148 d^{-1}. The K values were calculated as 1.22×10^{-7}, 2.61×10^{-3} and 2.36×10^{-3} mol/L for CA, DCIV and DCVII, respectively. These findings will be helpful for ensuring the safety and for aiding the quality assurance of cochineal dye products.
Keywords: cochineal, kermesic acid, anomerization

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Ito Y*1, Harikai N*2, Ishizuki K, Shinomiya K*2, Sugimoto N, Akiyama H: Spiroketalcarminic acid,

a novel minor anthraquinone pigment in cochineal extract used in food additives.

Chem Pharm Bull 2017;65:883–887.

Cochineal extract prepared from the scale insect *Dactylopus coccus* (American cochineal) has been used as a natural red dye for food, cosmetics, and pharmaceuticals. The major pigment in cochineal extract is carminic acid (CA), an anthraquinone glucoside, and several minor pigments have been previously reported. Our investigation aimed at establishing the safety of cochineal dye products using ultra performance liquid chromatography-photo diode array-electrospray ionization-time of flight (UPLC-PDA-ESI-TOF)/MS found an unknown minor pigment, spiroketalcarminic acid (1), in three commercial cochineal extract samples; cochineal extract used in food additives, carmine that is an aluminum salt of cochineal extract used as natural dye, and a research reagent of CA. The purification of 1 from cochineal extract involved sequential chromatographic techniques, including preparative reversed-phase HPLC. Two dimensional (2D)-NMR and mass analyses established the structure of 1 to be a novel anthraquinone with an unusual 6,5-spiroketal system instead of the *C*-glucosyl moiety of CA. The absolute stereochemistry of the spiroketal moiety in 1 was determined by nuclear Overhauser effect spectroscopy (NOESY) correlations and optical rotation. No data corresponding to 1 had previously been reported for extracts of dried cochineal insects and traditional art products dyed with cochineal extract, indicating that 1 is likely produced during the preparation of commercial cochineal extract.

Keywords: cochineal extract, *Dactylopus coccus*, carminic acid

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Katayama S*, Ohno F*, Mitani T*, Akiyama H, Nakamura S*: Rutinosylated Ferulic Acid Attenuates Food Allergic Response and Colitis by Upregulating Regulatory T Cells in Mouse Models.

J Agric. Food Chem., 2017:65;10730-10737

The purpose of this study was to screen the immunosuppressive phytochemicals inducing immune tolerance via enhanced TGF-β1secretion. In the screening test using THP-1-derived dendritic cells, a significant increase in TGF-β1levels was observed by treatment with ferulic acid(FA)and its glycosides, of which FA rutinoside(FAR) induced the highest level of TGF-β1secretion. Oral administration of FAR suppressed serum levels of IgE and histamine in ovalbumin (OVA)-sensitized mice and induced the differentiation of regulatory T(Treg) cells. Compared with the control, FAR treatment also demonstrated higher levels of TGF-β1secretion from splenic dendritic cells. FAR treatment attenuated the dextran sulfate sodium-induced colitis of model mice and induced Treg differentiation. These results suggest that FAR possesses potent immunomodulatory effects against allergic and intestinal inflammatory responses by inducing Treg differentiation. These findings are expected to contribute to the development of immunomodulatory agents for the prevention and treatment of food allergy and colitis.

Keywords: ferulic acid, immune tolerance, regulatory T cells

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Tatebe C, Ohtsuki T, Fujita T*, Nishiyama K*, Itoh S*, Sugimoto N, Kubota H, Tada A, Sato K, Akiyama H: Determination of starting materials, intermediates, and subsidiary colors in the color additive Food Red No. 106 (Sulforhodamine B) using high-performance liquid chromatography.

Food Chem. 2017;237:733-742.

The main subsidiary color of structure in Food Red No. 106 (R106) was identified to be a desethyl derivative (R106-SubA). High-performance liquid chromatography (HPLC) was performed for the quantitative determination of benzaldehyde-2,4-disulfonic acid, *N*, *N*-diethyl-m-aminophenol, leuco acid, pyrone acid, R106-SubA, etc. in R106. An ammonium acetate solution (20mM) and acetonitrile: water (7:3) were used to stabilize the retention time of the HPLC analytes. The linearity of the calibration curves was in the range of 0.05-10 μg/mL, with good correlation coefficients (R^2>0.9983). The recoveries of impurities at levels 0.1%, 0.5% and 1% ranged from 94.2% to 106.6% with relative standard deviations of 0.1%-1.0%. While surveying commercial R106, the amounts

obtained by area% determination were similar to those obtained by the calibration-curve determination. The area% determination by HPLC for the determinations of impurities in R106 is a simple and reliable method and can be applied in routine analysis.

Keywords: Food Red No. 106, sulforhodamine B, subsidiary color

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日本食品化学学会誌 2017;24:94-104.

Daily intakes of food additives such as sweeteners, preservatives, colors, and food manufacturing agents in adults (over 20 years old) in Japan were estimated using the market basket method for 2011-2013. A list of daily consumption of processed foods used for the estimation was prepared based on the special Japanese national survey study conducted in 2011. The food additives with high daily intake were estimated to be orthophosphoric acid (250 mg/day as phosphorus), condensed phosphoric acid (15.2 mg/day as phosphorus), and propylene glycol (14.1 mg/day). The estimated daily intakes of the food additives were compared with their acceptable daily intake (ADI) or maximum tolerable daily intake (MTDI) assessed by international risk assessment meetings or the Food Safety Commission of Japan. The ratios of the estimated daily intakes to ADI for sweeteners, preservatives, colors, antioxidants, fungicides, and propylene glycol were 0-4.9%. The ratio of the estimated daily intake to MTDI for phosphorus compounds (orthophosphoric acid and condensed phosphoric acid) was 6.9%.

Keywords: market basket method, food additives, acceptable daily intake (ADI)

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鈴木一平，大槻 崇，吉田充哉[*1]，吉田美佳[*1]，阿部 裕，久保田浩樹，建部千絵，多田敦子，矢野竹男[*2]，穐山浩，佐藤恭子：過酢酸製剤処理された生鮮食品中のオクタン酸分析法の開発と輸入生鮮食品中のオクタン酸量の調査．

日本食品化学学会誌 2017;24:25-31.

Octanoic acid (OA) is an ingredient of peracetic acid–based sanitizers (PAS), which are widely used in the sanitation of uncooked food. Since, OA can remain on PAS-treated foods, we developed a simple analytical method to determine OA levels in uncooked foods. The developed method involves straightforward solvent extraction with diethyl ether, derivatization with sulfuric acid/methanol and gas chromatography coupled with mass spectrometry. The recovery and relative standard deviation of OA ranged from 74.2 to 96.6% and 2.6 to 9.6%, respectively. The limit of quantification (LOQ) in foods was estimated to be 0.02 mg/kg. We applied the developed method to imported uncooked foods (56 beef samples, 34 vegetable samples and 89 fruit samples), and found that OA levels ranged from 0.34 to 0.53 mg/kg, the LOQ to 0.48 mg/kg and LOQ to 1.12 mg/kg, respectively. Most of the determined OA in imported uncooked foods could be considered to be derived from OA naturally contained in the foods.

Keywords: octanoic acid, food additives, peracetic-acid based sanitizer

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西﨑雄三，佐藤（増本）直子，中西章仁[*1]，橋爪雄志[*1]，タンジャマハマドゥ[*1]，山﨑太一[*2]，黒江美穂[*2]，沼田雅彦[*2]，井原俊英[*2]，杉本直樹，佐藤恭子：定量NMRに基づく相対モル感度を利用した加工食品中のヘスペリジンおよびモノグルコシルヘスペリジンの定量．

食品衛生学雑誌 2018;59:1-10.

基準物質と分析対象物質の混合試料を調製し，^{1}H-qNMRとHPLC/PDAの両方に付し，PDA検出器における両者の応答比を，^{1}H-qNMRから求めた物質量比で補正し，正確な相対モル感度（RMS）を算出する方法を検討した．メチルパラベン（MPB）を基準物

質，ヘスペリジン（Hes）とモノグルコシルヘスペリジン（MGHes）を分析対象としてRMS 1.25（Hes^{283nm}/MPB^{255nm}）および1.32（$MGHes^{283nm}$/MPB^{255nm}）を算出した．さらに，食品中のHesとMGHesの定量分析を，MPBを内標準物質としてRMSを適用したHPLC/PDAと従来法である絶対検量線法で実施した結果，両手法から得られる定量値の差はHesで2.0％以下，MGHesで3.5％以下であった．

Keywords：相対モル感度，^{1}H-qNMR，ヘスペリジン

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佐藤（増本）直子，西﨑雄三，斎藤直樹*，山﨑太一*，沼田雅彦*，井原俊英*，杉本直樹，佐藤恭子：qNMR及びHPLCによる機能性表示食品中の機能性関与成分ルテインの定量．

日本食品化学学会誌 2017;24:75-81.

A new system of functional foods, which was instituted by Consumer Affairs Agency, Government of Japan, has been put into effect in April 2015. In this system, foods containing functional substances, whose functions for the human body are demonstrated scientifically, are defined as “Foods with Function Claims”, and are allowed to be sold with the information about their functions. Since the amount of functional substances affects the quality and safety of these functional foods, the manufacturers are obliged to submit the accurate amount of functional substances in the foods with the analytical method. High performance liquid chromatography (HPLC) is usually adopted as the analytical method for the determination of functional substances because of its high selectivity and quantitative capability.

Lutein, one of natural carotenoids, is reported to have effect to prevent human macula from damages causing by light, so that lutein-containing Foods with Function Claims are sold with claiming the promotion of eye health. However, the reference material of lutein is not available in the reagent markets so that its accurate amount is difficult to be determined by HPLC and might result in leading to reduce the quality and safety of the Foods with Function Claims. In order to solve this problem, the contents of lutein in Foods with Function Claims determined by HPLC and ^{1}H-qNMR methods were compared. As the result, the amount determined by HPLC tended to be larger than that by ^{1}H-qNMR. One reason for this was that the absolute purity of lutein reagent was lower than the purity, which was calculated using already-known absorption coefficient of lutein. For more accurate calculation of purity of lutein reagent, the absorption coefficient of lutein was also determined by ^{1}H-qNMR method, revealing that absorption coefficient of lutein was 2591, which was larger than known value.

Keywords: lutein, foods with function claims, ^{1}H-qNMR

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日本食品化学学会誌 2017;24:10-15.

In this study, the relationship between the catechin content and antioxidant capacity of the tea extract, a natural food additive, was investigated to establish the quality standards based on the antioxidant capacity. The antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The antioxidant capacities were detected in 13 kinds of tea extracts among the tested 14 kinds of them. A correlation was found between the total amount of catechins (C, EC, GC, EGC, Cg, ECg, GCg, and EGCg) and the antioxidant capacity ($r = 0.975$, $n = 13$, $p < 0.01$). In addition, the contribution ratio of all kinds of catechins in the tea extract to the antioxidant capacity was 90%. These results suggest that the DPPH assay is a useful method to evaluate the antioxidant capacity in the tea extract for establishing the quality standards.

Keywords: antioxidant, DPPH assay, natural food additive

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Yoshimura M*, Ochi K*, Sekiya H*, Tamai E*, Maki J*, Tada A, Sugimoto N, Akiyama H, Amakura Y*: Identification of characteristic phenolic constituents in mousouchiku extract used as food additives.

Chem. Pharm. Bull. 2017;65:878–882.

Mousouchiku extract is prepared from the bamboo-sheath of *Phyllostachys heterocycla* MITF. (Poaceae), and is registered as a food manufacturing agent in the List of Existing Food Additives in Japan. This study describes the chromatographic evaluation of characteristic components of this extract to obtain the chemical data needed for standardized specifications. We isolated 12 known compounds from this extract: 5-hydroxymethyl-2-furfural, 4-hydroxybenzoic acid, trans-p-coumaric acid, trans-ferulic acid, *N,N'*-diferuloylputrescine, 4'-hydroxypropiophenone, β-arbutin, tachioside, isotachioside, 3,4'-dihydroxypropiophenone 3-*O*-glucoside, koaburaside, and (+)-lyoniresinol 9'-*O*-glucoside. Moreover, a new propiophenone glycoside, propiophenone 4'-*O*-(6-β-D-xylosyl)-β-D-glucoside (propiophenone 4'-*O*-primeveroside), was isolated. The structure of each isolated compound was elucidated based on NMR and MS data or direct HPLC comparisons with authentic samples. Among the isolates, (+)-lyoniresinol 9'-*O*-glucoside was found to be the major ingredients of the extract as observed using HPLC analysis. However, 2,6-dimethoxy-1,4-benzoquinone, which is considered the main constituent of mousouchiku extract, was only detected as a trace constituent and not isolated in this study.

Keywords: *Phyllostachys heterocycle*, propiophenone glycoside, lyoniresinol glucoside

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Kitamaki Y*, Saito N*, Yamazaki T*, Otsuka S*, Nakamura S*, Nishizaki Y, Sugimoto N, Numata M*, Ihara T*: Determination of PAHs in solution with a single reference standard by a combination of ^{1}H quantitative NMR spectroscopy and chromatography.

Anal. Chem. 2017;89:6963-6968.

We have applied a combination of ^{1}H quantitative NMR spectroscopy (^{1}H-qNMR) and chromatography (GC or LC) to establish reliable analytical methods (qNMR/GC and qNMR/LC) for organic compounds. In this method, a reference standard is used as an internal standard for both ^{1}H-qNMR and chromatography to estimate relative molar sensitivity (*RMS*) for analytes. The *RMS* values are calculated from the molar ratios between analytes and the reference standard obtained by ^{1}H-qNMR; and the response ratio between them obtained by chromatography. Concentrations of analytes in the organic solution can be simultaneously determined from the *RMS* and amount of the reference standard added in the sample solution. This analytical method is an innovative one because only one reference standard with International System of Units (SI)-traceable property value, purity, or concentration, is necessary to determine accurate concentrations of multiple organic components in organic solutions, without the respective certified reference standards for various analytes. To verify this method, a certified reference material, NIST SRM 1647f, was used. Among the 16 polycyclic aromatic hydrocarbons (PAHs) included in NIST SRM 1647f, naphthalene and benzo[a]pyrene were selected as analytes for this method, using 1,4-bis(trimethylsilyl)benzene-d_4 as the reference standard. Each quantitative value obtained by qNMR/GC and qNMR/LC agreed with each certified value within its expanded uncertainty.

Keywords: relative molar sensitivity, ^{1}H-qNMR, chromatography

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三浦亨*1，杉本直樹，渡邊龍一*2，末松孝子，高柳庸一郎，伊藤喜之，斎藤直樹，澤竜一，加藤毅，藤峰慶徳，小池亮，大福裕子*1，山田裕子*1，内海博明，鈴木敏之*2：^{1}H定量核磁気共鳴分光法の標準化のためのラウンドロビン試験による評価.

薬学雑誌 2017;137(12):1543-1553.

^{1}H quantitative NMR (^{1}H-qNMR) is known as a powerful tool for determination of analytes without the need for their identical standards, which is eligible to a primary rate method. ^{1}H-qNMR has been already stipulated to an assay for purity determination in Japanese Pharmacopoeia (JP), and then this technique has been also applied in several fields such as pharmaceutical and food sciences. However, there is little information about the accuracy of ^{1}H-qNMR so that the further applications into other fields such as industrial chemistry could be constricted. In this study, in order to assess the reliability of ^{1}H-qNMR, we designed the round-robin test of ^{1}H-qNMR under the basis of the measurement conditions described in JP. 1,4-Bis(trimethylsilyl)benzene-d_4 [1,4-BTMSB-d_4, 99.9

±0.6%（w/w）] and 3,5-bis(trifluoromethyl)benzoic acid [3,5-BTMFBA, 99.96±0.06%（w/w）], which are certified reference materials（CRMs）, were adopted to analyte and qNMR reference standard respectively for the accurate evaluation in this test. Six NMR instruments in 5 institutions optimized to ^{1}H-qNMR conditions provided the purity 1,4-BTMSB- d_4 within acceptable error range. This result represented that ^{1}H-qNMR has the capability to determine precisely the value of analyte in practical analytical field and to be set as official analytical method for purity determination or assay of concentration of organic compounds.

Keywords: ^{1}H-qNMR, absolute quantitation, round-robin test

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阿部 裕，山口未来，六鹿元雄，佐藤恭子，穐山浩：GC-MSを用いたフタル酸エステル測定において共存可塑剤が定量値へ与える影響．

日本食品化学学会誌 2017;24:119-124.

The effect of other plasticizers on the analysis of six types of phthalic acid esters（PAEs）, such as dibutyl phthalate, benzyl butyl phthalate, bis(2-ethylhexyl) phthalate, di-*n*-octyl phthalate（DNOP）, diisononyl phthalate（DINP）, diisodecyl phthalate, in a polyvinyl chloride product using gas chromatography-mass spectrometry（GC-MS）was evaluated. In the presence of other plasticizers whose signals were detected on the chromatogram in front of or overlapping those of the target PAEs, the quantified values were higher than the actual values. It was suspected to be due to the effect of these plasticizers in the test solution. These effects could be eliminated, and the exact quantitative values for PAEs other than DNOP and DINP were obtained by simply diluting the test solution more than twofold. However, the effect of diisononyl adipate or diisononyl-1,2-cyclohexanedicarboxylate on the quantitative values of DNOP and DINP could not be eliminated, even though the test solution was diluted fivefold.

Keywords: polyvinyl chloride, plasticizer, GC-MS

大野浩之*1，六鹿元雄，阿部智之*2，阿部裕，天野保希*3，石原絹代*4，大坂郁恵*5，大野春香*6，大野雄一郎*7，尾崎麻子*8，柿原芳輝*9，小林尚*10，櫻木大志*1，柴田博*11，城野克広*12，関戸晴子*13，高坂典子*14，竹中佑*15，但馬吉保*16，田中葵*17，田中秀幸*12，外岡大幸*18，中西徹*4，野村千枝*8，羽石奈穂子*19，早川雅人*20，三浦俊彦*21，山口未来，渡辺一成*20，佐藤恭子：器具・容器包装における蒸発残留物試験の試験室間共同試験（第1報）．

食品衛生学雑誌 2018;59:55-63.

水，4%酢酸および20%エタノールの3種類の浸出用液で調製した試験溶液を用い，器具・容器包装の蒸発残留物試験における試験室間共同試験を行い，公定法と公定法変法の性能を評価した．試験には23機関が参加し，濃度非明示の試験溶液9種類の蒸発残留物量を測定した．蒸発乾固の際の加熱装置として，公定法では水浴を，公定法変法ではホットプレートを使用した．ほとんどの試験機関では，蒸発乾固の際，試験溶液を乾固直前まで加熱したのち，余熱で乾固させていた．その結果，加熱装置に関わらず，両法の性能には大きな差はないことが判明した．それにより，公定法変法は公定法と同様に規格試験法として適用できると判断された．

Keywords：器具・容器包装，蒸発残留物，試験室間共同試験

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大野浩之*1，六鹿元雄，阿部智之*2，阿部裕，天野保希*3，石原絹代*4，大坂郁恵*5，大野春香*6，大野雄一郎*7，尾崎麻子*8，柿原芳輝*9，小林尚*10，櫻木大志*1，柴田博*11，城野克広*12，関戸晴子*13，高坂典子*14，竹中佑*15，但馬吉保*16，田中葵*17，田中秀幸*12，中西徹*4，野村千枝*8，羽石奈穂子*18，早川雅人*19，三浦俊彦*20，山口未来，山田恭平*21，渡辺一成*19，佐藤恭子：器具・容器包装における蒸発残留物試験の試験室間共同試験（第２報）．

食品衛生学雑誌 2018;59:64-71.

ヘプタンで調製した試験溶液を用い，油脂および脂肪性食品用器具・容器包装の蒸発残留物試験における試験室間共同試験を行い，公定法と公定法変法の性能を評価した．試験には23機関が参加し，濃度非明示の試験溶液9種類の蒸発残留物量を測定した．蒸発乾固の際の加熱装置として水浴を用いた場合を公定法とし，ホットプレートを使用した場合，ならびに蒸発乾固前の減圧濃縮を省略した場合を公定法変法とした．ほとんどの試験機関では，蒸発乾固の際，試験溶液を乾固直前まで加熱したのち，余熱で乾固させていた．その結果，加熱装置に関わらず，両法の性能には大きな差はないことが判明した．それにより，公定法変法は公定法と同様に規格試験法として適用できると判断された．さらに，95%エタノールおよびイソオクタンを浸出用液として用いた場合の性能についても検証したところ，それらの性能はヘプタンとほぼ同等であった．

Keywords：器具・容器包装，蒸発残留物，試験室間共同試験

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Asakura H, Kawase J*1, Ikeda T*2, Honda M*3, Sasaki Y, Uema M, Kabeya H*4, Sugiyama H*5, Igimi S*6, Takai S*7: Microbiological Quality Assessment of Game Meats at Retail in Japan.

J Food Prot. 2017;80:2119-2126.

Here we examined the prevalence of Shiga toxin-producing Escherichia coli (STEC) and *Salmonella* spp. and the distribution of indicator bacteria in 248 samples of game meats (120 venison and 128 wild boar) retailed in Japan. No *Salmonella* spp. were detected in any of the samples, whereas STEC OUT:H25 (*stx2d+, eae-*) was isolated from one deer meat sample, suggesting a possible source for human infection. Plate count assays indicated greater prevalence of coliforms and *E. coli* in wild boar meat than in venison, whereas their prevalence in processing facilities showed greater variation than in animal species. The 16S rRNA metagenomic analysis of 24 representative samples revealed that the abundances of *Acinetobacter* and *Arthrobacter* spp. significantly correlated with the prevalence of *E. coli,* and quantitative PCR analyses verified these correlations. To our knowledge, this is the first to characterize the diversity of microorganisms of game meats at retail in Japan, together with identification of dominant microbiota. Our data suggest the necessity of bottom-up hygienic assessment in areas of slaughtering and processing facilities to improve microbiological safety.

Keywords: game meat, microbiological quality, microbiota, shiga toxin-producing *E. coli* (STEC)

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Ohyama N*1, Torio M*1, Nakashima K*1, Koga Y*1, Kanno S*1, Nishio H*1, Nishiyama K*1, Sasazuki M*1, Kato H*2, Asakura H, Akamine S*1, Sanefuji M*1, Ishizaki Y*1, Sakai Y*1, Ohga S*1: A childhood-onset intestinal toxemia botulism during chemotherapy for relapsed acute leukemia.

Ann Clin Microbiol Antimicrob. 2017;16:61.

We report a 5-year-old boy, who developed general muscle weakness, constipation, ptosis and mydriasis during the third induction therapy for relapsed acute myeloid leukemia. Repeated bacterial cultures identified *Clostridium botulinum* producing botulinum neurotoxin A. Botulinum toxin A was isolated from his stools at 17, 21, and 23 days after the onset. Symptoms were self-limiting, and were fully recovered without anti-botulinum toxin globulin therapy. This is the second report of a pediatric case with cancer chemotherapy-associated intestinal toxemia botulism. Our case provides further evidence that the immunocompromised status due to anti-cancer treatments increases the risk for the development of botulism at all ages in childhood.

