Matsui K[*1], Nakamichi K[*1], Nakatani M[*1], Yoshida H, Yamashita S[*2], Yokota S[*1]: Lowly-buffered biorelevant dissolution testing is not necessarily biopredictive of human bioequivalence study outcome: Relationship between dissolution and pharmacokinetics.

Int J Pharm. 2023;631:122531. doi:10.1016/j.ijpharm.2022.122531.

It has been revealed that buffer capacity of aspirated human intraluminal fluid is much lower than that of *in vitro* compendial dissolution media. Since buffer capacity significantly alters the dissolution profile of certain drug products, dissolution testing in highly buffered media dictates poor predictability of *in vivo* drug performance. To mitigate this inconsistency, low buffer capacity medium was suggested as an *in vivo* representation (biorelevant dissolution testing). The purpose of this study was to characterize the dissolution profiles of enteric-coated drug products in different buffer capacity media in a flow through cell dissolution apparatus, and to evaluate the *in vivo* predictability of human bioequivalence study outcomes conducted in the fasted state. It was confirmed that the lower the buffer capacity of dissolution media, the higher the discriminatory power of esomeprazole magnesium hydrate enteric-coated pellets, reflecting human bioequivalence failure. In the meantime, two duloxetine hydrochloride enteric-coated pellets also exhibited distinct dissolution profiles in such a lowly buffered medium despite the fact that these two are bioequivalent in human. Biopharmaceutical and pharmacokinetic characteristics comparison suggested that low intestinal permeability and small systemic elimination rate of duloxetine hinders the clear impact of different dissolution profile on its *in vivo* performance. These data suggest that dissolution comparison in physiologically-relevant low buffer capacity media is not always indicative of human bioequivalence. Instead, biopharmaceutical and pharmacokinetic aspects must be taken into consideration to make biorelevant dissolution testing biopredictive.

Keywords: Bioequivalence, Buffer capacity, Dissolution testing

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Naoya Araki[*1], Tokio Morita, Takeshi Akiyoshi[*1], Hiroki Kataoka[*1], Kodai Yajima[*1], Kazuhiro Katayama[*2], Ayuko Imaoka[*1], Hisakazu Ohtani[*1]: Comparison of the inhibitory properties of the fruit component naringenin and its glycosides against OATP1A2 genetic variants.

Drug Metab Pharmacokinet. 2022;46:100454. doi:10.1016/j.dmpk.2022.100464.

Non-synonymous genetic variants of organic anion-transporting polypeptide (OATP) 1A2 with altered transport activity have been identified. Naringin and narirutin, which are found in grapefruit, and their aglycon naringenin inhibit OATP1A2. However, their inhibitory effects on OATP1A2 variants have not been investigated, nor has the influence of their molecular structure, such as the number of sugar moieties, on their inhibitory potency. This study aimed to investigate the inhibitory effects of naringenin, its monosaccharide glycoside prunin, and its disaccharide glycosides naringin and narirutin on fexofenadine (FEX) uptake by OATP1A2 variants (Ile13Thr, Asn128Tyr, Ala187Thr, and Thr668Ser). Naringin, narirutin, and prunin inhibited FEX (0.3 μM) uptake by all of the examined OATP1A2 variants in a concentration-dependent manner. Compared with those for the wild type, the inhibition constants (K_i) of naringin, narirutin, and prunin for the Ala187Thr variant were significantly increased by 3.36-fold, 7.55-fold, and 10.6-fold, respectively. Naringenin inhibited all of the OATP1A2 variants, except Ala187Thr, concentration-dependently. The order of inhibitory potency was as follows for all variants: aglycone > monosaccharide glycoside > disaccharide glycosides. These results suggest that the Ala187Thr variant is less vulnerable to inhibition by naringenin and its glycosides. Moreover, greater glycosylation of naringenin reduces its inhibitory potency against OATP1A2.

Keywords: citrus fruits, food-drug interaction, genetic variants

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Hongye Han[*1], Takeshi Akiyoshi[*1], Tokio Morita, Hiroki Kataoka[*1], Kazuhiro Katayama[*2], Kodai Yajima[*1], Ayuko Imaoka[*1], Hisakazu Ohtani[*1]: Comparison of the transport kinetics of fexofenadine and its pH dependency among OATP1A2 genetic variants.

Drug Metab Pharmacokinet. 2022;47:100470. doi:10.1016/j.dmpk.2022.100470.

Little is known about the influence of non-synonymous genetic variations in the organic anion-transporting polypeptide (OATP) 1A2 on the transport kinetics of its substrate fexofenadine. Moreover, the pH-dependency of fexofenadine uptake also remains unclear. This study aimed to evaluate the effects of genetic variants (Ile13Thr, Asn128Tyr, Glu172Asp, Ala187Thr, and Thr668Ser) on the OATP1A2-mediated uptake of fexofenadine at pH 6.3 and 7.4 and compare the pH dependency of OATP1A2-mediated uptake of fexofenadine and estrone 3-sulfate. The uptake clearances of 0.3 μM and 300 μM fexofenadine were compared with those of 0.3 μM and 300 μM estrone 3-sulfate at pH 6.3 and 7.4. Among the six variants examined, the Thr668Ser variant showed the highest fexofenadine uptake clearance (V_{max}/K_m); i.e., 4.53- and 6.28-fold higher uptake clearance than the wild type at pH 6.3 and 7.4, respectively. All variants exhibited significantly higher fexofenadine uptake at pH 6.3 than at pH 7.4. Compared with estrone 3-sulfate uptake, the uptake of 0.3 μM fexofenadine was less sensitive to pH. Our findings suggest that genetic variations in OATP1A2 may lead to altered intestinal absorption of fexofenadine, such as increased absorption in subjects bearing the Thr668Ser variant, which showed higher uptake activity.