Keywords: acute leukemia, antibiotics, chemotherapy, intestinal toxemia botulism

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Kawase J*1, Asakura H, Kurosaki M*1, Oshiro H*1, Etoh Y*2, Ikeda T*3, Watahiki M*4, Kameyama M*5, Hayashi F*1, Kawakami Y*1, Murakami Y*1, Tsunomori Y*1: Rapid and accurate diagnosis based on real-time PCR cycle threshold value for the identification of *Campylobacter jejuni, astA* gene-positive *Escherichia coli,* and *eae* gene-positive *E. coli.*

Jpn J Infect Dis. 2018;71:79-84.

We previously developed a multiplex real-time PCR assay (RFBS24 ver.5) for simultaneous detection of 24 foodborne bacterial targets. Here we analyzed 246 human clinical samples from 49 gastroenteritis outbreaks using the RFBS24 ver.5 and evaluated the correlation between the CT value of RFBS24 ver.5 and the culture results. The RFBS24 ver.5 was more sensitive than culture methods for *Campylobacter jejuni* and E*scherichia coli* harboring *astA* or *eae*, with positive predictive values of 45.5-87.0% and a kappa coefficient of 0.60-0.92, respectively. All RFBS24 ver.5-positive samples were culture-positive under the lower confidence interval limit of 95% or 99% for the CT of the culture-negative samples.

Keywords: *Campylobacter,* diarrheagenic *E. coli,* gastroenteritis outbreak

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Asakura H, Yamamoto S, Momose Y, Kato H*, Iwaki M*, Shibayama K*: Genome sequence of *Clostridium botulinum* strain Adk2012 associated with a foodborne botulinum case in Tottori Japan in 2012.

Genome Announc. 2017;5:e00872-17.

We report here a draft genome sequence of Clostridium botulinum Adk2012 responsible for a foodborne botulism case that occurred in Tottori, Japan, in 2012. Its genome size was 2,904,173 bp, with 46 rRNAs and 54 tRNAs, at a coverage of 14.5x.

Keywords: *Clostridium botulinum,* genome, foodborne infection

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Asakura H, Takahashi N*, Yamamoto S, Maruyama H*: Draft Genome sequence of *Campylobacter jejuni* CAM970 and *C. coli* CAM962 associated with a large outbreak in Fukuoka Japan 2016.

Genome Announc. 2017;5:e00508-17.

Here we report draft genome sequences of *Campylobacter jejuni* CAM970 and *C. coli* CAM962 associated with a large foodborne outbreak by undercooked chicken sushi in Fukuoka Japan on May 2016. Their genome sizes ranged at 1,690,901 or 1,704,736 bp, with 22-23 rRNA and 9 tRNA on coverage of 411-419x.

Keywords: *Campylobacter jejuni*, genome, foodborne

infection

* Fukuoka City Institute of Health and Environment

佐々木貴正，関口秀人*，永井英貴*：牛および豚の消化器におけるカンピロバクター分布．
獣医畜産新報 2017(6);70:445-450.

牛および豚の消化器におけるカンピロバクターの分布状況を調査した．牛では，盲腸内容物（91%），直腸内容物（81%），十二指腸内容物（67%）および胆汁（43%）のカンピロバクター分離率が高かった．肝臓内部と胆汁のカンピロバクター分離には関連性が見られた（フィッシャーの正確確率：$P < 0.01$）．肝臓内部と胆汁からカンピロバクターが分離された5頭について，両部位から分離された*C. jejuni*は同一であった．豚でも，盲腸内容物（100%）および直腸内容物（96%）のカンピロバクター分離率が高かった．胆汁および十二指腸内容物からカンピロバクターは分離されなかったが，1頭（4%）の肝臓内部から*C. lanienae*が分離された．以上の結果から，十分に加熱されていない牛及び豚の肝臓の摂食は，カンピロバクター食中毒のリスクを増加させることが示唆された．

Keywords：カンピロバクター，牛，豚，消化器，肝臓

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佐々木貴正，関口秀人*，小佐々隆志*，永井英貴*：牛の移動に伴うフルオロキノロン耐性カンピロバクターの伝播の可能性．
獣医畜産新報 2017(8);70:601-604.

肉用牛農場におけるフルオロキノロン（FQ）耐性カンピロバクターのリスク管理の基礎資料とするため，5年以上抗菌剤使用歴がない1肉用牛農場の協力の下，FQ不使用農場におけるFQ耐性カンピロバクターの持続的存在及び牛の移動に伴うFQ耐性カンピロバクターの伝播の可能性について調査した．2回の農場検査で合計32棟の牛から分離された*Campylobacter jejuni*の17株のうち9株（53%），*C. coli*の11株のうち7株（70%）がFQ耐性であった，さらに，農場検査から1～3か月後に農林水産省動物医薬品検査所へ移動して計13に行った搬入時検査において，分離された*C. jejuni*の9株のうち3株（33%），*C. coli*の2株すべてがFQ耐性であった．以上から，FQ不使用肉用牛農場においてFQ耐性カンピロバクターが持続的に存在する可能性および牛の移動に伴うFQ耐性カンピロバクター伝播の可能性が示唆された．

Keywords：カンピロバクター，牛，豚，フルオロキノロン耐性

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Sasaki Y, Haruna M*, Uema M, Noda M, Yamada Y*: Prevalence and phylogenetic analysis of hepatitis E virus in pigs in Japan.
Jpn J Infect Dis. 2018;71:75-78.

The number of reported cases of human hepatitis E virus (HEV) infection has increased since 2012. It is possible that the prevalence of HEV in pigs at slaughter age (approximately six months old) has increased in the last decade. Therefore, we investigated the current prevalence of HEV in pigs in Japan. Although HEV RNA was detected in rectal content samples from pigs aged from one to five months, no HEV RNA was detected in any samples from six-month-old pigs. The highest viral shedding rate (33%) was found in three-month-old pigs. This study shows that there has been no change in the prevalence of HEV in pigs at slaughter age, the prevalence of HEV by age group on pig farms, or the phylogenetic classification of HEV isolates in the last decade.

Keywords: hepatitis E virus, pig, Japan

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Nakayama T, Nguyen CH*[1], Phong QL*[2], Kawahara R*[3], Kumeda Y*[4], Sumimura Y*[5], Yamamoto Y*[5]: Consumption of edible ice contaminated with *Acinetobacter, Pseudomonas,* and *Stenotrophomonas* is a risk factor for fecal colonization with extended-spectrum beta-lactamase-producing *Escherichia coli* in Vietnam.
J Water Health. 2017;15:813-822.

The aim of this study was to evaluate the frequency with which edible ice served in restaurants is contaminated with antibiotic-resistant bacteria. Ice from restaurants in Vietnam and Japan was screened for bacteria capable of growing on agar containing cefotaxime. 40%, 39%, and 12% were identified as *Pseudomonas* spp., *Acinetobacter* spp., and *Stenotrophomonas maltophilia*, respectively. Meanwhile, of the six such strains isolated in Japan, five were identified as *Acinetobacter* spp. and one as

Pseudomonas spp. More than 10% of the *Acinetobacter* isolates exhibited cefotaxime, ceftazidime, and sulfa/trimethoprim resistance, while 21% of *Pseudomonas* and 14% of S. *maltophilia* isolates exhibited meropenem and sulfa/ trimethoprim resistance, respectively.
Keywords: *Acinetobacter*, edible ice, ESBL-producing bacteria, *Pseudomonas, Stenotrophomonas*

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Nakayama T, Kawahara R*1, Kumeda Y*2, Yamamoto Y*3: Extended-spectrum beta-lactamase-producing *Escherichia coli* contributes to the survival of cefotaxime-susceptible *E. coli* under high concentrations of cefotaxime by acquisition of increased AmpC expression.
FEMS Microbiol Lett. 2018;365:fny009

We investigated the effect of oral administration of ESBL-producing *E. coli* (TB19) and cefotaxime (CTX) on luminescence-emitting CTX-sensitive *E. coli* (X14). Mice were given water containing TB19 and X14. The mice were administered CTX and luminescent bacteria were monitored, following which luminescent bacteria were isolated from mouse feces. Luminescence continued to be detected in mice administered TB19 24 h after CTX ingestion. Fecal analysis revealed two types of luminescent colonies, cefoxitin-resistant *E. coli* (X14-R) and *Pseudomonas aeruginosa*. PFGE confirmed that X14-R was a clonal strain of X14, Overall, ESBL-E and cefotaxime promoted the expansion of cefoxitin-resistant E. coli.
Keywords: AmpC beta-lactamase-producing *Escherichia coli,* extended-spectrum beta-lactamase-producing *Escherichia coli,* luminescent *Escherichia coli,* cefotaxime

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Nakayama T, Kumeda Y*1, Kawahara R*2, Yamaguchi T*2, Yamamoto Y*3: Carriage of colistin-resistant, extended-spectrum beta-lactamase-producing *Escherichia coli* harboring the *mcr*-1 resistance gene after short-term international travel to Vietnam.
Infect Drug Resist. 2018;11:391-395.

The aim of this study was to determine whether a traveler on a short-term international trip to a developing country could bring *mcr*-1 back to their home country. Genotyping of ESBL-producing isolates showed that $bla_{\text{CTX-M-1}}$/bla_{TEM} (27.7%) and $bla_{\text{CTX-M-9}}$ (45.9%) were the most prevalent genotypes, while the most frequently detected phylogenetic group was D (41.9%) followed by B2 (23.0%). In a significant number of travel events, travelers brought ESBL-producing *E. coli* back to Japan and three events by three travelers carried *mcr*-1. ESBL-producing *E. coli* isolates harboring *mcr*-1 were identified as those carrying both $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-55}}$ and *mcr*-1. We have shown that even a short-term trip to some countries may result in ESBL-producing *mcr*-1-positive *E. coli* carriage by international travelers.
Keywords: traveler, ESBL-producing *E. coli, mcr*-1, Vietnam, Japan

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池原強*1，木下翼*1，黒川純花*1，中島志穂子*2，前川公彦*3，大城直雅，安元健*4：タンパク質脱リン酸化酵素２Ａ（PP2A）を利用した下痢性貝毒簡易検査法の評価.
日本水産学会誌 2017;83:367-372.

バキュロウイルス―昆虫細胞発現系を利用して生産した高純度で酵素活性が安定したタンパク質脱リン酸化酵素２Ａ（PP2A）を使用した下痢性貝毒簡易検査法（PP2A阻害法）について，脂質含量の異なるホタテガイ可食部を試料として評価を行った．検出限界，定量限界は，それぞれ0.0262 mg/kg, 0.0470 mg/kgであった．試料の脂質含量の違いは定量結果に影響を与えないことも示された．PP2A阻害法は高感度・再現性に優れた迅速・簡便な検査法であり，スクリーニング法として有効であると考えられる．
Keywords：OAs，PP2A阻害法，下痢性貝毒，スクリーニング法

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Ikehara T*1, Kuniyoshi K, Oshiro N, Yasumoto T*2: Biooxidation of ciguatoxins leads to species-specific toxin profiles.
Toxins. 2017;9:205.

Ciguatoxins (CTXs) contaminate fish worldwide and cause the foodborne illness ciguatera. In the Pacific, these toxins are produced by the dinoflagellate *Gambierdiscus toxicus,* which accumulates in fish through the food chain and undergoes oxidative modification, giving rise to numerous analogs. In this study, we examined the oxidation of CTXs in vitro with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using reference toxins, and found that CTX4A, CTX4B, and CTX3C, which are produced by the alga, are oxidized to the analogs found in fish, namely CTX1B, 52-epi-54-deoxyCTX1B, 54-deoxyCTX1B, 2-hydroxyCTX3C, and 2,3-dihydroxyCTX3C. This oxidation was catalyzed by human CYP3A4, fish liver S9 fractions, and microsomal fractions prepared from representative ciguateric fishes (*Lutjanus bohar, L. monostigumus,* and *Oplegnathus punctatus*). In addition, fish liver S9 fractions prepared from non-ciguateric fishes (*L. gibbus* and *L. fulviflamma*) in Okinawa also converted CTX4A and CTX4B to CTX1B, 54-deoxyCTX1B, and 52-epi-54-deoxyCTX1B in vitro. This is the first study to demonstrate the enzymatic oxidation of these toxins, and provides insight into the mechanism underlying the development of species-specific toxin profiles and the fate of these toxins in humans and fish.
Keywords: ciguatera, ciguatoxin, in vitro oxidation, fish liver S9, Cyp3A4

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Ogihara H*1, Suzuki H*2, Michishita M*3, Hatakeyama H*3, Okada Y: Effects of high hydrostatic pressure processing on the number of bacteria and texture of beef liver.
J Food Qual 2017;ID7835714

Providing beef liver for raw consumption was banned in Japan on July 1, 2012. To lift the ban, the establishment of effective countermeasures for safe raw consumption is necessary. In this study, we examined the effects of high hydrostatic pressure processing on raw beef liver. Beef liver samples subjected to 300 MPa of pressure or higher for 10 min at 25℃ became firmer and showed a paler color and were considered unsuitable for raw consumption. More than 3.0 log reductions of bacteria were seen after treatments at 400 and 500 MPa, but the treatment with lower pressure did not show enough microcidal effects for safe consumption. Histological and ultrastructural analysis revealed that high hydrostatic pressure processing increased mitochondrial swelling and reduce rough endoplasmic reticula in hepatocytes, and such changes might be related to the observed changes of texture in the treated raw beef liver.
Keywords: high hydrostatic pressure, bacteria

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Teramura H*1, Fukuda N*2, Okada Y, Ogihara H*2: Comparison of chromogenic selective media for the detection of *Cronobacter* spp. (*Enterobacter sakazakii*).
Biocontrol Sci. 2018;23:27-33.

The four types of chromogenic selective media that are commercially available in Japan were compared for establishing a Japanese standard method for detecting *Cronobacter* spp. based on ISO/TS 22964:2006. When assessed using 9 standard *Cronobacter* spp. strains and 29 non-*Cronobacter* strains, *Enterobacter sakazakii* isolation agar, Chromocult™ *Enterobacter sakazakii* agar, CHROMagar™ *E. sakazakii,* and XM-sakazakii agar demonstrated excellent inclusivity and exclusivity. Using the ISO/TS 22964:2006 method, the recovered numbers of 38 *Cronobacter* spp. strains, including 29 *C. sakazakii* isolates obtained from each medium, were equivalent indicating that there was no significant difference ($p > 0.05$) among the four types of chromogenic selective media. Thus, we demonstrated that these four chromogenic selective media are suitable alternatives for using in the standard ISO/TS 22964:2006 method in Japan for detecting *Cronobacter* spp.
Keywords: *Cronobacter* spp, *Enterobacter sakazakii*, chromogenic medium, detection, ISO/TS 22964:2006

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Imamura S*1, Kanezashi H*1, Goshima T*1, Haruna M*1, Okada T*2, Inagaki N*3, Uema M, Noda M, Akimoto K*1: Next-Generation Sequencing Analysis of the Diversity of Human Noroviruses in Japanese Oysters.
Foodborne Pathog Dis. 2017;14(8):465-471.

To obtain detailed information on the diversity of infectious norovirus in oysters (*Crossostrea gigas*), oysters obtained from fish producers at six different sites (sites A, B, C, D, E, and F) in Japan were analyzed once a month during the period spanning October 2015–February 2016. To avoid false-positive polymerase chain reaction (PCR) results derived from noninfectious virus particles, samples were pretreated with RNase before reverse transcription-PCR (RT-PCR). RT-PCR products were subjected to next-generation sequencing to identify norovirus genotypes in oysters. As a result, all GI genotypes were detected in the investigational period. The detection rate and proportion of norovirus GI genotypes differed depending on the sampling site and month. GII.3, GII.4, GII.13, GII.16, and GII.17 were detected in this study. Both the detection rate and proportion of norovirus GII genotypes differed depending on the sampling site and month. In total, the detection rate and proportion of GII.3 were highest from October to December among all detected genotypes. In January, the detection rates of GII.4 and GII.17 reached the same level as that of GII.3. The proportion of GII.17 was relatively lower from October to December, whereas it was the highest in January. To our knowledge, this is the first investigation on noroviruses in oysters in Japan, based on a method that can distinguish their infectivity.
Keywords: Japanese oyster, enzymatic pretreatment, infectious noroviruses, next-generation sequencing

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Imamura S*1, Kanezashi H*1, Goshima T*1, Suto A*2, Ueki Y*3, Sugawara N*3, Ito H*4, Zou B*5, Uema M, Noda M, Akimoto K*1: Effect of High-Pressure Processing on Human Noroviruses in Laboratory-Contaminated Oysters by Bio-Accumulation.
Foodborne Pathog Dis. 2017;14(9):518-523.

The contamination of oysters with human noroviruses poses a human health risk, since oysters are often consumed raw. In this study, human norovirus genogroup II was allowed to bio-accumulate in oysters, and then the effect of high-pressure processing (HPP) on human noroviruses in oysters was determined through a polymerase chain reaction (PCR)-based method with enzymatic pretreatment to distinguish infectious noroviruses. As a result, oysters could be artificially contaminated to a detectable level of norovirus genome by the reverse transcription-PCR. Concentrations of norovirus genome in laboratory-contaminated oysters were log normally distributed, as determined by the real-time PCR, suggesting that artificial contamination by bio-accumulation was successful. In two independent HPP trials, a 1.87 log10 and 1.99 log10 reduction of norovirus GII.17 genome concentration was observed after HPP at 400MPa for 5min at 25° C. These data suggest that HPP is a promising process of inactivation of infectious human noroviruses in oysters. To our knowledge, this is the first report to investigate the effect of HPP on laboratory-contaminated noroviruses in oysters.
Keywords: Japanese oyster, enzymatic pretreatment, high-pressure processing, norovirus

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Kanayasu-Toyoda T, Ishii-Watabe, Kikuchi Y, Kitagawa H, Suzuki H, Tamura H*1, Tada M, Suzuki T*1, Mizuguchi H*2, Yamaguchi T: Occludin as a functional marker of vascular endothelial cells on tube-forming activity.
J Cell Physiol. 2018;233:1700-11

Cell therapy using endothelial progenitor cells (EPCs) is a promising strategy for the treatment of ischemic diseases. Two types of EPCs have been identified: early EPCs and late EPCs. Late EPCs are able to form tube structure by themselves, and have a

high proliferative ability. The functional marker(s) of late EPCs, which relate to their therapeutic potential, have not been fully elucidated. Here we compared the gene expression profiles of several human cord blood derived late EPC lines which exhibit different tube formation activity, and we observed that the expression of occludin (OCLN) in these lines correlated with the tube formation ability, suggesting that OCLN is a candidate functional marker of late EPCs. When OCLN was knocked down by transfecting siRNA, the tube formation on Matrigel, the S phase+G_2/M phase in the cell cycle, and the spheroid - based sprouting of late EPCs were markedly reduced, suggesting the critical role of OCLN in tube formation, sprouting, and proliferation. These results indicated that OCLN plays a novel role in neovascularization and angiogenesis.

Keywords: late endothelial progenitor cell, occludin, therapeutic angiogenesis

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Wang L*1,2, Zhang S*2, Zheng D*2, Fujihara S*3, Wakabayashi A*4, Okahata K*4, Suzuki M*4, Saeki A*5, Nakamura H*6, Hara- Kudo Y*7, Kage-Nakadai E*2,8, Nishikawa Y*2: Prevalence of diarrheagenic *Escherichia coli* in foods and fecal specimens obtained from cattle, pigs, chickens, asymptomatic carriers and patients in Osaka and Hyogo, Japan.
Japanese Journal of Infectious Diseases. 2017;70:464-469

The source and routes of diarrheagenic *Escherichia coli* (DEC) remain poorly understood. To investigate the involvement of domestic animals in the dissemination of DEC, the prevalence of DEC in foods and fecal specimens from cattle, pigs, chickens, healthy carriers, and patients in Osaka and Hyogo, Japan was investigated using a multiplex real-time polymerase chain reaction assay. The most abundant virulence genes were astA and eae, which had a prevalence 46.8% and 27.4%, respectively. Additionally, stx1 (26.6%) and stx2 (45.9%) were prevalent in cattle feces, while est (8.5%) and elt (7.6%) were prevalent in pig feces. afaB was the second-most prevalent gene in patients and healthy carriers, and it had detection rates of 5.1% and 8.1%, respectively. In contrast, afaB was not detected in animal feces or foods, except for three porcine fecal samples. The aggR gene was more prevalent in humans than in foods or animal feces. Both Shiga toxin-producing *E. coli* and atypical enteropathogenic *E. coli* carried by cattle may be sources for diarrheal diseases in humans. Pigs may be a source for human enterotoxigenic *E. coli* infections, whereas humans are expected to be the reservoir for diffusely adhering *E. coli*, enteroaggregative *E. coli*, and enteroinvasive *E. coli*.

Keywords: diarrheagenic *Escherichia coli*, prevalence, real-time polymerase chain reaction

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Wang L*1,2, Hara-Kudo Y*3, Kage-Nakadai E*2 Nakamura H*4, Nishikawa Y*2: Prevalence, antimicrobial resistance and multiple-locus variable-number tandem-repeat analysis profiles of diarrheagenic *Escherichia coli* isolated from different retail foods.
Int J Food Microbiol. 2017;249:44-52

Diarrheagenic *E. coli* (DEC) isolates were recovered from local retail markets and the Osaka Municipal Central Wholesale Market in Japan. Retail food samples were collected for analysis in Osaka Japan from 2005 to 2008 and consisted of 32 beef, 28 pork, 20 poultry, 136 fish, 66 fruits and vegetables and 51 ready–to-eat (RTE) food samples. A total of 82 DEC strains were recovered from 64 (19%) food samples with the highest prevalence in poultry (100%, 20/20), followed by pork (54%, 15/28), beef (28%, 9/32), fruits and vegetables (12%, 8/66), fish (6.6%, 9/136) and RTE foods (5.9%, 3/51). Most of the strains belonged to *E.*

coli possessing the enteroaggregative *E. coli* (EAEC) heat-stable enterotoxin 1 (EAST1) gene (EAST1EC; n=62, P b 0.0001) and enteropathogenic *E. coli* (EPEC; n=16, P b 0.01), whereas only 1 strain belonged to Shiga toxin-producing *E. coli* (STEC), 1 to EAEC and 2 to enterotoxigenic *E. coli* (ETEC) strains. Of the 82DEC isolates, 22O and 13H serogroupswere detected, including some specific serogroups (O91, O103, O115, O119, O126, and O157) which have been associated with human diarrheal infections. Phylogenetic group A and B1 were predominant among the DEC isolates. Antimicrobial resistance to tetracycline was most common (49%), followed by nalidixic acid (28%), ampicillin (24%), sulfamethoxazole/trimethoprim (20%), and cephalothin (18%). Multiple-locus variable-number tandemrepeat analysis (MLVA) was used in this study for genotyping of DEC. The 82 isolates collected for this study showed 77 distinct MLVA profiles located among 3 branches. In conclusion, retail food samples in Japan were contaminated with DEC; EAST1EC, a putative DEC, were detected at high rates in poultry, pork and beef. Isolates resistant to N3 antimicrobials were found only in raw meat and fish. Food animals may act as the reservoir for multi-resistant bacteria. Due to the finding that nearly 1/3 of EAST1EC strains were resistant to N3 antimicrobials, additional surveillance for EAST1EC should be initiated.

Keywords: diarrheagenic *Escherichia coli*, prevalence, food

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日本食品微生物学会雑誌 2017;34(4):202-206

食品の物理的または化学的性質の影響で細菌学的試験の実施が困難になる食品の例として，固形化成分を含有する粉末清涼飲料を取り上げ，細菌数測定法の改善について検討した．告示法の細菌数測定法に従い検体の10倍乳剤を調製した場合，調製直後にゲル化し，混釈培養への供試が困難であるため，乳剤のゲル化を弱める目的で検体の100倍乳剤を調製し，乳剤調製から混釈培養まで５分以内で試験する方法を検討した．その結果，良好な回収率が得られ，告示法と同等の検出感度での細菌数の検査が実施できた．また，200倍乳剤を使用することも試験に適すると考えられた．本研究で示された優れた方法は，同様の性質を示す他の食品の細菌数測定にも応用が可能であると考えられる．

Keywords：粉末清涼飲料，固形化成分，細菌数測定法

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Oshikata C*1,2, Watanabe M, Saito A*3, Ishida M*4, Kobayashi S*4, Konuma R*5, Kamata Y*6, Terajima J, Cho J*7, Yanai M*4, Tsurikisawa N*1,2: Allergic bronchopulmonary mycosis caused by *Eurotium herbariorum* suffered after the Great East Japan Earthquake.
Prehosp Disaster Med. 2017;32(6):688-690

Case Presentation A 66-year-old, Japanese male, ex-smoker had been diagnosed with bronchial asthma when he was five years old; he achieved remission at the age of 13 years. He was displaced from his home during the Great East Japan Earthquake on March 11, 2011 and moved to temporary housing in Miyagi Prefecture in June 2011. Mycofloral surveillance detected high counts of *Eurotium* in the air of his bedroom, kitchen, and living room, with a maximal fungal count of 163,200 colony-forming units per cubic meter (CFU/m^3). Morphologic identification confirmed the isolates as *E. herbariorum*. The patient had positive reactions to E. herbariorum in skin prick testing and the presence of antigen-specific precipitating antibodies to E. herbariorum. Computed tomography of the chest in August 2013 revealed central bronchiectasis and bronchial wall thickening. The patient experienced late reactions after provocation testing with E. herbariorum. This report presents the rare case of a patient who developed allergic bronchopulmonary mycosis (ABPM) due to exposure to *E. herbariorum* during temporary housing

after the Great East Japan Earthquake.
Keywords: Allergic bronchopulmonary mycosis, Great East Japan Earthquake, *Eurotium herbarirum*

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Furukawa I*1, Ishihara T*1, Teranishi H*2, Saito S*3, Yatsuyanagi J*4, Wada E*4, Kumagai Y*4, Takahashi S*4, Konno T*4, Kashio H*4, Kobayashi A*5, Kato N*5, Hayashi KI*6, Fukushima K*6, Ishikawa K*6, Horikawa K*7, Oishi A*7, Izumiya H*8, Ohnishi T, Konishi Y*9, Kuroki T*1: Prevalence and Characteristics of *Salmonella* and *Campylobacter* in Retail Poultry Meat in Japan.
Jpn. J. Infect. Dis. 2017;70:239-47

This study was performed to determine the prevalence, antimicrobial susceptibility, and genetic relatedness of *Salmonella* enterica subsp. enterica and *Campylobacter* spp. in poultry meat, and to analyze the association of genetic types of these bacteria with their geographical distribution and antimicrobial resistance profiles. *Salmonella* and *Campylobacter* isolates have been detected, respectively, in 54 and 71 samples out of 100 samples tested. Nine *Salmonella* serotypes were found, including *S.* enterica subsp. enterica serovar Infantis (33%), Schwarzengrund (12%), Manhattan (9%), and others. *Campylobacter jejuni* and *C. coli* were detected in 64 (64%) and 14 (14%) samples, respectively. S. enterica subsp. enterica isolates were very frequently resistant to tetracycline (78.3%) and streptomycin (68.3%). Many *C. jejuni* and *C. coli* isolates were resistant to sulfamethoxazole/trimethoprim (90.5%), nalidixic acid (47.3%), ampicillin (45.9%), and ciprofloxacin (40.5%). Cluster analysis was performed for the Salmonella isolates using pulsed-field gel electrophoresis (PFGE) data. For *Campylobacter* isolates, the cluster analysis was based on both PFGE and comparative genomic fingerprinting. The molecular typing results were compared with the information about antimicrobial resistance and geographical locations in which the poultry meat was produced. This analysis revealed that *C. jejuni* strains with a particular genotype and antimicrobial resistance profile are spreading in specific areas of Japan.
Keywords: *Campylobacter, Salmonella,* PFGE

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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;116:425-7

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℃

for up to 16 months. Although the spores stored at −80℃ 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℃ 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, Sea-food

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Irikura D[*1], Saito M[*2], Sugita-Konishi Y[*3], Ohnishi T, Sugiyama KI, Watanabe M, Yamazaki A[*4], Izumiyama S[*5], Sato H[*4], Kimura Y[*4], Doi R[*6], Kamata Y[*4]: Characterization of *Sarcocystis fayeri's* actin-depolymerizing factor as a toxin that causes diarrhea.
Genes Cells 2017;22:825-35

Raw horsemeat has the potential to induce food poisoning which often presents with diarrheal symptoms. A sample of horsemeat was found to be infected with *Sarcocystis fayeri*, and a 15-kDa protein isolated from the cysts of *S. fayeri* was found to clearly show its diarrhea-inducing activity. A nested polymerase chain reaction was used to clone the cDNA of the 15-kDa protein. The deduced amino acid sequence showed homology to actin-depolymerizing factor (ADF). A recombinant 15-kDa protein depolymerized prepolymerized actins in a test tube. The 15-kDa protein possessed conserved amino acid sequences of ADF of *Toxoplasma gondii* and *Eimeria tenella*. These characteristics indicate that the 15-kDa protein of *S. fayeri* belongs to the ADF/cofilin protein family. The recombinant 15-kDa protein evoked fluid accumulation in the looped ileum, resulting in diarrhea, but it did not kill the cultured fibroblast cells, macrophages or intestinal mucosal cells. In addition, the culture supernatant of the macrophages treated with the recombinant 15-kDa protein killed the fibroblast L929 cells. This fact indicates that ADF of *S. fayeri* induced cytotoxic substances, such as tumor necrosis factor-α, according to the published reports. Although further experiments are needed now to elucidate the enterotoxic mechanism of *S. fayeri's* ADF, our findings may offer new insight into research on parasites and parasite-instigated food poisoning.
Keywords: *Sarcocystis*, Toxin, diarrhea

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Ohnishi T, Kubo A, Yoshinari T, Watanabe M: A *Kudoa septempunctata* antigen induces production of IgE in BALB/c mice.
Parasitol. Res. 2017;113:303-6

Kudoa septempunctata, a myxosporean parasite, is the causative agent of a foodborne illness associated with consumption of raw *Paralichthys olivaceus* (olive flounder). Because the lag phase of this illness is short (from 1 to 12 h), it is possible that an allergic response is relevant to this illness. To test whether a *K. septempunctata* antigen is the possible allergen, we injected a myxospore extract into BALB/c mice and measured IgE levels in serum. When the mice were injected with the myxospore extract, the total serum IgE concentration increased significantly after the second immunization as compared to the negative control. After the third immunization, total IgE concentration in the immunized mice reached 26.5 ng/ml and was almost equivalent to that of egg albumin-injected mice. Western blot analysis revealed that IgE antibodies-in serum samples that were collected from myxospore extract-injected mice-bound to at least two *Kudoa* proteins with molecular weight between 28 and 36 kDa. These results suggested that a *K. septempunctata* antigen is the allergen. Further studies are needed to clarify the contribution of allergy to the foodborne illness caused by *K. septempunctata*.
Keywords: *Kudoa*, Allergen, Foodborne illness

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大西貴弘，小原徹也*，新井沙倉，吉成知也，小西良子*：カンパチの生食に伴う有症苦情事例残品中の

*Unicapsula seriolae*寄生量の定量的解析の検討.
食品衛生学雑誌 2018;59:24-9

カンパチの生食に伴う有症苦情29事例の喫食残品中に含まれる*Unicapsula seriolae*の定量を行った．定量リアルタイムPCR（qRT-PCR）を用いて検体中の*U. seriolae* 18S rDNAを検出したところ，26検体で陽性となった．*U. seriolae* DNAが検出された事例の潜伏時間は１～12時間付近に集中（77%）していた．事例の発生に明瞭な季節性は認められなかった．患者の主な症状は下痢，嘔吐であった．*U. seriolae* DNAが検出された事例残品中の胞子数を測定したところ１グラム当たり1.9×105個から1.7×10^7個だった．しかし，市場で購入したカンパチから定量限界値以上の胞子は検出されなかったことから，事例の発生に*U. seriolae*が関与している可能性が示唆された．胞子数とDNAコピー数の相関性は低かったが，胞子を計数できた事例のDNAコピー数は１グラム当たり10^7コピー以上だった．喫食量が判明している11事例について摂取胞子数を推定したところ，最小で3.8×10^6個であった.

Keywords：*Kudoa*，*Unicapsula*，カンパチ

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Yoshinari T, Sugita-Konishi Y*, Ohnishi T, Terajima J: Inhibitory activities of blasticidin S derivatives on aflatoxin production by *Aspergillus flavus*.
Toxins (*Basel*). 2017;9:176

Blasticidin S (BcS) is a protein synthesis inhibitor which shows strong growth inhibitory activity against a number of microorganisms. However, BcS inhibited aflatoxin production by Aspergillus flavus without affecting its growth. In order to obtain information about the structure–activity relationship of BcS as an aflatoxin production inhibitor, BcS derivatives were prepared and their aflatoxin production inhibitory activities were evaluated. Among five derivatives, blasticidin S carboxymethyl ester, deaminohydroxyblasticidin S, and pyrimidinoblasticidin S showed inhibitory activity, while the others did not. The IC50 value for aflatoxin production of the carboxymethyl ester derivative was one-fifth of that of BcS although their antimicrobial activities were almost the same.

Keywords: aflatoxin, blasticidin S, inhibitor

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Gratz SW*[1], Dinesh R*[1], Yoshinari T, Holtrop G*[2], Richardson AJ*[1], Duncan G*[1], MacDonald S*[3], Lloyd A*[3], Tarbin J*[3]: Masked trichothecene and zearalenone mycotoxins withstand digestion and absorption in the upper GI tract but are efficiently hydrolyzed by human gut microbiota in vitro.
Mol Nutr Food Res. 2017;61:1600680

Masked mycotoxins were incubated with artificial digestive juices and absorption was assessed in differentiated Caco-2/TC7 cells. All masked mycotoxins were stable under upper GI tract conditions and no absorption was observed. Free trichothecenes were absorbed intact whereas free zearalenone compounds were absorbed and metabolized to undetected compounds by Caco-2/TC7 cells. Human gut microbiota efficiently hydrolyzed all masked mycotoxins. Trichothecenes were fully recovered as parent mycotoxins whereas 40-70% of zearalenone compounds were further metabolized to unknown metabolites. Our results demonstrate that masked trichothecenes will reach the colon intact to be released as parent mycotoxins by gut microbiota, hence contributing to mycotoxin exposure.

Keywords: masked mycotoxin, trichothecene, gut microbiota

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Furukawa T*, Yoshinari T, Sakuda S*: Intracellular superoxide level controlled by manganese superoxide dismutases affects trichothecene production in *Fusarium graminearum*.
FEMS Microbiol Lett. 2017;364:213

The intracellular superoxide level is a clue to clarification of the regulatory mechanism for mycotoxin production in *Fusarium graminearum*. In this study, we focused on two manganese superoxide dismutases (SODs) of the fungus, FgSOD2 and FgSOD3, to investigate the relationship of the superoxide level to trichothecene production. Recombinant FgSOD2 and FgSOD3 showed SOD activity, and they were localized mainly in the mitochondria and cytoplasm, respectively. Significant increases in the cytosolic and mitochondrial superoxide levels were observed in ΔFgSod2 and ΔFgSod3, respectively. These results

suggested that the cellular superoxide level affects trichothecene production in *F. graminearum*.
Keywords: *Fusarium graminearum*, manganese superoxide dismutase, trichothecene

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Tohya M[*1], Watanabe T[*1], Maruyama F[*2], Arai S, Ota A[*2], Athey TB[*3], Fittipaldi N[*3,4], Nakagawa I[*2], Sekizaki T[*1]: Comparative genome analyses of *Streptococcus suis* isolates from endocarditis demonstrate persistence of dual phenotypic clones. *PLoS One* 2016;11:e0159558

Many bacterial species coexist in the same niche as heterogeneous clones with different phenotypes; however, understanding of infectious diseases by polyphenotypic bacteria is still limited. In the present study, encapsulation in isolates of the porcine pathogen *Streptococcus suis* from persistent endocarditis lesions was examined. Coexistence of both encapsulated and unencapsulated *S. suis* isolates was found in 26 out of 59 endocarditis samples. The isolates were serotype 2, and belonged to two different sequence types (STs), ST1 and ST28. The genomes of each of the 26 pairs of encapsulated and unencapsulated isolates from the 26 samples were sequenced. The data showed that each pair of isolates had one or more unique nonsynonymous mutations in the cps gene, and the encapsulated and unencapsulated isolates from the same samples were closest to each other. Pairwise comparisons of the sequences of cps genes in 7 pairs of encapsulated and unencapsulated isolates identified insertion/deletions (indels) ranging from one to 104 bp in different cps genes of unencapsulated isolates. Capsule expression was restored in a subset of unencapsulated isolates by complementation in trans with *cps* expression vectors. Examination of gene content common to isolates indicated that mutation frequency was higher in ST28 pairs than in ST1 pairs. Genes within mobile genetic elements were mutation hot spots among ST28 isolates. Taken all together, our results demonstrate the coexistence of dual phenotype (encapsulated and unencapsulated) bacterial clones and suggest that the dual phenotypes arose independently in each farm by means of spontaneous mutations in cps genes.
Keywords: *Streptococcus suis*, endocarditis, genome analyses

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窪崎敦隆：細菌と真菌の同時検出を達成する群集構造解析条件とバリデーション標準品の検討. *医薬品医療機器レギュラトリーサイエンス* 2017;48: 346-351

第十七改正日本薬局方の参考情報として微生物迅速試験法が収載され，この中には，ハイスループット・シークエンシングが欧米薬局方に先駆けて記載された．本参考情報の中には，試験法の理念が書かれている一方で，具体的な実験条件は示されていないことから，本研究において，細菌だけではなく，真菌も解析できる実用的で簡便な同時網羅検出法について検討した．本研究では，一定量の微生物が含まれるように作製されている日本薬局方標準菌株商品BioBallを研究試料として用いた．細菌の16S rRNA V3-V4領域と真菌のITS1領域を増幅できるプライマーを設計し，非標的な増幅が起きない組合せを選択することでシーケンスライブラリを調整した．作製されたシーケンスライブラリは次世代シーケンサーMiSeqを用いて両端300塩基を決定して解析に供した．その結果，BioBallに含まれる6菌種について属レベルでの検出に成功し，具体的な解析手順を提示することが出来た．本研究成果は，欧米の薬局方で導入が進められているModern MicrobiologicalMethodsの議論に良い影響を与えると考えられた．
Keywords：微生物迅速試験，ハイスループット・シークエンシング，網羅検出法

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 receptor (ER) is a family of nuclear receptors (NRs) that regulates physiological effects such as reproduction and bone homeostasis. It has been reported that approximately 70% of human breast cancers are hormone-dependent and ER α-positive. Recently, novel anti-breast cancer drugs based on different mechanisms of action have been 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 the ERα through the ubiquitin-proteasome system. We also performed the computational docking analysis to predict the binding mode of PBP-NC10 to ERα.

Keywords: estrogen receptor, degradation inducer, diphenylheptane skeleton

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Misawa T, Tanaka K, Demizu Y, Kurihara M: Efficient synthesis of a multi-substituted diphenylmethane skeleton as a steroid mimetic.
Bioorganic and Medicinal Chemistry. 2017;27:2590-3

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, steroid, diphenylheptane skeleton

Okitsu K, Misawa T, Shoda T, Kurihara M, Demizu Y: Development of an ON/OFF switchable fluorescent probe targeting His tag fused proteins in living cells.
Bioorganic and Medicinal Chemistry. 2017;27: 3417-22

The fluorescent labeling of target proteins is useful for analyzing their functions and localization in cells, and several fluorescent probes have been developed. However, the fusion of tags such as green fluorescent protein (GFP) to target proteins occasionally affects their functions and/or localization in living cells. Therefore, an imaging method that uses short peptide tags such as hexa-histidine (the His tag) has been attracting increasing attention. Few studies have investigated ON/OFF switchable fluorescent probes for intracellular His-tagged proteins. We herein developed a novel ON/OFF switchable probe for imaging targeted intracellular proteins fused with a CH6 tag, which is composed of one cysteine residue and six histidine residues.

Keywords: on/off switchable probe, His tag, green fluorescent protein

Koba Y[*1], Ueda A[*1], Oba M[*1], Doi M[*2], Demizu Y, Kurihara M[*3], Tanaka M[*1]: Helical L-Leu-based peptides having chiral five-membered carbocyclic ring amino acids with an ethylene acetal moiety.
ChemistrySelect. 2017;2:8108-14

l-Leu-based heteropeptides having (R)-or (S)-chiral five-membered carbocyclic ring amino acids (Ac5c3EG) with an ethylene acetal moiety were prepared. A conformational analysis using FT-IR absorption, 1HNMR, and circular dichroism (CD) spectra revealed that l–Leu-based hexapeptides and nonapeptides having (R)- or (S)-Ac5c3EG formed right-handed (P) helical structures in solution. An X-ray crystallographic analysis of nonapeptides 5a and 5b showed similar right-handed (P) α-helical structures, without an intramolecular hydrogen bond of the peptide N–H・・・–O– (acetal) type.

Keywords: conformational analysis, helical structure, peptides

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Furukawa K[*1], Oba M[*1], Toyama K[*1], OPiyo O G[*1], Demizu Y, Kurihara M[*2], Doi M[*3], Tanaka M[*1]: Low-pH triggering changes in peptide secondary structures.
Organic and Biomolecular chemistry. 2017;15:6302-5

We developed a novel methodology using cyclic α, α-disubstituted α-amino acids (dAAs) with an acetal-side chain to control peptide secondary structures. The introduction of cyclic dAAs into peptides contributed to the stabilization of peptide secondary structures as a helix, while an acidic treatment of peptides resulted in a marked conformational change.

Keywords: low-pH, helical structure, conformation change

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Sugiyama T*1, Hasegawa G*1, Niikura C*1, Kuwata K*2, Imamura Y, Demizu Y, Kurihara M*3, Kittaka A*1:PNA monomers fully compatible with standard Fmoc-based solid-phase synthesis of pweudocomplementary PNA.
Bioorganic and Medicinal Chemistry Letters. 2017;27:3337-41

Here we report the synthesis of new PNA monomers for pseudocomplementary PNA (pcPNA) that are fully compatible with standard Fmoc chemistry. The thiocarbonyl group of the 2-thiouracil (sU) monomer was protected with the 4-methoxy-2-methybenzyl group (MMPM), while the exocyclic amino groups of diaminopurine (D) were protected with Boc groups. The newly synthesized monomers were incorporated into a 10-mer PNA oligomer using standard Fmoc chemistry for solid-phase synthesis. Oligomerization proceeded smoothly and the HPLC and MALDI-TOF MS analyses indicated that there was no remaining MMPM on the sU nucleobase. The new PNA monomers reported here would facilitate a wide range of applications, such as antigene PNAs and DNA nanotechnologies.
Keywords: nucleic acid, strand invasion, antigene

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Misawa T, Imamura M, Ozawa Y, Haishima K, Kurihara M, Kikuchi Y, Demizu Y: Development of helix-stabilized antimicrobial peptides composed of lysine and hydrophobic α,α-disubstituted α-amino acid residues.
Bioorganic and Medicinal Chemistry Letters. 2017;27:3950-53

Lysine-based amphipathic nonapeptides, including homochiral peptides [Ac-(l-Lys-l-Lys-Xaa)$_3$-NH$_2$ (Xaa = Gly, Ala, Aib, Ac5c, or Ac6c) and Ac-(d-Lys-d-Lys-Aib)$_3$-NH$_2$], a heterochiral peptide [Ac-(l-Lys-d-Lys-Aib)$_3$-NH$_2$], and a racemic mixture of diastereomeric peptides [Ac-(rac-Lys-rac-Lys-Aib)3-NH2] were designed and synthesized to investigate the relationship between their preferred secondary structures and their antimicrobial activity. Peptide 5, [Ac-(l-Lys-l-Lys-Ac6c)3-NH2] formed a stable α-helical structure and exhibited strong activity against Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa).
Keywords: α,α-disubstituted α-amino acid, helical peptide, antimicrobial activity

Maruyama H*1, Oikawa R*2, Hayakawa M*2, Takamori S*1, Kimura Y*2, Abe N*2, Tsuji G, Matsuda A*1, Shuto S*1, Ito Y*3, Abe H*2: Chemical ligation of oligonucleotides using an electrophilic phosphorothioester.
Nucleic acid research. 2017;45:7042-8

We developed a new approach for chemical ligation of oligonucleotides using the electrophilic phosphorothioester (EPT) group. A nucleophilic phosphorothioate group on oligonucleotides was converted into the EPT group by treatment with Sanger's reagent (1-fluoro-2,4-dinitrobenzene). EPT oligonucleotides can be isolated, stored frozen, and used for the ligation reaction. The reaction of the EPT oligonucleotide and an amino-modified oligonucleotide took place without any extra reagents at pH 7.0–8.0 at room temperature, and resulted in a ligation product with a phosphoramidate bond with a 39–85% yield. This method has potential uses in biotechnology and chemical biology.
Keywords: nucleic acid, chemical ligation, electrophilic phosphorothioester

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Ma X*1, Zhou J*1, Wang C*2, Carter-cooper B*2, Yang F*2, Larocque E*1, Fine J*1, Tsuji G, Chopra G*1, Lapidus G R*1, Shintim O H*1: Identification of New FLT3 Inhibitors That Potently Inhibit AML Cell Lines via an Azo Click-It/Staple-It Approach.
ACS Medicinal chemistry letters. 2017;45:7042-8

Acute myeloid leukemia (AML) is an aggressive malignancy with only a handful of therapeutic options. About 30% of AML patients harbor mutated FLT3 kinase, and thus, this cancer-driver has become a hotly pursued AML target. Herein we report a new

class of FLT3 inhibitors, which potently inhibit the proliferation of acute myeloid leukemia (AML) cells at nanomolar concentrations.

Keywords: acute myeloid leukemia, click-it/staple-it, FMS-like tyrosine kinase 3 inhibitor

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Kobayashi H, Misawa T, Matsuno K*, Demizu Y: Preorganized Cyclic α,α-Disubstituted α-Amino Acids Bearing Functionalized Side Chains That Act as Peptide-Helix Inducers.