Keywords: intestinal absorption, single nucleotide polymorphism, pH-dependent uptake

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Koide T, Hiyama Y: Analysis of over-granulated particles using near-infrared chemical imaging and attenuated total reflectance-infrared techniques.

International Journal of Pharmaceutics. 2022;617:121607. doi:10.1016/j.ijpharm.2022.121607

To elucidate the previously described mechanism of segregation caused by over-granulation, we analyzed over-granulated particles using the techniques of near-infrared chemical imaging (NIR-CI) and attenuated total reflectance infrared (ATR-IR). The same area of over-granulated particles was measured using both techniques. The distributions of the active ingredient, ethenzamide, and other additives in the over-granulated particles were compared. As ATR-IR chemical imaging easily identifies components and has higher spatial resolution than NIR-CI, it permitted a clearer observation of the distribution of ingredients, particularly in fine cornstarch particles. Using both techniques, segregation of components were observed as previously reported. Although lactose was barely observed in the ethenzamide-enriched regions, ethenzamide and cornstarch were observed in lactose-enriched regions. This suggests that only lactose aggregated and segregated from the other compounds during the process of granulation. Hydrophilic lactose aggregation is supposedly caused by the behavior of water during granulation. In conclusion, ATR-IR chemical imaging is an excellent analytical technique for obtaining the detailed distribution of components. Furthermore, fusion of ATR-IR chemical imaging and NIR-CI is a useful tool for understanding drug manufacturing processes and may be applicable to pharmaceutical manufacturing and quality control.

Keywords: image analysis, near-infrared spectroscopy, high shear granulation

Inoue M[*1], Osada T[*1], Hisada H[*1], Koide T, Fukami T[*1], Roy A[*2], Carriere J[*2]: Quantitative monitoring of cocrystal polymorphisms in model tablets using transmission low-frequency Raman spectroscopy.

Journal of Pharmaceutical Sciences. 2023;112:225－9. doi: 10.1016/j.xphs.2022.09.009

Cocrystallization is a technique for improving the physical properties of active pharmaceutical ingredients. However, cocrystals can transform into more stable polymorphs as well as dissociate to original materials. Therefore, an analytical technique is required to determine the polymorphic transformation quickly and accurately in tablets. The purpose of this study is to develop a method to monitor cocrystal polymorphs in model tablets using transmission low-frequency Raman spectroscopy. The tablets, consisting of only metastable polymorphs of caffeine-glutaric acid cocrystals, were stored under various relative humidity

levels. The composition of the cocrystal polymorphs were calculated from a calibration curve relating the actual composition to the predicted values calculated by partial least squares regression processing of low-frequency Raman spectra. The metastable form gradually converted to a stable form, and polymorphic phase transformation occurred with increasing relative humidity. Ninety-six percent of the metastable form converted into a stable form stored at 25℃ after 3 h at 95% RH. In conclusion, transmission low-frequency Raman spectroscopy can be used to quantitatively monitor cocrystal polymorphs. This technique is one of the candidate techniques to quantifiably evaluate the physico-chemical stability of cocrystal polymorphs in tablets.

Keywords: co-crystal, Raman spectroscopy, polymorph

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Yamamoto Y*1, Ougi K*2, Onuki Y*2, Fukami T*3, Koide T: Quality evaluation of humidified magnesium oxide tablet formulations with respect to disintegration time prolongation.

Chemical and Pharmaceutical Bulletin. 2023;71:165-74. doi: 10.1248/cpb.c22-00798

In the present study, we conducted a detailed evaluation of the effects of humidification on the quality of five types of commercial magnesium oxide (MgO) tablet formulations. When near-IR spectroscopy was performed, a peak derived from the first overtone of the stretching vibration of the hydroxyl group was observed at approximately 7200 cm^{-1} in a humidified MgO tablet formulation. To visually evaluate the effect of this humidification, a mapping image was created using microscopic IR spectroscopy. In the IR spectrum, a peak derived from the stretching vibration of the hydroxyl group appears at approximately 3700 cm^{-1}, so we created a mapping image using the absorbance ratio of 3700 and 3400 cm^{-1} as an index. In the mapping image of humidified MgO tablet formulations, many areas had a higher absorbance ratio than the dried tablet formulations. From these results, it is qualitatively confirmed that the MgO was changed to magnesium hydroxide ($Mg(OH)_2$) by humidification. Although these results were observed in the four types of MgO tablet formulations, only one type of tablet formulation was less affected by humidification. In addition, although most tablet formulations tended to prolong disintegration time due to humidification, there was almost no effect of humidification on the disintegration time in one type of tablet formulation, which had little change in the above evaluation. Thus, in most commercial MgO tablet formulations, humidification prolongs the disintegration time, and $Mg(OH)_2$ significantly contributes to this factor.