Journal of Organic Chemistry. 2017;82:10722-6

Preorganized cyclic α,α-disubstituted α-amino acids (dAA) bearing functionalized side chains that acted as peptide-helix inducers, which could be used for solid-phase peptide synthesis, were designed and synthesized. Furthermore, a helical octapeptide with the following amino acid sequence was prepared, and its preferred conformation was analyzed based on its CD spectra: Ac-X^1EYSAX^2KA-NH_2 (11: X^1 = Api^{C4N3}, X^2 = Ac6c). The side-chain azido functional group of peptide 11 was efficiently converted to various 1,2,3-triazole groups via Huisgen 1,3-dipolar cycloaddition reactions involving different types of alkynes. The new cyclic dAA derivatives, which combine the advantages of conformational preorganization and side-chain functional groups, should prove to be a useful tool for the further development of biologically active peptides.

Keywords: helical structure, postmodification, azido functional group

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Eto R, Oba M*1, Ueda A*1, Uku T*1, Doi M*2, Matsuo Y*1, Tanaka T*1, Demizu Y, Kurihara M*3, Tanaka M*1: Diastereomeric Right - and Left - Handed Helical Structures with Fourteen (R) - Chiral Centers.

Chemistry A European Journal. 2017;23:18120-4

The relationship between chiral centers and the helical-screw control of their peptides has already been reported, but it has yet to be elucidated in detail. A chiral four-membered ring α,α-disubstituted α-amino acid with a (R,R)-butane-2,3-diol acetal moiety at the γ-position, but no α-chiral carbon, was synthesized. X-ray crystallographic analysis unambiguously revealed that its homo-chiral heptapeptide formed right-handed (P) and left-handed (M) 3_{10}-helical structures at a ratio of 1:1. They appeared to be enantiomeric at the peptide backbone, but diastereomeric with fourteen (R)-configuration chiral centers. Conformational analyses of homopeptides in solution also indicated that diastereomeric (P) and (M) helices existed at approximately equal amounts, with a slight preference toward right-handedness, and they quickly interchanged at room temperature. The circumstances of chiral centers are important for the control of their helical-screw direction.

Keywords: chirality, helical structure, foldamers

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Misawa T, Kanda Y, Demizu Y: Rational design and synthesis of post-functionalizable peptide foldamers as helical templates.

Bioconjugate Chemistry. 2017;28:3029-35

In this study, we developed post-functionalizable helical peptides composed of Leu, Aib, and Azl residues. We show that the synthesized peptides 1 and 2 form helical structures, and may be modified using specific side chain or several functional groups by the click reaction without influencing their secondary structures.

Keywords: helical structure, post-functionalizable, click reaction

Sugano Y*, Sakata K, Nakamura K, Noguchi A, Nozomi F, Suzuki T*, Kondo K: Rapid identification method of *Omphalotus japonicas* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Shokuhin Eiseigaku Zasshi. 2017;58:113-123.

Omphalotus japonicus is a poisonous mushroom that grows in Japan. It can be mistaken for edible mushrooms (Shiitake, Hiratake and Mukitake), and if ingested, it causes food poisoning within 30 min to 1 hr. We established a rapid detection method using PCR-RFLP to identify *O. japonicus* by restriction digestion of the amplified ITS region. By using *Sau96*I,

*Bpu10*I, *Sfc*I or *Drd*I/*Hinc*II as a restriction enzyme, it was possible to rapidly identify and discriminate *O. japonicus* based on the fragment length. This study also provided a short PCR-RFLP system comprising amplification and digestion of a short 200-bp DNA fragment within the ITS region. The system could identify and discriminate *O. japonicus* after in vitro gastric digestion of native and heated mushroom samples as a model of food poisoning. In addition, a confirmatory assay using real-time PCR was developed to achieve more sensitive detection of *O. japonicus*.

Keywords: *Omphalotus japonicus*, PCR-RFLP, short PCR-RFLP

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Kondo K, Nakamura K, Ishigaki T, Sakata K, Obitsu S, Noguchi A, Fukuda N, Nagasawa E*, Teshima R, Nishimaki-Mogami T: Molecular phylogenetic analysis of new *Entoloma rhodopolium*-related species in Japan and its identification method using PCR-RFLP.

Scientific Reports. 2017;7:14942.

Poisonous *Entoloma rhodopolium* and other similar species including edible E. sarcopum are morphologically diverse. People mistake poisonous species for edible species. Classification and the detection method of these species need to be defined. The morphological and phylogenetic studies have been reported in northern Europe. In Japan, the genetic study remains unsolved. Thus, phylogenetic analysis of *E. rhodopolium* was conducted using ITS and RPB2 sequences, and the result was compared with that of European species. Japanese *E. rhodopolium* was classified into three clades, none of which belonged to the true *European E. rhodopolium* and other known species. Three species were defined as new species. Entoloma rhodopolium clade-I (named *E. lacus*) was genetically close to but morphologically separated from *E. majaloides*. Clade-II (*E. subrhodopolium*) was classified to the same group as *E. sinuatum* and *E. subsinuatum*, but distinct from these species. Clade-III was segregated from known *Entoloma* species including *E. lupinum*, and named *E. pseudorhodopolium*. Based on the classification, a simple identification method PCR-RFLP was developed to discriminate between poisonous species and edible *E. sarcopum*, which is very similar in morphology. The study can help to clarify the taxonomy of complex *E. rhodopolium*-related species, and to prevent food poisoning.

Keywords: mushroom, PCR, *Entoloma rhodopolium*

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Shoji M*, Adachi R, Akiyama H: Japanese food allergen labeling regulation: An update.

J AOAC Int. 2018;101(1):8-13.

The Japanese food allergen labeling regulation was designed to match real Japanese food allergy circumstances and also to be enforced effectively; thus, (1) regulated food allergens were selected by prevalence and seriousness according to food allergy surveys in Japan; (2) the detection criterion for ELISA monitoring, 10 μg food allergen protein/g (or mL) food, was set up as the threshold value to regulate commercial prepackaged foods; and (3) official food allergen analytical methods, which can determine the threshold value accurately, were developed. These three points are distinctive from other countries. Furthermore, as an on-going project, the regulation has been amended according to food allergy circumstances and requirements of society. This paper presents recent changes regarding the Japanese food allergen labeling regulation. To date, the Japanese food allergen labeling regulation has been enforced for more than 15 years and seems to be working effectively. Now would be an opportune time to review the regulation for its next level of development.

Keywords: food allergy, food allergen labeling, Japanese regulation system

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日本食品化学学会誌. 2017;24(3):88-93.

Three kinds of ELISA kits for wheat protein were evaluated by a collaborative inter-laboratory trial conducted between ten participating Japanese laboratories to determine their effectiveness for

quantifying wheat gluten levels in rice flour. Samples of rice flour were prepared with wheat gluten levels set at 0 g/g, 1.0 μg/g, 2.0 μg/g, 3.0 μg/g, and 5.0 μg/g. The samples underwent preliminary validation at three laboratories prior to the inter-laboratory evaluation. A replicate analysis of the samples was performed at each of the 10 laboratories. Each of the three ELISA kits showed sufficient RSDR values (6.8-18.0%) and demonstrated high recoveries (83-100%). The RSDr values for the results of all samples measured were less than 5.8. Results from this study suggest that all three ELISA kits can be applied as precise and reliable tools for the determination of wheat gluten levels in rice flour.

Keywords: rice flour, wheat gluten level, ELISA

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Tamehiro N*, Nishida K*, Yanobu-Takanashi R*, Goto M*, Okamura T*, Suzuki H*: T-cell activation RhoGTPase-activating protein plays an important role in TH17-cell differentiation.

Immunol Cell Biol. 2017;95(8):729-735.

T-cell activation RhoGTPase-activating protein (TAGAP) is a GTPase-activating protein specific for RhoA that is exclusively expressed in activated T cells. Genome-wide association studies and metagenome SNPs analyses have indicated that TAGAP is associated with the pathogenesis of multiple autoimmune diseases, including psoriasis, rheumatoid arthritis, Crohn's disease, celiac disease and multiple sclerosis. However, the precise function of TAGAP remains unclear. Because T_H17 cells contribute to TAGAP-associated autoimmune diseases, we hypothesized that TAGAP plays key roles in the differentiation and/or function of T_H17 cells. To evaluate this hypothesis, we analyzed the effect of TAGAP on T_H17 differentiation in vitro and established a line of TAGAP-deficient mice. We found that TAGAP was required for T_H17 differentiation in vitro and that the loss of TAGAP in mice ameliorated the clinical features of experimental autoimmune encephalomyelitis, indicating that TAGAP is critical for disease progression. We also demonstrated that TAGAP interacts with RhoH, an adapter protein that interacts with lck and ZAP70 in proximal TCR signaling. TAGAP competes with ZAP70 for RhoH binding, thereby inhibiting TCR-associated signal transduction. Consistent with these findings, TCR-induced ERK activation was increased in TAGAP-deficient T cells. Because the upregulation of TCR signaling inhibits Th17 differentiation, TAGAP may prevent TCR signaling activity from reaching the limit of the induction of T_H17 cells. Collectively, our findings indicate that TAGAP is a novel factor required for T_H17-cell differentiation and that TAGAP potentially represents a novel target of autoimmune disease therapies.

Keywords: T_H17-cell, TAGAP, Rho

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Teno N*1, Iguchi Y*1, Yamashita Y*1, Mori N*1, Une M*1, Nishimaki-Mogami T, Gohda K*2: Discovery and optimization of benzimidazole derivatives as a novel chemotype of farnesoid X receptor (FXR) antagonists.

Bioorg Med Chem. 2017;25(6):1787-1794.

We describe here a novel chemotype with substituted benzimidazole scaffold for nonsteroidal farnesoid X receptor (FXR) antagonists starting from the identification of a screening hit, BB-4. Structure diversity in four regions A-D of BB-4 or 1 is discussed. In particular, regions A and C had an effect on an antagonism against FXR as demonstrated by the derivatives represented by 7 and 15, respectively. Thus, compound 19 arising from the combination of regions A and C underscored an important fact on antagonism against FXR, also showing the reduced small heterodimer partner and the increased cholesterol 7α-hydroxylase expression levels.

Keywords: antagonist, FXR

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Teno N*1, Yamashita Y*1, Iguchi Y*1, Fujimori K*1, Une M*1, Nishimaki-Mogami T, Hiramoto T*1, Gohda K*2 : Nonacidic chemotype possessing N-acylated piperidine moiety as potent farnesoid X receptor (FXR) antagonists.

ACS Med Chem Lett. 2018;9(2):78-83.

Farnesoid X receptor (FXR) plays a major role in the control of cholesterol metabolism. Antagonizing transcriptional activity of FXR is an effective means to treat the relevant metabolic syndrome. Some of antagonists so far have the charged functions; however, they may negatively affect the pharmacokinetics. We describe herein a structure-activity relationship (SAR) exploration of nonacidic FXR antagonist 6 focusing on two regions in the structure and biological evaluation of nonacidic 10 with the characteristic N-acylated piperidine group obtained from SAR studies. As the robust affinity to FXR is feasible with our nonacidic analogue, 10 is among the most promising candidates for in vivo testing.

Keywords: antagonist, FXR

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Song I*, Tanaka R*, Aso M*, Sakamoto Y*, Maeda M*, Ochiai M*, Saito Y, Maekawa K, Kumagai Y*: Influences of long-term, high-dose acetaminophen administration on liver function markers in healthy Japanese adults.

臨床薬理 2017;48:153-159.

Background: Acetaminophen is widely used as an analgesic and antipyretic; however, acetaminophen overdose is known to cause hepatic injury. However, minor and self-limiting alanine aminotransferase (ALT) elevation unrelated to hepatic injury is occasionally observed in individuals receiving high-dose acetaminophen. The aim of this study was to evaluate the changes in liver function markers induced by long-term, high-dose acetaminophen administration.

Methods: Acetaminophen (3000 mg/day) or placebo was repeatedly administered to 242 healthy Japanese adults for 28 days. Plasma samples collected on Day 1 were used to measure the pharmacokinetics of acetaminophen. Liver function was monitored in terms of aspartate aminotransferase (AST), ALT, alkaline phosphatase (ALP), total bilirubin (T-Bil), and high mobility group box 1 (HMGB-1) levels for 35 days, from the day of the first dose. Subjects were withdrawn from the study if their AST, ALT, or ALP levels exceeded twice the respective upper limit of normal (2×ULN).

Results: From a total of 242 subjects, 202 and 40 subjects were assigned to the acetaminophen group and the placebo group, respectively. Twelve subjects in the acetaminophen group (6.0%) were withdrawn owing to ALT elevation over 2×ULN; no subjects were withdrawn from the placebo group. During the study period, ALT was higher in the acetaminophen group than in the placebo group, and increased from Day 7 to 14 after the start of administration. However, no evidence of hepatic injury owing to acetaminophen was observed, and the ALT elevation was attenuated after Day 14. Moreover, no correlation was observed between maximum ALT and levels of HMGB-1, a novel biomarker candidate for hepatic injury, during the study period. These findings led us to conclude that the ALT elevation was not caused by hepatic injury.

Conclusion: ALT elevation >2×ULN was observed in 6.0% of subjects in the acetaminophen group. However, no subjects developed hepatic injury, and ALT levels started to return to the normal values even during continued administration. The phenomenon of adaptation may be involved in these changes.

Keywords: acetaminophen, biomarkers, drug-induced liver injury

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Maekawa K, Adachi M*1, Matsuzawa Y, Zhang Q*2, Kuroki R*3, Saito Y, Shah MB*4,5: Structural basis of single-nucleotide polymorphisms in cytochrome P450 2C9.

Biochemistry 2017;56:5476-5480.

Single-nucleotide polymorphisms in drug-metabolizing cytochrome P450 (CYP) enzymes are important contributors to interindividual differences in drug metabolism leading to adverse drug reactions. Despite their extensive characterization and importance in pharmacogenetics of clinical drugs, the structural basis of CYP polymorphisms has remained

scant. Here we report the crystal structures of human CYP2C9 and its polymorphic variants, *3 (I359L) and *30 (A477T), with an antihypertensive drug losartan. The structures show distinct interaction and occupation of losartan in the active site, the access channel, and the peripheral binding site. The I359L substitution located far from the active site remarkably altered the residue side chains near the active site and the access channel, whereas the T477 substitution illustrated hydrogen-bonding interaction with the reoriented side chain of Q214. The results yield structural insights into the reduced catalytic activity of the CYP2C9 variants and have important implications for understanding genetic polymorphisms in CYP-mediated drug metabolism.

Keywords: crystal structure, CYP2C9, genetic polymorphisms

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Aoyama T*1, Ishida Y*1,2, Kaneko M*1,3, Miyamoto A*1, Saito Y, Tohkin M*4, Kawai S*5, Matsumoto Y*1: Pharmacokinetics and pharmacodynamics of meloxicam in east asian populations: The role of ethnicity on drug response.
CPT Pharmacometrics Syst Pharmacol. 2017;6:823-832.

We aimed to reanalyze the differences in the pharmacokinetics (PKs) of meloxicam in East Asian populations based on a population approach using previously published data and to investigate the factors found in population PK analysis that affect the pharmacodynamics (PDs) of meloxicam. Population PK analysis was performed in 119 healthy male subjects (30 Japanese, 30 Chinese, 29 Korean, and 30 white) under strictly controlled trial conditions with regulated meals and a single lot of the drug. We found that CYP2C9 genotype and lean body mass were statistically significant predictors of clearance and volume of distribution, respectively. A statistical significant difference in the PK parameters between ethnic groups could not be identified. Simulations using PK/PD models showed that CYP2C9 genotype is the factor that affects the PDs of meloxicam. The genetic polymorphisms highlighted in this study would be beneficial for conducting clinical trials in East Asians with similar genetic backgrounds.

Keywords: East Asians, meloxicam, population pharmacokinetics

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Takeyama M, Sai K, Imatoh T, Segawa K, Hirasawa N*, Saito Y: Influence of japanese regulatory action on denosumab-related hypocalcemia using japanese adverse drug event report database.
Biol Pharm Bull. 2017:40:1447-53

The anti-receptor activator of nuclear factor kappa-B ligand antibody, Denosumab (DEN), was approved in April 2012 in Japan, but a Dear Healthcare Professional Letter of Rapid Safety Communication was released in September, 2012 by the regulatory authority because of the severe hypocalcemia risks. Currently, the effectiveness of this regulatory action has not been evaluated. This study aimed to assess its impact on DEN-induced hypocalcemia using the Japanese Adverse Drug Event Report database (JADER). The changes of reporting odds ratio (ROR) of hypocalcemia for DEN and zoledronic acid (ZOL, a reference drug) were compared between the pre- (Pre, April 2012 to September 2012) and post- (Post 1, October 2012 to September 2013 and Post 2, October 2013 to September 2014) periods of the regulatory action. A decrease in the hypocalcemia ROR was observed for DEN in the post-periods, especially Post 2. Multivariate logistic regression analysis showed a significant decrease in hypocalcemia signal in Post 1 ($p=0.0306$ vs. Pre) and Post 2 ($p=0.0054$ vs. Pre). ZOL caused no significant changes in ROR of hypocalcemia. This study suggests that the regulatory action against hypocalcemia in DEN effectively decreased hypocalcemia signal. Further studies using medical information databases are needed to confirm this result.

Keywords: denosumab, regulatory action, Japanese

Adverse Drug Event Report database

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Imatoh T, Sai K, Hori K*, Segawa K, Kawakami J*, Kimura M*, Saito Y: Development of a novel algorithm for detecting glucocorticoid-induced diabetes mellitus using a medical information database.

J Clin Pharm Ther. 2017;42:215-220

We conducted a pharmacoepidemiological study to develop an algorithm for detecting GIDM using MID. We selected 1214 inpatients who were newly prescribed with a typical glucocorticoid, prednisolone, during hospitalization from 2008 to 2014 from an MID of Hamamatsu University Hospital in Japan. GIDM was screened based on fasting blood glucose (FBG) and haemoglobin A1c (HbA1c) levels according to the current Japan Diabetes Society (JDS) DM criteria, and its predictability was evaluated by an expert's review of medical records. We investigated further candidate screening factors using receiver operating characteristics analysis. Sixty-three inpatients were identified by the JDS DM criteria. Of these, 33 patients were definitely diagnosed as having GIDM by expert's review (positive predictive value = 52.4%). To develop a highly predictive algorithm, we compared the characteristics of inpatients diagnosed with definite GIDM and those diagnosed as non-GIDM. The maximum levels of HbA1c in patients with GIDM were significantly higher than those of patients with non-GIDM (66.9 mmol/mol vs. 58.7 mmol/mol, $P < 0.001$). The patients with GIDM had significantly higher relative increase in maximum level of HbA1c (RIM-HbA1c) than those with non-GIDM (0.3 vs. 0.03, $P < 0.001$). However, we did not observe a significant difference in those of fasting blood glucose (FBG) levels. We applied the RIM-HbA1c as a second screening factor to improve the detection of GIDM. It showed that a 13% increase in RIM-HbA1c separated patients with from patients without GIDM. Our results suggest that monitoring changes in HbA1c levels is important for detecting GIDM and adds to current diagnostic criteria for type 2 DM.

Keywords: glucocorticoid-induced diabetes mellitus, detection algorithm, pharmacoepidemiological study

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Imatoh T, Sai K, Fukazawa C*, Hinomura Y*, Nakamura R, Okamoto-Uchida Y, Segawa K, Saito Y: Association between infection and severe drug adverse reactions: an analysis using data from the Japanese Adverse Drug Event Report database.

Eur J Clin Pharmacol. 2017;73:1643-1653

We aimed to determine the associations between infections and drug-induced interstitial lung disease (DILD), rhabdomyolysis, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), or drug-induced liver injury (DILI) using a spontaneous adverse drug event reporting database in Japan. The reported cases were classified into three categories (anti-infectious drug group, concomitant infection group, and non-infection group) based on the presence of anti-infectious drugs (either as primary suspected drug or concomitant drug) and infectious disease. We assessed the association between four severe ADRs and the presence and seriousness of infection using logistic regression analysis. We identified 177,649 cases reported in the study period (2009-2013). Logistic regression analysis showed significant positive associations between infection status and onset of SJS/TEN or DILI compared to the non-infection group. By contrast, there were negative or no associations between infection and DILD or rhabdomyolysis. A significantly positive association between infection and SJS/TEN seriousness. This study suggested that infection plays an important role in the development of SJS/TEN and DILI. For the patients with infection and/ or anti-infectious drugs, careful monitoring for severe ADRs, especially SJS/TEN, might be needed.

Keywords: severe adverse reaction, infection, pharmacoepidemiological study

* JAPIC

Saito K, Ohno Y*, Saito Y.: Enrichment of resolving power improves ion-peak quantification on a lipidomics platform.

J Chromatogr B Analyt Technol Biomed Life Sci. 2017;1055-1056:20-28

In this study, we delineated the importance of MS resolving power on the ion-peak quantification of lipids

using an Orbitrap Fusion instrument and established a liquid chromatography-based, high-performance lipidomics platform. The ion-peak recognition of several lipids in human plasma, such as LPC(15:0), LPE(22:5), and PC(35:0), was clearly improved by increasing the MS resolving power. In addition, we evaluated the impact of resolving power on the quantitative detection of lipids by automatic ion-peak recognition with calculation of the coefficient of variance (CV). The extracted ions obtained from human plasma were automatically annotated by Compound Discoverer software with manual confirmation of standards or MS2/MS3 fragments (class- and acyl side chain-specific ions and neutral losses). Quantitative evaluation of 499 lipids in human plasma in terms of their CV values clearly demonstrated an improvement in the quantitative performance by enriching the resolving power. Moreover, we evaluated our new lipidomics platform with enriched MS resolving power (setting of 240,000, full width at half maximum at m/z 200). Because automatic annotation by TraceFinder software overlooks several lipid ions, we further manually annotated additional lipid ions, which were confirmed by standards or MS2/MS3 fragments. Eventually, our platform detected 967 lipids encompassing 34 lipid classes, which were confirmed with standards or MS2/MS3 fragments. Of these lipids, 922 scored <20% of the CV values. Taken together, enriching the resolving power improved ion-peak quantification on our novel lipidomics platform, which enabled us to detect broad-spectrum lipids from human plasma.

Keywords: global semi-quantification, lipidomics, mass spectrometry

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Goda K*, Kobayashi A*, Takahashi A*, Takahashi T*, Saito K, Maekawa K, Saito Y, Sugai S*: Evaluation of the potential risk of drugs to induce hepatotoxicity in human-relationships between hepatic steatosis observed in non-clinical toxicity study and hepatotoxicity in humans.

Int J Mol Sci. 2017;18(4)

In the development of drugs, we sometimes encounter fatty change of the hepatocytes (steatosis) which is not accompanied by degenerative change in the liver in non-clinical toxicity studies. In this study, we investigated the relationships between fatty change of the hepatocytes noted in non-clinical toxicity studies of compound X, a candidate compound in drug development, and mitochondrial dysfunction in order to estimate the potential risk of the compound to induce drug-induced liver injury (DILI) in humans. We conducted in vivo and in vitro exploratory studies for this purpose. In vivo lipidomics analysis was conducted to investigate the relationships between alteration of the hepatic lipids and mitochondrial dysfunction. In the liver of rats treated with compound X, triglycerides containing long-chain fatty acids, which are the main energy source of the mitochondria, accumulated. Accumulation of these triglycerides was considered to be related to the inhibition of mitochondrial respiration based on the results of in vitro mitochondria toxicity studies. In conclusion, fatty change of the hepatocytes (steatosis) in non-clinical toxicity studies of drug candidates can be regarded as a critical finding for the estimation of their potential risk to induce DILI in humans when the fatty change is induced by mitochondrial dysfunction.

Keywords: drug-induced liver injury, lipidomics, mitochondrial dysfunction, steatosis

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Umehara K*, Sun Y*, Hiura S*, Hamada K*, Itoh M*, Kitamura K*, Oshima M*, Iwama A*, Saito K, Anzai N*, Chiba K*, Akita H*, Furihata T*: A new conditionally immortalized human fetal brain pericyte cell line: establishment and functional characterization as a promising tool for human brain pericyte studies.

Mol Neurobiol. 2017.

While pericytes wrap around microvascular endothelial cells throughout the human body, their highest coverage rate is found in the brain. Brain pericytes actively contribute to various brain functions, including the development and stabilization of the blood-brain barrier (BBB), tissue regeneration, and brain inflammation. Accordingly, detailed characterization of the functional nature of brain pericytes is important for understanding the mechanistic basis of brain physiology and pathophysiology. Herein, we report on the development of a new human brain pericyte cell line, hereafter

referred to as the human brain pericyte/conditionally immortalized clone 37 (HBPC/ci37). Developed via the cell conditionally immortalization method, these cells exhibited excellent proliferative ability at 33℃. However, when cultured at 37℃, HBPC/ci37 cells showed a differentiated phenotype that was marked by morphological alterations and increases in several pericyte-enriched marker mRNA levels, such as platelet-derived growth factor receptor β. It was also found that HBPC/ci37 cells possessed the facilitative ability of in vitro BBB formation and differentiation into a neuronal lineage. Furthermore, HBPC/ci37 cells exhibited the typical "reactive" features of brain pericytes in response to pro-inflammatory cytokines. To summarize, our results clearly demonstrate that HBPC/ci37 cells possess the ability to perform several key brain pericyte functions while also showing the capacity for extensive and continuous proliferation. Based on these findings, it can be expected that, as a unique human brain pericyte model, HBPC/ci37 cells have the potential to contribute to significant advances in the understanding of human brain pericyte physiology and pathophysiology.

Keywords: blood-brain barrier, brain inflammation, conditionally immortalized cell

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Sanjo H*1, Komeya M*1, Sato T*1, Abe T*1, Katagiri K*1, Yamanaka H*1, Ino Y*1, Arakawa N, Hirano H*1, Yao T*1, Asayama Y*2, Matsuhisa A*2, Yao M*1, Ogawa T*1: In vitro mouse spermatogenesis with an organ culture method in chemically defined medium. *PLoS One*. 2018; 13(2):e0192884

We previously reported the successful induction and completion of mouse spermatogenesis by culturing neonatal testis tissues. The culture medium consisted of α-minimum essential medium (α-MEM), supplemented with Knockout serum replacement (KSR) or AlbuMAX, neither of which were defined chemically. In this study, we formulated a chemically defined medium (CDM) that can induce mouse spermatogenesis under organ culture conditions. It was found that bovine serum albumin (BSA) purified through three different procedures had different effects on spermatogenesis. We also confirmed that retinoic acid (RA) played crucial roles in the onset of spermatogonial differentiation and meiotic initiation. The added lipids exhibited weak promoting effects on spermatogenesis. Lastly, luteinizing hormone (LH), follicle stimulating hormone (FSH), triiodothyronine (T3), and testosterone (T) combined together promoted spermatogenesis until round spermatid production. The CDM, however, was not able to produce elongated spermatids. It was also unable to induce spermatogenesis from the very early neonatal period, before 2 days postpartum, leaving certain factors necessary for spermatogenic induction in mice unidentified. Nonetheless, the present study provided important basic information on testis organ culture and spermatogenesis in vitro.

Keywords: testis, organ culture, spermatogenesis

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Ibi M*1, Liu J*1, Arakawa N, Kitaoka S*1, Kawaji A*1, Matsuda KI*1, Iwata K*1, Matsumoto M*1, Katsuyama M*1, Zhu K*1, Teramukai S*2, Furuyashiki T*1, Yabe-Nishimura C*1: Depressive-like behaviors are regulated by NOX1/NADPH oxidase by redox modification of NMDA Receptor 1. *J Neurosci*. 2017; 37(15):4200-4212

Involvement of reactive oxygen species (ROS) has been suggested in the development of psychiatric disorders. NOX1 is a nonphagocytic form of NADPH oxidase whose expression in the nervous system is negligible compared with other NOX isoforms. However, NOX1-derived ROS increase inflammatory pain and tolerance to opioid analgesia. To clarify the role of NOX1 in the brain, we examined depressive-like behaviors in mice deficient in *Nox1* (*Nox1*$^{-/Y}$). Depressive-like behaviors induced by chronic social defeat stress or administration of corticosterone (CORT) were significantly ameliorated in *Nox1*$^{-/Y}$ Generation of ROS was significantly elevated in the prefrontal cortex (PFC) of mice administrated with CORT, while NOX1 mRNA was upregulated only in the ventral tegmental area (VTA) among brain areas responsible for emotional behaviors. Delivery of miRNA against NOX1 to VTA restored CORT-induced depressive-like behaviors in wild-type (WT) littermates. Administration of CORT to WT, but not to *Nox1*$^{-/Y}$, significantly reduced transcript levels

of brain-derived neurotrophic factor (*bdnf*), with a concomitant increase in DNA methylation of the promoter regions in *bdnf* Delivery of miRNA against NOX1 to VTA restored the level of BDNF mRNA in WT PFC. Redox proteome analyses demonstrated that NMDA receptor 1 (NR1) was among the molecules redox regulated by NOX1. In cultured cortical neurons, hydrogen peroxide significantly suppressed NMDA-induced upregulation of BDNF transcripts in NR1-expressing cells but not in cells harboring mutant NR1 (C744A). Together, these findings suggest a key role of NOX1 in depressive-like behaviors through NR1-mediated epigenetic modification of *bdnf* in the mesoprefrontal projection. SIGNIFICANCE STATEMENT NADPH oxidase is a source of reactive oxygen species (ROS) that have been implicated in the pathogenesis of various neurological disorders. We presently showed the involvement of a nonphagocytic type of NADPH oxidase, NOX1, in major depressive disorders, including behavioral, biochemical, and anatomical changes in mice. The oxidation of NR1 by NOX1-derived ROS was demonstrated in prefrontal cortex (PFC), which may be causally linked to the downregulation of BDNF, promoting depressive-like behaviors. Given that NOX1 is upregulated only in VTA but not in PFC, mesocortical projections appear to play a crucial role in NOX1-dependent depressive-like behaviors. Our study is the first to present the potential molecular mechanism underlying the development of major depression through the NOX1-induced oxidation of NR1 and epigenetic modification of *bdnf*.

Keywords: BDNF, NADPH oxidase, NMDA receptor

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Okamoto-Uchida Y, Nakamura R, Sai K, Imatoh T, Matsunaga K*1, Aihara M*2, Saito Y: Effect of infectious diseases on the pathogenesis of Stevens-Johnson syndrome and toxic epidermal necrolysis.
Biol Pharm Bull. 2017;40(9):1576-1580

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe cutaneous adverse drug reactions. Recent studies have revealed that the prevalence of SJS/TEN is associated with genetic backgrounds, such as polymorphisms in human leukocyte antigens (HLAs). However, non-genetic factors contributing to the etiology of SJS/TEN are largely unknown. This study aimed to assess the involvement of concurrent infection on the pathological states of SJS/TEN, examining the severity of cutaneous symptoms and ocular involvement as well as the time to onset in drug-induced SJS/TEN patients. We recruited 257 Japanese SJS/TEN patients from June 2006 to September 2013 through a nationwide case collection network and participating hospitals and reviewed the clinical information including patient backgrounds, primary disease and medication status. Association between infection and pathological states of SJS/TEN was assessed using univariate and multivariate analyses. The concurrent infectious group of SJS/TEN patients showed a significantly higher rate of exhibiting severer dermatological and ophthalmological phenotypes and an earlier onset of SJS/TEN than the non-infectious group. Our results suggest that the infection could be a risk factor to cause severer symptoms and earlier onset of SJS/TEN.

Keywords: Stevens-Johnson syndrome, epidemiological study, infection

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Yamasaki T*1, Deki-Arima N*1, Kaneko A*2, Miyamura N*1, Iwatsuki M*1, Matsuoka M*3, Fujimori-Tonou N*4, Okamoto-Uchida Y, Hirayama J*1, Marth JD*5, Yamanashi Y*6, Kawasaki H*7, Yamanaka K*8, Penninger JM*9, Shibata S*2, Nishina H*1: Age-dependent motor dysfunction due to neuron-specific disruption of stress-activated protein kinase MKK7.
Scientific Reports. 2017;s41598

c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase family and controls various physiological processes including apoptosis. A specific upstream activator of JNKs is the mitogen-activated protein kinase kinase 7 (MKK7). It has been reported that MKK7-JNK signaling plays an important regulatory role in neural development, however, post-developmental functions in the nervous system have not been elucidated. In this study, we generated neuron-specific Mkk7 knockout mice (MKK7 cKO),

which impaired constitutive activation of JNK in the nervous system. MKK7 cKO mice displayed impaired circadian behavioral rhythms and decreased locomotor activity. MKK7 cKO mice at 8 months showed motor dysfunctions such as weakness of hind-limb and gait abnormality in an age-dependent manner. Axonal degeneration in the spinal cord and muscle atrophy were also observed, along with accumulation of the axonal transport proteins JNK-interacting protein 1 and amyloid beta precursor protein in the brains and spinal cords of MKK7 cKO mice. Thus, the MKK7-JNK signaling pathway plays important roles in regulating circadian rhythms and neuronal maintenance in the adult nervous system.

Keywords: MKK7, nervous system, circadian rhythm

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Okamoto-Uchida Y, Nakamura R, Matsunaga K*1, Aihara M*2, Saito Y: Specific association of the rs6500265 and rs9933632 SNPs in Japanese patients with antipyretic analgesic-related Stevens–Johnson syndrome and toxic epidermal necrolysis with severe ocular involvements.

Pharmacogenetics and Genomics, 2018;28:95-98

A recent study using the microarray for single-nucleotide polymorphisms (SNPs) genotyping specifically designed for the Japanese population in combination with genome-wide imputation showed the association of several SNPs with cold medicine-related Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) with severe ocular complications. However, it remains to be determined whether these polymorphisms are associated with the onset of antipyretic analgesic (AA)-related SJS/TEN, the progression of severe ocular involvements (SOIs), or both AA-related SJS/TEN and SOI phenotypes. To gain a better understanding of the features of these genetic markers, we compared the allele and carrier frequencies of these SNPs among our original SJS/TEN patient groups: (a) AA-related SJS/TEN with SOIs, (b) AA-related SJS/TEN without SOIs, and (c) AA-unrelated SJS/TEN with SOIs. AA-related SJS/TEN with SOIs were found to be associated significantly with both rs6500265 [allele frequency: odds ratio (OR): 2.18; 95% confidence interval (CI): 1.30-3.65; P=0.0052; carrier frequency: OR: 2.52; 95% CI: 1.33-4.78; P=0.058] and rs9933632 (allele frequency: OR: 2.28: 95% CI: 1.37-3.79; P=0.0032; carrier frequency: OR: 2.76; 95% CI: 1.46-5.22; P=0.0031). In contrast, allele and carrier frequencies of these SNPs in patients with AA-related SJS/TEN without SOIs or with SOIs not treated with any AAs were comparable with those in healthy Japanese controls. Collectively, our findings indicate that the rs6500265 and rs9933632 SNPs could be specific markers for AA-related SJS/TEN with SOIs, suggesting that certain genetic backgrounds contribute toward the etiology of this complex syndrome.

Keywords: Stevens-Johnson syndrome, single nucleotide polymorphisms, severe ocular involvements

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Komeij Y*1, Okiyama Y, Mochizuki Y*2,3, Fukuzawa K*3,4: Explicit solvation of a single-stranded DNA, a binding protein, and their complex: a suitable protocol for fragment molecular orbital calculation.

Chem-Bio Informatics Journal. 2017;17:72–84

Fragment molecular orbital (FMO) calculations were performed for explicitly solvated single-stranded DNA (ssDNA), ssDNA binding protein, and their complex in order to assess the solvent effects on the solutes and thereby to find optimal solvation conditions for FMO calculation. A series of solvated structures were generated with different solvent thicknesses. The structures were subjected to FMO calculation at MP2/6-31G* to obtain the net charges and internal energies of the solutes and the solute–solvent interaction energies as functions of the solvent thickness. In all cases, the properties showed complete or marginal convergence at ca. 6 Å, regardless whether or not the system charge was neutralized.

This suggested that the first and second solvent shells mainly determine the electronic structure of a solute while the outer solvent including ions has only minor effects, consistent with several preceding reports. In light of this, and considering safety as a factor, we conclude that a solvent shell thickness of ca. 8 Å suffices for FMO calculation of the solutes.

Keywords: fragment molecular orbital (FMO) method, explicit solvent, single-stranded DNA

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Nakano T, Mochizuki Y*1,2, Fukuzawa K*2,3, Okiyama Y, Watanabe C*4: A preliminary study of correction for inter fragment interaction energy (IFIE) between fragments sharing bond detached atom (BDA).

Journal of Computer Aided Chemistry. 2017;18:143–148

Recently, the fragment molecular orbital (FMO) method has attracted considerable attention as an electronic structure calculation scheme applicable to macromolecular systems. As a major advantage, a list of inter fragment interaction energies (IFIEs) are straightforwardly obtained from the FMO calculations. It has been well recognized that the IFIE-based analyses are useful to grasp the nature of interactions in the given target system in practical applications. However, there exists a severe limitation that the value of IFIE between covalently bonded fragments takes an abnormally large value (about –15 hartree), and this should degrade the usability of FMO calculations in several cases. In this paper, we examined a correction method to solve this problem, based on the fictitious dissociation processes.

Keywords: fragment molecular orbital (FMO) method, inter fragment interaction energy (IFIE), IFIE correction

Keywords: lipidomics, ether-phosphatidylcholine, sever ocular complications

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Watanabe C*1, Watanabe H*1, Fukuzawa K*2,3, Parker LJ*4,5, Okiyama Y*1, Yuki H*1, Yokoyama S*4, Nakano H*6, Tanaka S*7, Honma T*1: Theoretical analysis of activity cliffs among benzofuranone-class Pim1 inhibitors using the fragment molecular orbital method with molecular mechanics Poisson–Boltzmann surface area (FMO+MM-PBSA) approach.

Journal of Chemical Information and Modeling. 2017;57:2996–3010

Significant activity changes due to small structural changes (i.e., activity cliffs) of serine/threonine kinase Pim1 inhibitors were studied theoretically using the fragment molecular orbital method with molecular mechanics Poisson－Boltzmann surface area (FMO+MM-PBSA) approach. This methodology enables quantum-chemical calculations for large biomolecules with solvation. In the course of drug discovery targeting Pim1, six benzofuranone-class inhibitors were found to differ only in the position of the indole-ring nitrogen atom. By comparing the various qualities of complex structures based on X-ray, classical molecular mechanics (MM)-optimized, and quantum/molecular mechanics (QM/MM)-optimized structures, we found that the QM/MM-optimized structures provided the best correlation (R2 = 0.85) between pIC50 and the calculated FMO+MM-PBSA binding energy. Combining the classical solvation energy with the QM binding energy was important to increase the correlation. In addition, decomposition of the interaction energy into various physicochemical components by pair interaction energy decomposition analysis suggested that CH－π and electrostatic interactions mainly caused the activity differences.

Keywords: fragment molecular orbital (FMO) method, Pim1, Poisson–Boltzmann surface area (PBSA)

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Mochizuki Y*1,2, Sakakura K*3, Akinaga Y*4, Kato K*5, Watanabe H*6, Okiyama Y, Nakano T, Komeiji Y*7, Okusawa A*8, Fukuzawa K*9, Tanaka S*10: Current status of ABINIT-MP as a FMO program and related works with machine learning.
Journal of Computational Chemistry, Japan. 2017;16:119–122

We have been developing the ABINIT-MP program for the fragment molecular orbital (FMO) method. The list of inter-fragment interaction energies (IFIEs) is available from FMO calculations and is useful in analyzing the nature of interactions in a given target system. In this Letter, we summarize the current status of ABINIT-MP and also the machine-learning assisted analyses of IFIE data.
Keywords: fragment molecular orbital (FMO) method, interaction energy, machine learning

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Yasuhiko Y, Hirabayashi Y, Ono R: LTRs of Endogenous Retroviruses as a Source of Tbx6 Binding Sites.
Frontiers in Chemistry. 2017 Jun 15;5:34.

Retrotransposons are abundant in mammalian genomes and can modulate the gene expression of surrounding genes by disrupting endogenous binding sites for transcription factors (TFs) or providing novel TFs binding sites within retrotransposon sequences. Here, we show that a (C/T)CACACCT sequence motif in ORR1A, ORR1B, ORR1C, and ORR1D, Long Terminal Repeats (LTRs) of MaLR endogenous retrovirus (ERV), is the direct target of Tbx6, an evolutionary conserved family of T-box TFs. Moreover, by comparing gene expression between control mice ($Tbx6^{+/-}$) and Tbx6-deficient mice ($Tbx6^{-/-}$), we demonstrate that at least four genes, Twist2, Pitx2, Oscp1, and Nfxl1, are down-regulated with Tbx6 deficiency. These results suggest that ORR1A, ORR1B, ORR1C and ORR1D may contribute to the evolution of mammalian embryogenesis.
Keywords: endogenous retroviruses, retrotransposon, transcription factors

Oka SI*1, Hirata T*1, Suzuki W*1, Naito D*1, Chen Y*2, Chin A*1, Yaginuma H*1, Saito T*1, Nagarajan N*1, Zhai P*1, Bhat S*1, Schesing K*1, Shao D*1, Hirabayashi Y, Yodoi J*3, Sciarretta S*4, Sadoshima J*1. Thioredoxin-1 maintains mTOR function during oxidative stress in cardiomyocytes.
J Biol Chem, 2017;292:18988-19000.

Thioredoxin 1 (Trx1) is a 12 kDa oxidoreductase that catalyzes thiol-disulfide exchange reactions to reduce proteins with disulfide bonds. As such, Trx1 helps protect the heart against stresses, such as ischemia and pressure overload. Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth, metabolism and survival. We have previously shown that mTOR activity is increased in response to myocardial ischemia-reperfusion injury. However, whether Trx1 interacts with mTOR to preserve heart function remains unknown. Using a substrate-trapping mutant of Trx1 (Trx1C35S), we here show that mTOR is a direct interacting partner of Trx1 in the heart. In response to H_2O_2 treatment in cardiomyocytes, mTOR exhibited a high molecular weight shift in non-reducing SDS-PAGE in a 2-mercaptoethanol sensitive manner, suggesting that mTOR is oxidized and forms disulfide bonds with itself or other proteins. The mTOR oxidation was accompanied by reduced phosphorylation of endogenous substrates, such as S6 kinase (S6K) and

4E-Binding Protein 1 (4EBP1) in cardiomyocytes. Immune complex kinase assays disclosed that the H_2O_2 treatment diminished mTOR kinase activity, indicating that mTOR is inhibited by oxidation. Of note, Trx1 overexpression attenuated both H_2O_2-mediated mTOR oxidation and inhibition, whereas Trx1 knockdown increased mTOR oxidation and inhibition. Moreover, Trx1 normalized H_2O_2- induced downregulation of metabolic genes and stimulation of cell death, and an mTOR inhibitor abolished Trx1-mediated rescue of gene expression. H_2O_2-induced oxidation and inhibition of mTOR were attenuated when Cys1483 of mTOR was mutated to phenylalanine. These results suggest that Trx1 protects cardiomyocytes against stress by reducing mTOR at Cys1483, thereby preserving the activity of mTOR and inhibiting cell death.

Keywords: heart, mammalian target of rapamycin (mTOR), redox regulation

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Ideta-Otsuka M*, Igarashi K*, Narita, M*, Hirabayashi Y. Epigenetic Toxicity of Environmental Chemicals Upon Exposure During Development - Bisphenol A and Valproic Acid May Have Epigenetic Effects.

Food Chem Toxicol 109, no. Pt 1 (Nov 2017):812-16.

As of 2017, chemical substances registered in Chemical Abstracts Service (CAS) exceed 100 million, which is increasing yearly. The safety of chemical substances is adequately managed by regulations based on scientific information from toxicity tests. However, there are substances reported to have "biological effects" even though they are judged to be nontoxic in conventional toxicity tests. Therefore, it is necessary to consider a new concept on toxicity, "epigenetic toxicity". In this review, we explain about epigenetic toxicity using bisphenol A (BPA) and valproic acid (VPA) as examples. We also discuss the problems associated with the judgment of epigenetic toxicity. Currently, epigenetic changes can only be detected by biochemical methods, which are labor-intensive. Therefore, we are developing reporter mice that can be used to detect epigenetic toxicity during conventional toxicity tests. In addition, we consider that linking epigenomic changes with phenotypic changes is important, because causality is important for toxicity evaluation. Therefore, we are developing an artificial epigenome-editing technology. If we can develop a safety-assessment system by incorporating epigenetic evaluation into toxicity tests, we can increase the safety of both food and environmental chemical substances. The practical application of such a new safety-assessment system will be increasingly important in the future.

Keywords: developmental exposure, Valproic Acid, Bisphenol A

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Loomis D*, Guyton KZ*, Grosse Y*, El Ghissassi F*, Bouvard V*, Benbrahim-Tallaa L*, Guha N*, Vilahur N*, Mattock H*, Straif K*, International Agency for Research on Cancer Monograph Working G. Carcinogenicity of benzene.

Lancet Oncol, 2017;18:1574-1575.

In October, 2017, a Working Group of 27 scientists from 13 countries met at the International Agency for Research on Cancer (IARC) in Lyon, France, to finalise their evaluation of the carcinogenicity of benzene. This assessment will be published in Volume 120 of the IARC Monographs.

Keywords: International Agency for Research on Cancer (IARC), Benzene, cancer Monograph

* International Agency for Research on Cancer

Fueta Y*1, Sekino Y*2, Yoshida S*3, Kanda Y, Ueno S*1: Prenatal exposure to valproic acid alters the development of excitability in the postnatal rat hippocampus.

Neurotoxicology. 2018;65:1-8.

Prenatal valproic acid (VPA) exposure is a well-known animal model of autism spectrum disorder (ASD) that produces alterations in embryonic and adult neurogenesis as well as adolescent/adulthood neurobehavioral phenotypes. However, the effects of prenatal VPA exposure on neural network excitability, especially during the synaptogenic period around eye opening, are not fully understood. In this study, we

orally administered VPA (300 mg/kg) to pregnant Wistar rats on gestation day 15 and subsequently performed field potential recording in the CA1 area of hippocampal slices obtained from control (saline-exposed) and VPA-exposed rat pups between postnatal day (PND) 13 and PND18. In control slices, we observed an abrupt enhancement of stimulation-dependent responses including population spike (PS) amplitudes and field excitatory postsynaptic potential (fEPSP) slopes at PND16, which coincided with the average day of eye opening. In contrast, VPA-exposed pups exhibited delayed eye opening (PND17) and gradual rather than abrupt increases in PS amplitudes and fEPSP slopes over the duration of the synaptogenic period. We next investigated the involvement of ambient GABA (γ-aminobutyric acid) in PS generation using bicuculline methiodide (BMI), a GABA type A (GABAA) receptor antagonist. In control slices, BMI enhanced PS amplitudes during PND14-15 (before eye opening) and had little effect thereafter during PND16-17; a subsequent regression model analysis of BMI ratios (the ratio of PS amplitudes in the presence and absence of BMI) indicated a possible developmental change between these periods. In contrast, almost identical regression models were obtained for BMI ratios during PND14-15 and PND16-17 in the VPA-exposed group, indicating the absence of a developmental change. Our results suggest that prenatal VPA exposure accelerates the development of hippocampal excitability before eye opening. Moreover, our experimental model can be used as a novel approach for the evaluation of developmental neurotoxicity.

Keywords: Developmental neurotoxicity, Electrophysiology, Hippocampus, Prenatal exposure, Slice preparation, Valproic acid

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Fueta Y*, Ishidao T*, Ueno S*, Yoshida Y*, Kanda Y, Hori H*: Prenatal exposure to 1-bromopropane causes delayed adverse effects on hippocampal neuronal excitability in the CA1 subfield of rat offspring.

J Occup Health. 2018;60:74-9.

Neurotoxicity of 1-bromopropane (1-BP) has been reported in occupational exposure, but whether the chemical exerts developmental neurotoxicity is unknown. We studied the effects of prenatal 1-BP exposure on neuronal excitability in rat offspring. We exposed dams to 1-BP (700 ppm, 6 h a day for 20 days) and examined hippocampal slices obtained from the male offspring at 2, 5, 8, and 13 weeks of age. We measured the stimulation/response (S/R) relationship and paired-pulse ratios (PPRs) of the population spike (PS) at the interpulse intervals (IPIs) of 5 and 10 ms in the CA1 subfield. Prenatal 1-BP exposure enhanced S/R relationships of PS at 2 weeks of age; however, the enhancement diminished at 5 weeks of age until it reached control levels. Prenatal 1-BP exposure decreased PPRs of PS at 2 weeks of age. After sexual maturation, however, the PPRs of PS increased at 5-ms IPI in rats aged 8 and 13 weeks. Our findings indicate that prenatal 1-BP exposure in dams can cause delayed adverse effects on excitability of pyramidal cells in the hippocampal CA1 subfield of offspring.

Keywords: 1-Bromopropane, Delayed adverse effect, Electrophysiology, Excitability, Prenatal exposure, Rat hippocampal slices

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Izumi-Nakaseko H*1, Kanda Y, Nakamura Y*1, Hagiwara-Nagasawa M*1, Wada T*1, Ando K*1, Naito AT*1, Sekino Y*2, Sugiyama A.*1: Development of correction formula for field potential duration of human induced pluripotent stem cell-derived cardiomyocytes sheets.

J Pharmacol Sci. 2017;135:44-50.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been used in many studies to assess proarrhythmic risks of chemical compounds. In those studies, field potential durations (FPD) of hiPSC-CMs have been corrected by clinically used Fridericia's and/or Bazett's formulae, however, the rationale for the use of these formulae has not been well established. In the present study, we developed a correction formula for experiments using hiPSC-CMs. First, we analyzed the effect of beating rate on FPD in the hiPSC-CMs sheets with electrical stimuli and a

HCN channel inhibitor zatebradine. Next, we examined the relationship between the electrophysiological properties and the expression levels of ion channel genes in the cell sheets. Zatebradine slowed the beating rate and allowed to analyze FPD changes at various pacing cycle lengths. Rate-dependent change in the repolarization period was smaller in the cell sheets than that reported on the human hearts, which can be partly explained by lower gene expression level of hKCNJ2 and hKCNE1. Thus, non-linear equation for correcting FPD in the cell sheet; FPDc = FPD/RR0.22 with RR given in second was obtained, which may make it feasible to assess net repolarization delay by various chemical compounds with a chronotropic action.

Keywords: Correction formula, Field potential duration, Human induced pluripotent stem cell-derived cardiomyocytes

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Ishida K*1, Aoki K*1, Takishita T*1, Miyara M*1, Sakamoto S*1, Sanoh S*1, Kimura T*2, Kanda Y, Ohta S*1, Kotake Y.*1: Low-Concentration Tributyltin Decreases GluR2 Expression via Nuclear Respiratory Factor-1 Inhibition.
J Pharmacol Sci. 2017;135:44-50.

Tributyltin (TBT), which has been widely used as an antifouling agent in paints, is a common environmental pollutant. Although the toxicity of high-dose TBT has been extensively reported, the effects of low concentrations of TBT are relatively less well studied. We have previously reported that low-concentration TBT decreases α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptor subunit 2 (GluR2) expression in cortical neurons and enhances neuronal vulnerability to glutamate. However, the mechanism of this TBT-induced GluR2 decrease remains unknown. Therefore, we examined the effects of TBT on the activity of transcription factors that control GluR2 expression. Exposure of primary cortical neurons to 20 nM TBT for 3 h to 9 days resulted in a decrease in GluR2 mRNA expression. Moreover, TBT inhibited the DNA binding activity of nuclear respiratory factor-1 (NRF-1), a transcription factor that positively regulates the GluR2. This result indicates that TBT inhibits the activity of NRF-1 and subsequently decreases GluR2 expression. In addition, 20 nM TBT decreased the expression of genes such as cytochrome c, cytochrome c oxidase (COX) 4, and COX 6c, which are downstream of NRF-1. Our results suggest that NRF-1 inhibition is an important molecular action of the neurotoxicity induced by low-concentration TBT.

Keywords: GluR2, neuronal vulnerability, nuclear respiratory factor-1, tributyltin

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Li M*1, Kanda Y, Ashihara T*2, Sasano T*1, Nakai Y*3, Kodama M*1, Hayashi E*1, Sekino Y*4, Furukawa T*1, Kurokawa J.*5: Low-Concentration Tributyltin Decreases GluR2 Expression via Nuclear Respiratory Factor-1 Inhibition.
J Pharmacol Sci. 2017;135:44-50.

Human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes hold great potentials to predict pro-arrhythmic risks in preclinical cardiac safety screening, although the hiPSC cardiomyocytes exhibit rather immature functional and structural characteristics, including spontaneous activity. Our physiological characterization and mathematical simulation showed that low expression of the inward-rectifier potassium (IK1) channel is a determinant of spontaneous activity. To understand impact of the low IK1 expression on the pharmacological properties, we tested if transduction of hiPSC-derived cardiomyocytes with KCNJ2, which encodes the IK1 channel, alters pharmacological response to cardiac repolarization processes. The transduction of KCNJ2 resulted in quiescent hiPSC-derived cardiomyocytes, which need pacing to elicit action potentials. Significant prolongation of paced action potential duration in KCNJ2-transduced hiPSC-derived cardiomyocytes was stably measured at 0.1 μM E-4031, although the same concentration of E-4031 ablated firing of non-treated hiPSC-derived cardiomyocytes. These results in single cells were confirmed by mathematical simulations. Using the hiPSC-derived cardiac sheets with KCNJ2-transduction, we also investigated effects of a range of drugs on field potential duration recorded at 1 Hz. The KCNJ2 overexpression in hiPSC-derived

cardiomyocytes may contribute to evaluate a part of QT-prolonging drugs at toxicological concentrations with high accuracy.

Keywords: arrhythmias, mathematical simulation, iPS cells

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Izumi-Nakaseko H*1, Nakamura Y*1, Wada T*1, Ando K*1, Kanda Y, Sekino Y*2, Sugiyama A.*1: Characterization of human iPS cell-derived cardiomyocyte sheets as a model to detect drug-induced conduction disturbance.
J Pharmacol Sci. 2017;135:44-50.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been used in many studies to assess proarrhythmic risks of chemical compounds. In those studies, field potential durations (FPD) of hiPSC-CMs have been corrected by clinically used Fridericia's and/or Bazett's formulae, however, the rationale for the use of these formulae has not been well established. In the present study, we developed a correction formula for experiments using hiPSC-CMs. First, we analyzed the effect of beating rate on FPD in the hiPSC-CMs sheets with electrical stimuli and a HCN channel inhibitor zatebradine. Next, we examined the relationship between the electrophysiological properties and the expression levels of ion channel genes in the cell sheets. Zatebradine slowed the beating rate and allowed to analyze FPD changes at various pacing cycle lengths. Rate-dependent change in the repolarization period was smaller in the cell sheets than that reported on the human hearts, which can be partly explained by lower gene expression level of hKCNJ2 and hKCNE1. Thus, non-linear equation for correcting FPD in the cell sheet; FPDc = FPD/RR0.22 with RR given in second was obtained, which may make it feasible to assess net repolarization delay by various chemical compounds with a chronotropic action.

Keywords: iPS, cardiomyocyte, field potential duration

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Hirata N*1, Yamada S*1, Sekino Y*2, Kanda Y: Tobacco nitrosamine NNK increases ALDH-positive cells via ROS-Wnt signaling pathway in A549 human lung cancer cells.
The Journal of Toxicological Sciences. 2017;42:193-204.

Epidemiological studies suggest that lung cancer, which is a major cause of cancer death, has a critical association with cigarette smoking. Tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in cigarette smoke is a major risk factor for carcinogenesis. However, the mechanisms by which NNK promotes cancer development have not been fully elucidated. Growing evidence suggests that lung cancer originates from cancer stem cells (CSCs), which are a minor population of lung cancer cells. In the present study, we investigated the effects of NNK on the CSCs in A549 human lung cancer cells using flow cytometry with aldehyde dehydrogenase (ALDH), a functional marker of CSCs. We found that NNK increased the proportion of ALDH-positive cells in a dose-dependent manner. A Wnt inhibitor PNU74654 reduced NNK-induced expression levels of Wnt target gene Dkk1 and increase in ALDH-positive cells. We next examined the signaling pathway that mediates the NNK-induced increase in ALDH-positive cells via Wnt signaling. DCF assay revealed that NNK induced reactive oxygen species (ROS) production. The ROS scavenger N-acetylcysteine (NAC) inhibited the NNK-induced Wnt activation and increase in ALDH-positive cells. These data suggest that NNK-induced ROS activate the Wnt signaling pathway in A549 cells. These findings would provide new insights into the role of NNK in the lung CSCs.

Keywords: cancer stem cells, NNK, ROS

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Satoh T*1, Sugiura S*1, Shin K*1, Onuki-Nagasaki R*1, Ishida S, Kikuchi K*2, Kakiki M*2, Kanamori*1: A multi-throughput multi-organ-on-a-chip system on a plate formatted pneumatic pressure-driven medium circulation platform. *Lab Chip.* 2017;18:115-25

This paper reports a multi-throughput multi-organ-on-a-chip system formed on a pneumatic pressure-driven medium circulation platform with a microplate-sized format as a novel type of microphysiological system. The pneumatic pressure-driven platform enabled parallelized multi-organ experiments (i.e. simultaneous operation of multiple multi-organ culture units) and pipette-friendly liquid handling for various conventional cell culture experiments, including cell seeding, medium change, live/dead staining, cell growth analysis, gene expression analysis of collected cells, and liquid chromatography-mass spectrometry analysis of chemical compounds in the culture medium. An eight-throughput two-organ system and a four-throughput four-organ system were constructed on a common platform, with different microfluidic plates.

Keywords: cell-based assay, organs-on-a-chip, pneumatic pressure-driven medium circulation platform

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Irie T, Trussell LO*: Double-Nanodomain Coupling of Calcium Channels, Ryanodine Receptors, and BK Channels Controls the Generation of Burst Firing. *NEURON*. 2017;96:856-870

Action potentials clustered into high-frequency bursts play distinct roles in neural computations. However, little is known about ionic currents that control the duration and probability of these bursts. We found that, in cartwheel inhibitory interneurons of the dorsal cochlear nucleus, the likelihood of bursts and the interval between their spikelets were controlled by Ca^{2+} acting across two nanodomains, one between plasma membrane P/Q Ca^{2+} channels and endoplasmic reticulum (ER) ryanodine receptors and another between ryanodine receptors and large-conductance, voltage- and Ca^{2+}-activated K^{+} (BK) channels. Each spike triggered Ca^{2+}-induced Ca^{2+} release (CICR) from the ER immediately beneath somatic, but not axonal or dendritic, plasma membrane. Moreover, immunolabeling demonstrated close apposition of ryanodine receptors and BK channels. Double-nanodomain coupling between somatic plasma membrane and hypolemmal ER cisterns provides a unique mechanism for rapid control of action potentials on the millisecond timescale.

Keywords: ryanodine receptor, burst firing, CICR

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Irie T, Kawakami T, Sato K, Usami M: Sub-toxic concentrations of nano-ZnO and nano-TiO_2 suppress neurite outgrowth in differentiated PC12 cells. *J Toxicol Sci*. 2017;42:723-729

Nanomaterials have been extensively used in our daily life, and may also induce health effects and toxicity. Nanomaterials can translocate from the outside to internal organs, including the brain. For example, both nano-ZnO and nano-TiO_2 translocate into the brain via the olfactory pathway in rodents, possibly leading to toxic effects on the brain. Although the effects of nano-ZnO and nano-TiO_2 on neuronal viability or neuronal excitability have been studied, no work has focused on how these nanomaterials affect neuronal differentiation and development. In this study, we investigated the effects of nano-ZnO and nano-TiO_2 on neurite outgrowth of PC12 cells, a useful model system for neuronal differentiation. Surprisingly, the number, length, and branching of differentiated PC12 neurites were significantly suppressed by the 7-day exposure to nano-ZnO (in the range of 1.0×10^{-4} to 1.0×10^{-1} microg/mL), at which the cell viability was not affected. The number and length were also significantly inhibited by the 7-day exposure to nano-TiO_2 (1.0×10^{-3} to 1.0 microg/mL), which did not have cytotoxic effects. These results demonstrate that the neurite outgrowth in differentiated PC12 cells was suppressed by sub-cytotoxic concentrations of nano-ZnO or nano-TiO_2.

Keywords: nanomaterials, neuron, PC12

Suzuki I, Cho YM, Hirata T, Toyoda T, Akagi J, Nakamura Y*1, Sasaki A*1, Nakamura T*1, Okamoto S*2, Shirota K*3, Suetome N*3, Nishikawa A, Ogawa K: Toxic effects of 4-methylthio-3-butenyl isothiocyanate (*Raphasatin*) in the rat urinary bladder without genotoxicity. *J Appl Toxicol*. 2017;37:485-94.

We recently reported that 4-methylthio-3-butenyl isothiocyanate (MTBITC) exerts chemopreventive effects on the rat esophageal carcinogenesis model at a low dose of 80 ppm in a diet. In contrast, some

isothiocyanates (ITCs) have been reported to cause toxic effects, promotion activity, and/or carcinogenic potential in the urinary bladder of rats. In the present study, we investigated whether MTBITC had toxic effects in the urinary bladder similar to other ITCs, such as phenethyl ITC (PEITC). First, to examine the early toxicity of MTBITC, rats were fed a diet supplemented with 100, 300 or 1,000 ppm MTBITC for 14days. Treatment with 1,000 ppm MTBITC caused increased organ weights and histopathological changes in the urinary bladder, producing lesions similar to those of 1,000 ppm PEITC. In contrast, rats treated with 100 or 300 ppm MTBITC showed no signs of toxicity. Additionally, we performed in vivo genotoxicity studies to clarify whether MTBITC may exhibit a carcinogenic potential through a genotoxic mechanism in rats. Rats were treated with MTBITC for 3days at doses of 10, 30 or 90 mg kg^{-1} body weight by gavage, and comet assays in the urinary bladder and micronucleus assays in the bone marrow were performed. No genotoxic changes were observed after treatment with MTBITC at all doses. Overall, these results suggested that the effects of MTBITC in the rat urinary bladder are less than those of PEITC, but that MTBITC could have toxic effects through a nongenotoxic mechanism in the urinary bladder of rats at high doses.

Keywords: 4-methylthio-3-butenyl isothiocyanate, urinary bladder, genotoxicity

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Hirata T, Cho YM, Toyoda T, Akagi J, Suzuki I, Nishikawa A, Ogawa K: Lack of *in vivo* mutagenicity of 1,2-dichloropropane and dichloromethane in the livers of *gpt* delta rats administered singly or in combination.

J Appl Toxicol. 2017;37:683-91.

1,2-Dichloropropane (1,2-DCP) and dichloromethane (DCM) are possible causative agents associated with the development of cholangiocarcinoma in employees working in printing plant in Osaka, Japan. However, few reports have demonstrated an association between these agents and cholangiocarcinoma in rodent carcinogenicity studies. Moreover, the combined effects of these compounds have not been fully elucidated. In the present study, we evaluated the *in vivo* mutagenicity of 1,2-DCP and DCM, alone or combined, in the livers of *gpt* delta rats. Six-week-old male F344 *gpt* delta rats were treated with 1,2-DCP, DCM or 1,2-DCP + DCM by oral administration for 4 weeks at the dose (200 mg kg^{-1} body weight 1,2-DCP and 500 mgkg^{-1} body weight DCM) used in the carcinogenesis study performed by the National Toxicology Program. *In vivo* mutagenicity was analyzed by *gpt* mutation/ Spi^- assays in the livers of rats. In addition, gene and protein expression of CYP2E1 and GSTT1, the major enzymes responsible for the genotoxic effects of 1,2-DCP and DCM, were analyzed by quantitative polymerase chain reaction and western blotting. *Gpt* and Spi^- mutation frequencies were not increased by 1,2-DCP and/or DCM in any group. Additionally, there were no significant changes in the gene and protein expression of CYP2E1 and GSTT1 in any group. These results indicated that 1,2-DCP, DCM and 1,2-DCP + DCM had no significant impact on mutagenicity in the livers of *gpt* delta rats under our experimental conditions.

Keywords: 1,2-dicloropropane, dichloromethane, *gpt* delta rat

Cho YM, Hasumura M, Imai T, Takami S, Nishikawa A, Ogawa K: Horseradish extract promotes urinary bladder carcinogenesis when administered to F344 rats in drinking water.

J Appl Toxicol. 2017;37:853-62.

Horseradish extract (HRE), consisting mainly of a mixture of allyl isothiocyanate and other isothiocyanates, has been used as a food additive. To evaluate the potential hazards of HRE, a 104-week chronic study, a 2-week analysis of cell proliferation in the urinary bladder and a medium-term promotion bioassay of HRE were conducted with administration at concentrations of up to 0.04% HRE in the drinking water to male F344 rats. In the 104-week chronic study with 32 male rats per group, no treatment-related increases in the incidences of neoplastic lesions in any organ, including urinary bladder, were observed, except for simple hyperplasia in the urinary bladder in rats treated with HRE at concentrations of more than 0.01% (5.0 mg kg^{-1} body weight day^{-1}). In

the promotion study, HRE treatment after *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine initiation caused a clear increase in papillary or nodular hyperplasia, papilloma, and urothelial carcinoma of the urinary bladder in the groups given HRE for 13 weeks at doses higher than 0.005%, 0.01%, and 0.04% (2.7, 5.4 and 20.5 mg kg^{-1} body weight day^{-1}), respectively. In the 2-week cell proliferation analysis, treatment with HRE at concentrations greater than 0.005% (3.9 mg kg^{-1} body weight day^{-1}) caused transient increases in 5-bromo-2'-deoxyuridine labeling indices in the urothelium. Although clear tumor induction was not observed, administration of relatively low-dose HRE increased cell proliferation in the urothelium and exerted obvious promoting effects on rat urinary bladder carcinogenesis. Further studies are needed to elucidate the mode of action of HRE in the rat urinary bladder to facilitate data extrapolation from the present study and provide insights into risk assessment.

Keywords: horseradish extract, isothiocyanate, urinary bladder

Komine C[*1], Nakajima S[*1], Kondo Y[*2], Horii Y[*1], Yoshida M, Kawaguchi M[*1]: Effects of neonatal 17α-ethinyloestradiol exposure on female-paced mating behaviour in the rat.

J Appl Toxicol. 2017;37:996-1003.

Correct perinatal oestrogen levels are critical for sexual differentiation. For example, perinatal exposure to oestrogen causes masculinization and defeminization of the brain in female rats and also induces delayed effects after maturation characterized by early onset of abnormal oestrus cycling. However, the mechanisms underlying the above effects of oestrogen remain to be fully determined. 17α-ethinyloestradiol (EE), a common synthetic oestrogen widely used in oral contraceptives, binds specifically to oestrogen receptors. In this study, we demonstrated the effects of a single neonatal injection of high- or low-dose EE on reproductive behaviours. Female rats within 24h after birth were subcutaneously injected with sesame oil, EE (0.02, 2 mg kg-1) and 17β-oestradiol (E2) (20 mg kg-1). Between 11 and 15 weeks of age, sexual behaviour was tested twice in a paced mating situation. Latency to enter, lordosis and soliciting behaviour were recorded. Both high-dose EE- and E2-treated females showed a significantly lower lordosis quotient, decreased soliciting behaviours, increased rejection and fighting numbers. Accessibility to males was also delayed by neonatal E2 exposure, although it was shortened by high-dose EE exposure. In contrast, low-dose EE-treated females did not exhibit impaired sexual behaviour. These results suggest that single neonatal exposure to a high dose of EE or E2 disturbs the normal development of the female brain, resulting in impaired sexual behaviours in a female-paced mating situation. Besides, the differences noted between high-dose EE- and E2-treated females might be caused by different affinities of the oestrogen receptors, metabolic rates or mechanisms of action.

Keywords: 17α-ethinyloestradiol, lordosis reflection, sexual behaviour

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Cho YM, Mizuta Y, Akagi J, Toyoda T, Sone M, Ogawa K: Size-dependent acute toxicity of silver nanoparticles in mice.

J Toxicol Pathol. 2018;31:73-80.

In this study, we aimed to evaluate changes in the acute toxicity of intraperitoneally administered silver nanoparticles (AgNPs) of varying sizes in BALB/c mice. Seven-week-old female BALB/c mice were intraperitoneally administered AgNPs measuring 10, 60, or 100 nm in diameter (0.2 mg/mouse) and then sacrificed 1, 3, or 6 h after treatment. In mice administered 10 nm AgNPs, reduced activity and piloerection were observed at 5 h post administration, and lowered body temperature was observed at 6 h post administration, with histopathological changes of congestion, vacuolation, single cell necrosis, and focal necrosis in the liver; congestion in the spleen; and apoptosis in the thymus cortex. These histopathological changes were not evident following administration of either 60 or 100 nm AgNPs. These results suggested that smaller AgNPs, e.g., those measuring 10 nm in diameter, had higher acute toxicity in mice.

Keywords: silver nanoparticle, size dependency, acute toxicity

Nonaka M[*], Amakasu K[*], Saegusa Y[*], Naota M[*], Nishimura T[*], Ogawa K, Nishikawa A: Non-neoplastic lesions found only in the two-year

bioassays but not in shorter toxicity studies of rats.
Regul Toxicol Pharmacol. 2017;86:199-204.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has been conducting a prospective evaluation period to validate the criteria for waiving some carcinogenicity studies in rats. Before the waiving strategy is practiced in ICH, it is crucial to elucidate whether non-neoplastic lesions are found only in 2-year rat carcinogenicity studies. To confirm possible importance of 2-year bioassays for evaluating chronic toxicity but not carcinogenicity, we retrospectively surveyed 59 pharmaceuticals approved by the Ministry of Health, Labour and Welfare (MHLW) from 2007 to 2010 in Japan for non-neoplastic lesions observed in carcinogenicity studies. Non-neoplastic histopathological lesions observed only in 2-year carcinogenicity studies but not in 6-month chronic toxicity studies using rats were compared with clinical adverse drug reactions (ADRs). Thirteen non-neoplastic lesions that may correlate with clinical ADRs were classified into three categories: Category 1, lesions not predictable from other nonclinical data except those from 2-year rat carcinogenicity studies; Category 2, lesions predictable mainly from chronic toxicity studies; Category 3, lesions predictable mainly from pharmacological actions. In the present survey, non-neoplastic lesions only found in 2-year rat carcinogenicity studies were neither significant in terms of frequency and severity nor useful for clinical risk management.

Keywords: carcinogenicity study, non-neoplastic lesion, ICH

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Takasu S, Ishii Y, Yokoo Y, Tsuchiya T, Kijima A, Kodama Y, Ogawa K, Umemura T: *In vivo* reporter gene mutation and micronucleus assays in *gpt* delta mice treated with a flame retardant decabromodiphenyl ether.
Mutat Res Genet Toxicol Environ Mutagen. 2017;816-817:7-11.

Polybrominated diphenyl ethers (PBDEs), a class of brominated flame retardants, have been widely used as additive flame retardants. Recently, the use of brominated flame retardants has been restricted or prohibited under various legislative acts because of the persistence, bioaccumulation potential, and toxicity of these compounds. However, there are also additional concerns regarding environmental contamination and human exposure to PBDEs resulting from informal recycling technology. Decabromodiphenyl ether (decaBDE), one type of PBDE, has carcinogenic potential in the livers of rodents. Although one study has shown that decaBDE exerts genotoxic effects, the other *in vitro* and *in vivo* studies were negative for such effects. Thus, it remains unknown whether genotoxic mechanisms are involved in decaBDE-induced hepatocarcinogenesis in rodents. In this study, to explore the genotoxicity of decaBDE in mice, particularly in the context of carcinogenesis, we performed micronucleus assays in the bone marrow and reporter gene mutation assays in the liver using *gpt* delta mice treated with decaBDE at carcinogenic doses for 28days. Our results demonstrated negative results in micronucleus tests and reporter gene mutation assays. Thus, decaBDE did not exert genotoxic effects at carcinogenic target sites and did not show positive results in conventional *in vivo* genotoxicity tests in mice for 4-week treatment. Overall, comprehensive evaluation using *in vivo* genotoxicity data in rats and our data indicated that nongenotoxic mechanisms may be responsible for decaBDE-induced hepatocarcinogenesis.

Keywords: decabromodiphenyl ether, *gpt* delta mouse, *in vivo* genotoxicity

Hirata T, Cho YM, Suzuki I, Toyoda T, Akagi J, Nakamura Y[*1], Numazawa S[*2], Ogawa K: 4-Methylthio-3-butenyl isothiocyanate mediates nuclear factor (erythroid-derived 2)-like 2 activation by regulating reactive oxygen species production in human esophageal epithelial cells.
Food Chem Toxicol. 2018;111:295-301.

4-Methylthio-3-butenyl isothiocyanate (MTBITC) extracted from daikon (*Raphanus sativus*), which shows antimutagenicity, may have applications as an effective chemopreventive agent in several cancers; however, few reports have described the associated mechanisms. We investigated whether MTBITC induced cytoprotective genes, including phase II enzymes, in Het-1A human esophageal epithelial cells. HMOX1, NQO1, and GCLC mRNA levels and

nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein levels were increased in Het-1A cells exposed to 10 μM MTBITC. Reactive oxygen species (ROS) tended to increase when Het-1A cells were treated with MTBITC, and the increases in ROS and Nrf2 expression in the cells treated with MTBITC were completely abolished by treatment with N-acetyl-L-cysteine. We also examined the relationships between Nrf2 activation and mitogen-activated protein kinase (MAPK) signaling by western blot analysis. MTBITC induced extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 phosphorylation in Het-1A cells; however, MTBITC did not affect the relationship between Nrf2 activation and MAPK responses. In the present study, we found that MTBITC induced Nrf2 activation and cytoprotective genes via ROS production in Het-1A cells. These results suggest that MTBITC may have the potential for preventing esophageal carcinogenesis through modification of carcinogen metabolism by phase II enzyme induction via ROS production.

Keywords: 4-methylthio-3-butenyl isothiocyanate, Nrf2, reactive oxygen species

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Ishii Y, Kuroda K, Matsushita K, Yokoo Y, Takasu S, Kijima A, Nohmi T, Ogawa K, Umemura T: Phosphorylation of protein phosphatase 2A facilitated an early stage of chemical carcinogenesis. *Toxicol Appl Pharmacol.* 2017;336:75-83.

Protein phosphatase 2A (PP2A) is a serine-threonine phosphatase that regulates cell signaling pathways. Its inactivation is correlated with tumor malignancy, possibly due to the effects on cell differentiation and malignant cell transformation. Therefore, it has been noted that PP2A could be a promising target for cancer therapy. In our previous study of the hepatocarcinogen estragole (ES), cell proliferation may be required to convert ES-specific DNA adducts to mutations. To explore the trigger for cell proliferation, *gpt* delta rats were administered ES by gavage at doses of 3, 30 and 300 mg/kg/day for 4weeks. ES-induced cell proliferation and gene mutations were observed at only the high dose whereas ES-specific DNA adducts were detected in a dose-dependent manner. Western blot analyses revealed activation of the Akt and ERK pathways without activation of upstream regulators, such as c-Raf, PKC and, PI3K. Phosphorylation of the PP2A C subunit at Tyr307 was found along with phosphorylation of Src. The overall data might imply that PP2A inactivation is responsible for cell cycle progression through activation of the Akt and ERK pathways at high doses of ES. Based on γ-H2AX immunohistochemistry and Western blot analysis for Rad51 protein, the resultant mutation spectra showed large deletion mutations that might result from double strand breaks of DNA. Thus, it is likely that inactivation of PP2A resulted in acceleration and exacerbation of gene mutations. We conclude that PP2A might contribute to an early stage of chemical carcinogenesis, suggesting that PP2A could be a molecular target of primary cancer prevention.

Keywords: DNA adduct, estragole, PP2A

Miyake Y*1, Tokumura M*1, Nakayama H*1, Wang Q*1, Amagai T*1, Ogo S*2, Kume K*3, Kobayashi T*4, Takasu S, Ogawa K, Kannan K*5: Simultaneous determination of brominated and phosphate flame retardants in flame-retarded polyester curtains by a novel extraction method.
Sci Total Environ. 2017;601-602:1333-9.

The use of novel brominated flame retardants (BFRs) and phosphate-based flame retardants (PFRs) has increased as substitutes for hexabromocyclododecane (HBCD) in many consumer products. To facilitate collection of data on chemicals used as flame retardants in textiles and fabrics, we developed an analytical method using liquid chromatography interfaced with tandem mass spectrometry (LC-MS/MS). We compared two extraction methods, one involving ultrasonic extraction (traditional method) using dichloromethane, toluene or acetone and the other encompassing complete dissolution of textile with 25% 1,1,1,3,3,3-hexafluoro-2-propanol/chloroform. The dissolution method extracted up to 204 times more BFRs and PFRs than the traditional ultrasonic extraction. Tris (2,3-dibromopropyl) isocyanurate (TDBP-TAZTO), triphenylphosphine oxide (TPhPO), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tricresyl phosphate (TCsP), and triphenyl phosphate (TPhP) were found in 40 flame-retarded curtain samples purchased from

Japanese market in 2014. TDBP-TAZTO was detected in polyester curtains for the first time. Some of the flame-retarded curtain samples did not contain any of the known target analytes, which suggested the presence of other unknown flame retardants in those fabrics.

Keywords: brominated flame retardant, indoor environment, phosphate flame retardant

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Akagi J, Yokoi M*1,2, Cho YM, Toyoda T, Ohmori H*1, Hanaoka F*1,3, Ogawa K: Hypersensitivity of mouse embryonic fibroblast cells defective for DNA polymerases η, ι and κ to various genotoxic compounds: Its potential for application in chemical genotoxic screening.
DNA Repair. 2018;61:76-85.

Genotoxic agents cause modifications of genomic DNA, such as alkylation, oxidation, bulky adduct formation, and strand breaks, which potentially induce mutations and changes to the structure or number of genes. Majority of point mutations are generated during error-prone bypass of modified nucleotides (translesion DNA synthesis, TLS); however, when TLS fails, replication forks stalled at lesions eventually result in more lethal effects, formation of double-stranded breaks (DSBs). Here we compared sensitivities to various compounds among mouse embryonic fibroblasts derived from wild-type and knock-out mice lacking one of the three Y-family TLS DNA polymerases (Polη, Polι, and Polκ) or all of them (TKO). The compounds tested in this study include genotoxins such as methyl methanesulfonate (MMS) and nongenotoxins such as ammonium chloride. We found that TKO cells exhibited the highest sensitivities to most of the tested genotoxins, but not to the non-genotoxins. In order to quantitatively evaluate the hypersensitivity of TKO cells to different chemicals, we calculated ratios of half-maximal inhibitory concentration for WT and TKO cells. The ratios for 9 out of 10 genotoxins ranged from 2.29 to 5.73, while those for 5 nongenotoxins ranged from 0.81 to 1.63. Additionally, the two markers for DNA damage, ubiquitylated proliferating cell nuclear antigen and γ-H2AX after MMS treatment, were accumulated in TKO cells more greatly than in WT cells. Furthermore, following MMS treatment, TKO cells exhibited increased frequency of sister chromatid exchange compared with WT cells. These results indicated that the hypersensitivity of TKO cells to genotoxins resulted from replication fork stalling and subsequent DNA double-strand breaks, thus demonstrating that TKO cells should be useful for evaluating chemical genotoxicity.

Keywords: translesion synthesis, genotoxicity, Polη, Polι, Polκ

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Kuwata K, Inoue K, Ichimura R, Takahashi M, Kodama Y, Shibutani M*1, Yoshida M*2: β-Catenin mutations are not involved in early-stage hepatocarcinogenesis induced by protoporphyrinogen oxidase inhibitors in mice.
Toxicol Pathol. 2017;45:493-505.

We previously reported the contribution of constitutive androstane receptor (CAR) in cytotoxicity-related hepatocarcinogenesis induced by oxadiazon (OX) or acifluorfen (ACI), two pesticides categorized as Protoporphyrinogen oxidase (PROTOX) inhibitors. The molecular characteristics of preneoplastic and neoplastic lesions induced by OX and ACI were immunohistochemically compared to those by phenobarbital (PB), a typical CAR activator, in wild-type (WT) and CAR-knockout (CARKO) mice after diethylnitrosamine initiation. We focused on changes in β-catenin and its transcriptional product glutamine synthetase (GS). In PB-promoted foci and adenomas, nuclear accumulation of mutated β-catenin was increased with high frequency. PB treatment also increased the multiplicity and area of GS-positive foci and adenomas in WT mice. No foci and adenomas showed nuclear accumulation of β-catenin and expression of GS in CARKO mice, similar to both genotypes of mice treated with OX and ACI. Interestingly, hepatocellular carcinoma induced in ACI-treated WT mice showed nuclear accumulation

of β-catenin and were positive for GS. Our results indicated that β-catenin mutations were not involved in early-stage hepatocarcinogenesis induced by PROTOX inhibitors in mice, although activation of β-catenin and CAR is important in PB-induced tumorigenesis. The significant differences in molecular profiles suggested involvements of multiple MoAs for hepatocarcinogenesis induced by PROTOX inhibitors.
Keywords: acifluorfen, constitutive active/androstane receptor, β-catenin

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Zhang H*, Taya K*, Nagaoka K*, Yoshida M, Watanabe G*: Neonatal exposure to 17α-ethynyl estradiol (EE) disrupts follicle development and reproductive hormone profiles in female rats.
Toxicol Lett. 2017;276:92-9.

Toxic effects induced by exposure to endocrine-disrupting chemicals during fetal and neonatal periods can be irreversible and exert effects throughout an animal's entire life. Our previous study showed that neonatal exposure to 17α-ethynyl estradiol (EE) induced irregular estrous cycle in adults. To uncover the reason for the delayed effect after neonatal exposure to EE, reproductive parameters including ovarian weight, ovarian steroidogenesis, and hormonal profiles were investigated in developing female rats. Ovarian weight decreased at postnatal days (PND) 14 and 21 after neonatal exposure to EE. Ovarian histology at PND21 showed that the ratio of follicles with a diameter >300μm decreased and the ratio of follicles with a diameter of 100-150μm increased in EE-treated ovaries, indicating that neonatal exposure to EE retarded follicular development. Moreover, the expression of P450arom increased at PND14 and the expressions of inhibin/activin subunits βA and βB decreased at PND21 in EE-treated ovaries. Consistent with the expression of P450arom, circulating levels of 17β-estradiol increased at PND14 in EE-treated animals. Furthermore, the circulating levels of luteinizing hormone (LH) also increased at PND14 in the treated animals. Although the expression of Kiss1 did not change in the anteroventral periventricular nucleus (AVPV) of the hypothalamus between controls and EE-treated rats, the expression of Kiss1 was reduced in the arcuate nucleus (ARC) of the hypothalamus at PND14. Based upon those results, we suggest that neonatal exposure to EE disrupted the system regulating the interactions between the reproductive hormones and follicle development in pre-pubertal rats, which may result in reproduction dysfunction in adulthood.
Keywords: 17α-ethynyl estradiol, endocrine-disrupting chemical, steroid hormone

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Zhang H*, Taya K*, Nagaoka K*, Yoshida M, Watanabe G*: 4-Nitrophenol (PNP) inhibits the expression of estrogen receptor β and disrupts steroidogenesis during the ovarian development in female rats.
Environ Pollut. 2017;229:1-9.

4-nitrophenol (PNP), isolated from diesel exhaust particles, has estrogenic and anti-androgenic activities, and affects the hypothalamus-pituitary-gonad axis in male rats. However, the effect of PNP on the reproduction of the female rats is still unknown. The aim of the study was to investigate the effect of neonatal PNP exposure on the ovarian function of female rats. The neonatal female rats were exposed to PNP (10 mg/kg, subcutaneously injection), the ovary and serum samples were collected at postnatal day (PND) 7, 14 and 21. The results showed that the ratio of primordial and primary follicles increased whereas the ratio of antral follicles decreased in the PNP treated ovaries at PND21. Even though no abnormality was observed in cyclicity, there was a significantly delayed timing of vaginal opening in PNP treated rats. The ovarian expression of steroidogenic enzymes including StAR, P450scc, P450c17 and P450arom increased at PND14 in the PNP treated rats compared with the control rats. In consistent with the gene expression, the concentration of estradiol-17β showed the similar pattern. However, PNP exposure failed to cause any significant change in the expression of steroidogenic enzymes in cultured neonatal ovaries. Furthermore, PNP suppressed the expression of estrogen receptor β (ERβ), but not estrogen receptor α (ERα), in cultured ovaries or developmental ovaries. These results suggested that PNP might directly affect the expression of ERβ in the rat ovaries, resulting in the

disrupted steroidogenesis during ovarian development and the delayed puberty.

Keywords: 4-nitrophenol, neonate, steroidogenesis

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Wada K*, Katoh Y*, Ohnuma-Koyama A*, Takahashi N*, Yamada M, Matsumoto K*: 2-Nitroanisole-induced oxidative DNA damage in *Salmonella typhimurium* and in rat urinary bladder cells.

Mutat Res 2017;816-7:18-23.

2-Nitroanisole (2-NA) is used in the manufacturing of azo dyes and causes cancer, mainly in the urinary bladder. Several bladder carcinogens were reported to induce oxidative DNA damage. Thus, we examined the potential induction of oxidative DNA damage by 2-NA using bacterial strain YG3008, a *mutM*$_{ST}$-deficient derivative of strain TA100, and was found that YG3008 was more sensitive to 2-NA than TA100. For further investigation, we performed the comet assay using the urinary bladder and liver of rats, with and without human 8-oxoguanine DNA-glycosylase 1 (hOGG1). Simultaneously, we conducted a micronucleus test using bone marrow from rats to assess the genotoxicity of 2-NA *in vivo*. 2-NA was administered orally to male Fischer 344 rats for 3 consecutive days with 2-NA at doses of 125, 250, and 500 mg/kg; a group treated with the combination of 2-NA and glutathione-SH (GSH); a negative control group; and a positive control group. The comet assay without hOGG1 detected no DNA damage in the liver or urinary bladder, and the micronucleus test did not show clastogenic effects in bone marrow cells. However, the comet assay with hOGG1 was positive in the urinary bladder samples, indicating the induction of oxidative DNA damage in the urinary bladder for the group treated with 2-NA at 500 mg/kg. Moreover, an antioxidant of GSH significantly reduced oxidative DNA damage caused by 2-NA. These results indicate that oxidative DNA damage is a possible mode of action for carcinogenesis in the urinary bladder of rats treated with 2-NA.

Keywords: 2-nitroanisole, bladder carcinogen, oxidative damage

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Sugiyama K, Furusawa H, Grúz P, Honma M: Detection of epigenetic mutagens including anthracene-derived compounds using yeast *FLO1* promoter GFP reporter gene assay.

Mutagenesis. 2017;32:429-435.

Recently, we have reported that the FLO1-mediated flocculation levels of yeast are affected by an epigenetic mutagen, alizarin. Alizarin promoted flocculation and reduced the bulk levels of histone H3 in yeast cells. Since alizarin has been known to possess carcinogenesis-promoting properties, it is important to estimate the effect of alizarin-related compounds on epigenome as measured by the flocculation of yeast. In this study, we examined the effects of two anthracene-derived compounds other than alizarin on the flocculation level of yeast. Purpurin significantly promoted the flocculation in a dose-dependent manner. While, quinizarin had a weaker promoting effect than purpurin. The strain treated with purprin showed *FLO1* mRNA upregulation and reduced histone H3 expression similarly to alizarin. We also confirmed that the purprin-treated cells frequently exhibited abnormally shaped nuclei. Moreover, fluorescence intensities of green fluorescent protein (GFP) reporter under the *FLO1* promoter control were dose-dependently increased by purprin and alizarin in the yeast. Taken together, these results suggest that the GFP reporter gene system utilising the *FLO1* promoter is useful for the detection of epigenetic mutagens including anthracene-derived compounds.

Keywords: epigenetic mutagen, yeast, *FLO1*

Grúz P, Shimizu M*, Sugiyama K, Honma M: Mutagenicity of ω-3 fatty acid peroxidation products in the Ames test. *Mutat Res*. 2017;819:14-19.

Polyunsaturated fatty acids (PUFA) represent one of the main building blocks of cellular membranes and their varying composition impacts lifespan as well as susceptibility to cancer and other degenerative diseases. Increased intake of ω-3 PUFA is taught to compensate for the abundance of ω-6 PUFA in modern human diet and prevent cardiocirculatory diseases. However, highly unsaturated PUFA of marine and seed origin easily oxidize to aldehydic products which form DNA adducts. With increased PUFA consumption it is prudent to re-evaluate ω-3 PUFA safety and the genotoxic hazards of their

metabolites. We have used the standard Ames test to examine the mutagenicity of 2 hexenals derived from lipid peroxidation of the common ω-3 PUFA in human diet and tissues. Both 4-hydroxyhexenal and 2-hexenal derived from the ω-3 docosahexaenoic and α-linolenic acid, respectively, induced base substitutions in the TA104 and TA100 Ames strains in a dose dependent manner. Their mutagenicity was dependent on the Y-family DNA polymerase RI and they did not induce other types of mutations such as the -2 and -1 frameshifts in the TA98 and TA97 strains. Our results expand previous findings about the mutagenicity of related ω-3 peroxidation product 4-oxohexenal and raise alert that overuse of ω-3 rich oils may have adverse effect on genome stability.

Keywords: ω-3 fatty acid, Ames test, DNA polymerase RI, peroxidation

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Gadaleta D*1, Porta N*1, Vrontaki E*1,2, Manganelli S*1, Manganaro A*3, Sello G*4, Honma M, Benfenati E*1: Integrating computational methods to predict mutagenicity of aromatic azo compounds.

J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2017;2;35(4):239-257.

Azo dyes have several industrial uses. However, these azo dyes and their degradation products showed mutagenicity, inducing damage in environmental and human systems. Computational methods are proposed as cheap and rapid alternatives to predict the toxicity of azo dyes. A benchmark dataset of Ames data for 354 azo dyes was employed to develop three classification strategies using knowledge-based methods and docking simulations. Results were compared and integrated with three models from the literature, developing a series of consensus strategies. The good results confirm the usefulness of in silico methods as a support for experimental methods to predict the mutagenicity of azo compounds.

Keywords: azo dyes, mutagenicity, computational methods

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Sugiyama K, Furusawa H, Grúz P, Honma M: Functional role of DNA methylation at the *FLO1* promoter in budding yeast.

FEMS Microbiol. Lett. 2017; 364

We have previously reported that the transformation of the budding yeast with plasmids encoding the human DNA methyltransferases *DNMT1* and *DNMT3B* cDNAs induces the mRNA of flocculin gene *FLO1* and the flocculation phenotype. In the present study, we evaluated the effect of *DNMT* inhibitor in the transformed yeasts using a *FLO1* promoter-based green fluorescent protein (GFP) reporter gene assay. The DNMT inhibitor, 5-aza-2'-deoxycytidine (5AZ), decreased GFP fluorescence driven by *FLO1* promoter in *DNMT*-genes transformed yeast (*DNMT* yeast). Surprisingly, the GFP activity driven by cytosine-phosphate-guanine (CpG) motif-reduced *FLO1*promoter decreased both in DNMTs gene-transformed and control strains. Yeast cells transformed with expression vector encoding a maintenance enzyme *DNMT1* cDNA showed a flocculation phenotype that was associated with an enhanced mRNA level of *FLO1*. Bisulfite sequencing revealed methylated CpG sites at the *FLO1* promoter in a control strain not expressing any *DNMT* transgenes, and no detectable methylation at the sites was observed in cells treated with 5AZ. These results suggest that the *FLO1* promoter is endogenously de novo methylated leading to the activation of *FLO1* gene transcription. Furthermore, the methylation level at the *FLO1* promoter is responsible for the significant differences in *FLO1* promoter-driven expression of GFP in *DNMT* yeast.

Keywords: DNA methylation, *FLO1* promoter, yeast

Masumura K, Toyoda-Hokaiwado N, Niimi N, Grúz P, Wada NA*, Takeiri A*, Jishage KI*, Mishima M*, Nohmi T: Limited ability of DNA polymerase kappa to suppress benzo[*a*]pyrene-induced genotoxicity *in vivo*.

Environ Mol Mutagen. 2017;58:644-653.

DNA polymerase kappa (Polk) is a specialized DNA polymerase involved in translesion DNA synthesis. To understand the protective roles against genotoxins *in*

vivo, we established inactivated Polk knock-in *gpt* delta (inactivated Polk KI) mice that possessed reporter genes for mutations and expressed inactive Polk. In this study, we examined genotoxicity of benzo[*a*] pyrene (BP) to determine whether Polk actually suppressed BP-induced genotoxicity as predicted by biochemistry and *in vitro* cell culture studies. Seven-week-old inactivated Polk KI and wild-type (WT) mice were treated with BP at doses of 5, 15, or 50 mg/(kg·day) for three consecutive days by intragastric gavage, and mutations in the colon and micronucleus formation in the peripheral blood were examined. Surprisingly, no differences were observed in the frequencies of mutations and micronucleus formation at 5 or 50 mg/kg doses. Inactivated Polk KI mice exhibited approximately two times higher *gpt* mutant frequency than did WT mice only at the 15 mg/kg dose. The frequency of micronucleus formation was slightly higher in inactivated Polk KI than in WT mice at the same dose, but it was statistically insignificant. The results suggest that Polk has a limited ability to suppress BP-induced genotoxicity in the colon and bone marrow and also that the roles of specialized DNA polymerases in mutagenesis and carcinogenesis should be examined not only by *in vitro* assays but also by *in vivo* mouse studies. We also report the spontaneous mutagenesis in inactivated Polk KI mice at young and old ages.

Keywords: DNA polymerase kappa, translesion DNA synthesis, mutation frequency

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Petkov, PI[*1], Schultz TW[*2], Honma M, Kirilov K[*1], Kotov S[*1], Mekenyan OG[*1]: Predicting *in vitro* genotoxicity by mouse lymphoma L5178Y thymidine kinase mutation assay (MLA): Accounting for simulated metabolic activation of chemicals.
Computational Toxicology. 2017;4:45-53.

The mouse lymphoma L5178Y thymidine kinase locus gene mutation assay (MLA) is typically part of regulatory batteries of methods used for *in vitro*evaluation of substances eliciting small gene mutations, and gross structural alterations at the chromosomal level. In an effort to make the MLA endpoint amenable to category formation to support assessments of mutagenesis, a model with 52 DNA and 32 protein binding alerts particular to the MLA endpoint has been developed. Each alert is supported by a mechanistic justification, an alert-specific training set and an alert performance evaluation. Subsequently, these alerts have been used in combination with an *in vitro* rat liver S9 fraction metabolic simulator (the TIssue MEtabolism Simulator (TIMES) platform) to evaluate MLA mutagenicity of likely metabolites of MLA negative parent compounds. The resulting system provides for transparent *in silico* identification of structural and general parametric requirements, as well as, binding mechanisms in parent chemicals and their simulated metabolites. When applied to the training sets, performance of the TIMES_MLA models, both with and without rat liver S9 activation show high (≥80%) sensitivity, specificity and concordance, which is consistent with the repeatability and reproducibility of experimental MLA results.

Keywords: MLA, alert-based profiling, metabolic simulation

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Horibe A[*1], Odashima S[*1], Hamasuna N[*1], Morita T, Hayashi M[*2]: Weight of contribution of *in vitro* chromosomal aberration assay for evaluation of pesticides: Experience of risk assessment at the Food Safety Commission of Japan.
Regul Toxicol Pharmacol. 2018:95:133-41

Among the set of safety evaluations for pesticides, genotoxicity assay data are mandatory. The standard test battery outcomes are used for mechanistic consideration of carcinogenicity, if any. As a rule, if a certain substance is carcinogenic and the mechanism of it includes genotoxicity, the Food Safety Commission might decide it is not possible to establish the acceptable daily intake of that pesticide. Therefore, the information about genotoxicity is critical for potentially carcinogenic chemicals, whether the applied substance will be adopted and permitted for use or not as pesticides. It is important to assess fairly, carefully, and transparently, but feasible, rapid, and efficient assessment also should be taken into account. Therefore, needless to say, the assay(s) should have

the sensitivity to detect potent mutagens. It is also important to be aware that the required data set should be consisted of reliable assays without certain assay(s) that give(s) false positive information or offer less of a contribution for the safety assessment.

Keywords: *in vitro* chromosomal aberration assay, *in vivo* micronucleus assay, pesticides

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Narita K*1, Ishii Y*1, Vo PTH*1, Nakagawa F*1, Ogata S*2, Yamashita K*3, Kojima H, Itagaki H*1: Improvement of human cell line activation test (h-CLAT) using short-time exposure methods for prevention of false-negative results.
J Toxicol Sci. 2018;43(3):229-240.

Recently, animal testing has been affected by increasing ethical, social, and political concerns regarding animal welfare. Several *in vitro* safety tests for evaluating skin sensitization, such as the human cell line activation test (h-CLAT), have been proposed. However, similar to other tests, the h-CLAT has produced false-negative results, including in tests for acid anhydride and water-insoluble chemicals. In a previous study, we demonstrated that the cause of false-negative results from phthalic anhydride was hydrolysis by an aqueous vehicle, with IL-8 release from THP-1 cells, and that short-time exposure to liquid paraffin (LP) dispersion medium could reduce false-negative results from acid anhydrides. In the present study, we modified the h-CLAT by applying this exposure method. We found that the modified h-CLAT is a promising method for reducing false-negative results obtained from acid anhydrides and chemicals with octanol-water partition coefficients ($LogK_{ow}$) greater than 3.5. Based on the outcomes from the present study, a combination of the original and the modified h-CLAT is suggested for reducing false-negative results. Notably, the combination method provided a sensitivity of 95% (overall chemicals) or 93% (chemicals with $LogK_{ow} > 2.0$), and an accuracy of 88% (overall chemicals) or 81% (chemicals with $LogK_{ow} > 2.0$). We found that the combined method is a promising evaluation scheme for reducing false-negative results seen in existing *in vitro* skin-sensitization tests. In the future, we expect a combination of original and modified h-CLAT to be applied in a newly developed *in vitro* test for evaluating skin sensitization.

Keyword: false-negative results, skin sensitization test, h-CLAT

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Daniel AB*1, Strickland J*1, Allen D*1, Casati S*2, Zuang V*2, Barroso J*2, Whelan M*2, Régimbald-Krnel MJ*3, Kojima H, Nishikawa A, Park HK*4, Lee JK*4, Kim TS*4, Delgado I*5, Rios L*6, Yang Y*7, Wang G*8, Kleinstreuer N*9: International regulatory requirements for skin sensitization testing.
Regul Toxicol Pharmacol. 2018;Jun 95:52-65.

Skin sensitization test data are required or considered by chemical regulation authorities around the world. These data are used to develop product hazard labeling for the protection of consumers or workers and to assess risks from exposure to skin-sensitizing chemicals. To identify opportunities for regulatory uses of non-animal replacements for skin sensitization tests, the needs and uses for skin sensitization test data must first be clarified. Thus, we reviewed skin sensitization testing requirements for seven countries or regions that are represented in the International Cooperation on Alternative Test Methods (ICATM). We noted the type of skin sensitization data required for each chemical sector and whether these data were used in a hazard classification, potency classification, or risk assessment context; the preferred tests; and whether alternative non-animal tests were acceptable. An understanding of national and regional regulatory requirements for skin sensitization testing will inform the development of ICATM's international strategy for the acceptance and implementation of non-animal alternatives to assess the health hazards and risks associated with potential skin sensitizers.

Keywords: non-animal methods, regulatory requirements, skin sensitization testing

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Tsukumo H*1, Matsunari N*2, Yamashita K*3, Kojima H, Itagaki H*1: Lipopolysaccharide interferes with the use of the human Cell Line Activation Test to determine the allergic potential of proteins.
J Pharmacol Toxicol Methods. 2018; Feb 10;92:34-42.

It was believed that high molecular weight molecules including proteins cannot penetrate the skin. However, protein penetration through disrupted/ruptured skin has been reported recently, thus carrying the potential for inducing an allergic response. We used the human Cell Line Activation Test (h-CLAT), an *in vitro* skin sensitization test, to assess the allergic potential of proteins by measuring levels of CD86 and CD54 in the human monocytic leukemia cell line THP-1. Six allergens including ovalbumin (OVA) and human serum albumin (HSA; negative control) upregulated CD86 and/or CD54; a false-positive result was obtained using HSA. This was caused by lipopolysaccharide (LPS) contamination. Naturally derived materials often include LPS at various concentrations and may influence protein induction of CD86 and CD54. Additionally, polymyxin B, an LPS inhibitor, could not completely overcome the effect of LPS. Therefore, if test proteins contain ≥0.1 EU/mL LPS, their allergenic potency will not be assessed accurately using h-CLAT. These data show that naturally occurring materials or those derived from living organisms should be evaluated for their LPS content. It is important to confirm the applicability of *in vitro* methods such as h-CLAT for assessing the allergenic potency of naturally occurring proteins; our findings can be a foundation for future studies.
Keyword: h-CLAT, lipopolysaccharide, skin sensitization test

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Matsumoto M, Furukawa M*, Kobayashi K, Iso T, Igarashi T, Yamada T, Hirose A: A 28-day repeated oral-dose toxicity study of insecticide synergist N-(2-ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide in rats.
Fundam Toxicol Sci. 2018;5:1-11

N-(2-Ethylhexyl)-1-isopropyl-4-methylbicyclo[2.2.2]oct-5-ene-2,3-dicarboximide (Synepirin 500; CAS: 13358-11-7) is used as a synergist, a chemical that makes pesticide ingredients more effective. People can be exposed to Synepirin 500 by using insecticides containing this chemical or from residues in food. The Japanese government chose this chemical as a target substance in its existing chemical testing program. Crl:CD(SD) rats were administered 0, 40, 200, and 1,000 mg/kg/day Synepirin 500 by gavage for 28 days, followed by a 14 day recovery period. Diarrhea or soft feces were observed in both sexes at 1,000 mg/kg/day. Absolute and/or relative liver weights significantly increased at ≥40 mg/kg/day in females and at ≥200 mg/kg/day in males. Absolute and/or relative thyroid weights significantly increased in both sexes at 1,000 mg/kg/day. These changes were still significant at the end of the recovery period in females. Significantly prolonged prothrombin time and activated partial thromboplastin time were observed in males receiving ≥40 mg/kg/day. Histopathological changes in the liver and thyroid were observed in both sexes at 1,000 mg/kg/day. On the basis of the effects on the liver, the level of the lowest observed adverse effect from repeated doses of Synepirin 500 was judged to be 40 mg/kg/day for rats.
Keywords: Synepirin 500, Synergist, OECD TG 407

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Narita K*, Vo PTH*, Yamamoto K*, Kojima H,

Itagaki H*: Preventing false-negatives in the *in vitro* skin sensitization testing of acid anhydrides using interleukin-8 release assays.

Toxicol In Vitro. 2017;Aug 42:69-75.

In vitro safety tests may be used as replacements for animal tests owing to their accuracy and high-throughput performance. However, several *in vitro* skin sensitization tests produce false-negative results such as acid anhydride. Here, we investigated the relationship between false-negative results of acid anhydride and its hydrolysis by aqueous vehicle. Differences in the pattern of hydrolysis for phthalic anhydride (PAH) due to addition of 1 drop of stock solution of PAH in liquid paraffin (LP) dispersion medium and PAH in DMSO were analyzed in a cell-free system. The results showed that use of LP dispersion medium stabilized the concentration of PAH in water over 5min by sustained-release, although almost all PAH converted to phthalic acid in water within 5min using DMSO. Additionally, treatment of THP-1 cells with PAH and phthalic acid using LP dispersion medium for 5min resulted in a 32-fold increase in IL-8 release for PAH as compared with that in the vehicle control. In contrast, for PAH using aqueous vehicle and phthalic acid using LP dispersion medium, there were no significant increases in IL-8 release. Similarly, using LP dispersion medium, trimellitic anhydride significantly increased IL-8 release was observed.

Keywords: false-negative, skin sensitization test, IL-8

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Akagi T*1, Nagura M*1,2, Hiura A*1, Kojima H, Akashi M*1: Construction of Three-Dimensional Dermo-Epidermal Skin Equivalents Using Cell Coating Technology and Their Utilization as Alternative Skin for Permeation Studies and Skin Irritation Tests.

Tissue Eng Part A. 2017;Jun;23(11-12):481-490.

In vitro generated human skin equivalents are generating interest as promising tools in basic study, as alternatives to animal testing, and for clinical applications in regenerative medicine. For prediction of skin irritation and corrosion, three-dimensional human skin equivalents consisting of differentiated human keratinocytes (KCs) have been developed and some models have been internationally accepted. However, more delicate assessments using full-thickness skin models, such as skin sensitization tests, cannot be performed due to the lack of a dermis containing fibroblasts or appendages. In a previous study, we developed dermo-epidermal human skin equivalents (DESEs) using a cell coating technique, which employs cell surface coating by layer-by-layer assembled extracellular matrix (ECM) films. The DESEs with dermis consisting of normal human dermal fibroblasts (NHDFs) and epidermis consisting of human KCs were easily fabricated by using this technology. In this study, the constructed DESEs were evaluated as an alternative skin for skin permeation and irritation tests. A good relationship of permeability coefficient of chemicals was observed between the DESEs and human skin data. We investigated whether the DESEs, a new *in vitro* skin model, are capable of identifying skin irritant and nonirritant substances among 20 reference chemicals. It was confirmed that the DESEs are applicable to skin irritation testing as defined in the European Centre for the Validation of Alternative Methods (ECVAM) Performance Standard (OECD Test Guideline 439). We further studied the construction of DESEs with density-controlled blood capillary networks using human umbilical vein endothelial cells (HUVECs). The results suggest that DESEs allowing incorporation of skin appendages are more promising alternatives to animal testing and can be applied to the design of physiologically relevant *in vitro* skin models.

Keywords: alternatives to animal testing, human skin equivalents, layer-by-layer assembly

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Ogihara T*1,2 Arakawa H*1,3, Jomura T*4, Idota Y*1, Koyama S*1, Yano K*1, Kojima H: Utility of human hepatocyte spheroids without feeder cells for evaluation of hepatotoxicity.

J Toxicol Sci. 2017;42(4):499-507

We investigated the utility of three-dimensionally cultured hepatocytes (spheroids) without feeder cells (Sph(f-)) for the prediction of drug-induced

liver injury (DILI) in humans. Sph(f−) and spheroids cultured on feeder cells (Sph(f+)) were exposed to the hepatotoxic drugs flutamide, diclofenac, isoniazid and chlorpromazine at various concentrations for 14 days, and albumin secretion and cumulative leakages of toxicity marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and γ-glutamyl transpeptidase (γ-GTP), were measured. The cumulative AST, LDH or γ-GTP leakages from Sph(f−) were similar to or greater than those from Sph(f+) for all drugs tested, although ALT leakages showed no consistent difference between Sph(f+) and Sph(f−). In the case of Sph(f−), significant correlations among all the toxicity markers except for γ-GTP were observed. As regards the drug concentrations causing 1.2-fold elevation of enzyme leakage ($F_{1.2}$), no consistent difference between Sph(f+) and Sph(f−) was found, although several $F_{1.2}$ values were undetermined, especially in Sph(f+). The IC_{50} of albumin secretion and $F_{1.2}$ of AST leakage from Sph(f−) were equal to or lower than those of Sph(f+) for all the tested drugs. These results indicate that feeder cells might contribute to resistance to hepatotoxicity, suggesting DILI could be evaluated more accurately by using Sph(f−). We suggest that long-term exposure of Sph(f−) to drugs might be a versatile method to predict and reproduce clinical chronic toxicity, especially in response to repeated drug administration.

Keywords: feeder cells, human hepatocytes, spheroid

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Arakawa H*1,2, Kamioka H*1, Jomura T*3, Koyama S*1, Idota Y*1, Yano K*1, Kojima H, Ogihara T*1,4: Preliminary Evaluation of Three-Dimensional Primary Human Hepatocyte Culture System for Assay of Drug-Metabolizing Enzyme-Inducing Potential.

Biol Pharm Bull. 2017;40(7):967-974

Drug-induced liver injury (DILI) is a common reason for withdrawal of candidate drugs from clinical trials, or of approved drugs from the market. DILI may be induced not only by intact parental drugs, but also by metabolites or intermediates, and therefore should be evaluated in the enzyme-induced state. Here, we present a protocol for assay of drug-metabolizing enzyme-inducing potential using three-dimensional (3D) primary cultures of human hepatocytes (hepatocyte spheroids). Hepatocyte spheroids could be used up to 21d after seeding (pre-culture for 7d and exposure to inducer for up to 14d), based on preliminary evaluation of basal activities of CYP subtypes and mRNA expression of the corresponding transcription factor and xenobiotic receptors (aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR)). After 2d exposure of hepatocyte spheroids to omeprazole, phenobarbital and rifampicin (typical inducers of CYP1A2, 2B6 and 3A4, respectively), CYP1A2, 2B6 and 3A4 mRNA expression levels were significantly increased. The mRNA induction of CYP2B6 remained reasonably stable between days 2 and 14 of exposure to inducers, while induction of both CYP1A2 and 3A4 continued to increase up to day 14. These enzyme activities were all significantly increased compared with the control until day 14. Our findings indicate that our 3D hepatocyte spheroids system would be especially suitable for long-term testing of enzyme activity induction by drugs, either to predict or to verify clinical events.

Keywords: drug-induced liver injury, primary human hepatocyte, spheroid

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Tanabe S, Kobayashi K, Matsumoto M, Serizawa H*, Igarashi T, Yamada T, Hirose A: Toxicity of repeated 28-day oral administration of acenaphthylene in rats.

Fundam Toxicol Sci. 2017;4:247-259

AIM: To assess the toxicity of acenaphthylene,

Sprague–Dawley rats were repeatedly administered with the chemical *via* oral gavage at daily doses of 0, 4, 20, or 100 mg/kg/day for 28 days, followed by a 14-day recovery period. Decreases in body weight, food consumption, and body weight gain were observed in males and females in the 100 mg/kg/day group. Additionally, increases in water consumption and urine volume, and decreases in osmolality were observed in both males and females in this group. Moreover, this highest dose was linked to decreases in the reticulocyte percentage and increases in platelet counts in males and females, and females additionally exhibited increases in the hemoglobin concentration, mean corpuscular hemoglobin concentration, and activated partial thromboplastin time. Meanwhile, total cholesterol and phospholipid levels were elevated in males and females treated with 100 mg/kg/day acenaphthylene, with males additionally displaying increased total protein and albumin levels. Increased relative liver weights and changes in liver histopathology were observed in males and females treated with 20 or 100 mg/kg/day acenaphthylene. Additionally, organ weight and/or histopathological changes were observed in the thymus, heart, femoral and sternal bones including bone marrow, urinary bladder, kidneys, spleen, and adrenal gland in both sexes, in the stomach in males, and in the uterus, ovaries, and mesenteric lymph nodes in females in the 100 mg/kg/day group. Some changes exhibited plasticity in the recovery period. Based on these results, the no-observed-effect-level of acenaphthylene after repeated 28-day oral administration was 4 mg/kg/day.

Keywords: acenaphthylene, body weight loss, hematological toxicity

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Tanabe S, Ohara M*, Ito M*, Noda A*, Kobayashi K, Matsumoto M, Hirose A: Toxicity in repeated 28-day oral administration of *N*-phenyl-1-naphthylamine in rats.

Fundam Toxicol Sci. 2017;4:207-218

AIM: To assess the toxicity of N-phenyl-1-naphthylamine, Sprague Dawley rats were repeatedly administered with the chemical by oral gavage daily at doses of 0, 4, 20, 100, and 500 mg/kg/day for 28 days, followed by a 14-day recovery period. A significant decrease or decreasing trend of red blood cell counts, hemoglobin concentration, hematocrit, and mean corpuscular hemoglobin concentration and a significant increase in reticulocyte counts were observed at a dose of 500 mg/kg in both male and female rats. Increase in blood urea nitrogen and sodium levels was observed in male rats that received 500 mg/kg; increase in serum total protein, albumin, and calcium levels and in albumin/globulin ratio were observed in female rats that received 500 mg/kg. Increase in relative liver weight in female rats that received 100 mg/kg and increase in the absolute and relative liver weights in both male and female rats that received 500 mg/kg were observed; increases in the absolute and relative spleen weights and absolute kidney weight in female rats that received 500 mg/kg were observed. Hypertrophy of centrilobular hepatocyte and extramedullary hematopoiesis in the spleen were observed in both male and female rats at doses of 100 and 500 mg/kg. Renal tubular dilatation and papillary necrosis were observed in both male and female rats that received 500 mg/kg. These changes had the reversible trend in the recovery period. Based on these results, the no-observed-effect-level of N-phenyl-1-naphthylamine after repeated daily oral administration for 28 days was determined to be 20 mg/kg/day for both sexes.

Keywords: N-phenyl-1-naphthylamine, liver toxicity, hypertrophy of centrilobular hepatocyte

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Fukuhara K*, Ohno A, Kikura-Hanajiri R: A metabolic study on the biochemical effects of chiral illegal drugs in rats using ^{1}H-NMR spectroscopy.

Yakugaku Zasshi. 2017;137(9):1147-1154

Considering the pharmacological effects of chiral drugs, enantiopure drugs may differ from their racemic mixture formulation in efficacy, potency, or adverse effects. Levomethorphan (LVM) and Dextromethorphan (DXM) act on the central nervous system and exhibit different pharmacological features. LVM, the l-stereoisomer of methorphan, shows many similarities to opiates such as heroin, morphine and codeine, including the potential for addiction, while the

d-stereoisomer, DXM, does not have the same opioid effect. In the present study, NMR-based metabolomics were performed on the urine of rats treated with these stereoisomers, and showed significant differences in metabolic profiles. In urine within 24 h after treatment of these samples, levels of citrate, 2-oxoglutarate, creatine, and dimethylglycine were higher in LVM-treated rats than in DXM-treated rats. While urinary levels of hippurate and creatinine gradually increased over 72 h in DXM-treated rats, these metabolites were decreased in the urine by 48-72 h after treatment with LVM. The levels of these changed metabolites may provide the first evidence for different cellular responses to the metabolism of stereoisomers.

Keywords: dextromethorphan, levomethorphan, NMR

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Yamada T, Hirose A: Case study on the use of an integrated approach to testing and assessment for the repeated-dose toxicity of phenolic benzotriazoles, Organisation for Economic Co-operation and Development (OECD), Series on Testing & Assessment No. 271. 1-44, 2017.

Phenolic benzotriazoles are UV absorbers added to various polymer products to protect against UV degradation. In total, there are around two dozen different phenolic benzotriazoles on the market. Several substances of this group have been described as emerging contaminants with properties of concern for environmental and human health. The phenolic benzotriazole category was previously assessed by the United States Environmental Protection Agency (EPA) High Production Volume (HPV) Challenge Program (U.S. EPA, 2009), National Toxicology Program (NTP) Chemical Information Review (NTP, 2011), and Government of Canada (Environment and Climate Change in Canada and Health Canada, 2016). However, these assessments did not include a detailed examination of the structure-toxicity relationships. A weight-of-evidence approach was used to assess the persistence of certain phenolic benzotriazoles (Brandt et al., 2016), but read-across assessment has not yet been attempted for the repeated-dose toxicity endpoint.

This case study focuses on repeated-dose toxicity endpoints for more detailed category assessment of structurally similar but unevaluated phenolic benzotriazoles. Transcriptomic profiles were generated for some category members and then integrated into the assessment. This case study is intended to address how read-across can be applied to screening assessments under the CSCL.

Keywords: IATA, repeated-dose toxicity