Keywords: disintegrant, magnesium oxide, magnesium hydroxide

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Katsutoshi Yamaguchi*1, Ryo Mizoguchi*1, Kohsaku Kawakami*2,3, Tamaki Miyazaki: Influence of the crystallization tendencies of pharmaceutical glasses on the applicability of the Adam-Gibbs-Vogel and Vogel-Tammann-Fulcher equations in the prediction of their long-term physical stability.

Int J Pharm. 2022;626:122158. doi: 10.1016/j.ijpharm.2022.122158

Amorphization is a powerful approach for improving the aqueous solubility and bioavailability of poorly water-soluble compounds. However, it can cause chemical and physical instability, the latter of which can lead to crystallization during storage, diminishing the solubility advantage of the amorphous state. As there is no standard method for predicting the physical stability of amorphous materials, a long-term stability study is needed in drug development. This study investigated the correlation between the physical stability of amorphous compounds and molecular mobility based on the assumption that physical stability is governed by the diffusional motion of a molecule. Model compounds were evaluated for crystallization onset time, structural relaxation time, fragility, and fictive temperature. The crystallization onset time of acetaminophen glass correlated with its relaxation time calculated from the Adam-Gibbs-Vogel equation; however, that of felodipine glass correlated with the relaxation time calculated from the Vogel-Tammann-Fulcher equation. The different crystallization tendencies of these compounds can be explained by the differences in the rate limiting steps

in their crystallization processes, indicating the importance of distinguishing the critical process associated with crystallization. These findings will be useful for more accurate prediction of long-term physical stability of amorphous materials.

Keywords: amorphous drug, crystallization, stability prediction

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Nakao M*1, Takechi-Haraya Y, Ohgita T*2, Saito H*2, Demizu Y, Izutsu K, Sakai-Kato K*1: Analysis of the interaction of cyclosporine congeners with cell membrane models.

Journal of Pharmaceutical and Biomedical Analysis. 2022;218:114874. doi: 10.1016/j.jpba.2022.114874

Owing to the relatively high molecular weight of macrocyclic peptides, investigation of the cellular uptake mechanism is required for the efficient design of macrocyclic peptides as potential drugs. We have previously reported, using HPLC, that cyclosporine A, a model macrocyclic peptide, and its congeners B, C, and D had different lipophilicity despite differing by only one amino acid. In the present study, we investigated how this difference in lipophilicity affected the interaction of the congeners with cell membranes. The circular dichroism spectra showed that the secondary structures were similar between the four congeners even at high temperature. The molar ellipticity of the four congeners in the presence of liposomes, as a cell membrane model, differed, and cyclosporines D and A showed lower molar ellipticity, while cyclosporine C exhibited higher molar ellipticity. Fluorescent spectra analysis using Laurdan indicated that liposome hydration was decreased in the presence of the cyclosporines, especially cyclosporines D and A. HPLC analysis also quantitatively showed that the amount of cyclosporine molecules internalized in HpG2 cells was the largest for cyclosporine D. We determined, using spectroscopy and HPLC, that the intensity of the interaction of the congeners with cell membranes was overall correlated with the lipophilicity derived from the side chains of each congener. Our results will contribute to the design of new macrocyclic peptides with favorable drug properties.

Keywords: cyclosporine, circular dichroism, fluorescent spectrum

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Yang Z*1, Nill K*2, Takechi-Haraya Y, Playford MP*1, Nguyen D*1, Yu Z-X*1, Pryor M*1, Tang J*1, Rojulpote KV*1, Mehta NN*1, Wen H*1, Remaley AT*1: Differential effect of dietary supplementation with a soybean oil enriched in oleic acid versus linoleic acid on plasma lipids and atherosclerosis in LDLR-deficient mice.

International Journal of Molecular Sciences. 2022;23:8385. doi: 10.3390/ijms23158385

Diets enriched in monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs) are associated with reduced cardiovascular risk. A modified soybean oil enriched in oleic acid (MSO) has been developed for its improved chemical stability to oxidation, but its effect on cardiovascular disease is unknown. Low-density lipoprotein receptor knock-out mice were fed a Western diet supplemented with linoleic acid-rich conventional soybean oil (CSO) or high-oleic MSO for 12 weeks. The CSO diet decreased plasma lipid levels and cholesterol content, while the MSO diet reduced atherosclerotic plaque size and the n-6/n-3 PUFA ratio in the liver. MSO, but not CSO, reduced atherosclerosis development in LDLR-KO mice independent of changes in plasma lipids.

Keywords: polyunsaturated fatty acid, monounsaturated fatty acid, atherosclerosis

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Takechi-Haraya Y, Usui A, Izutsu K, Abe Y: Atomic force microscopic imaging of mRNA-lipid nanoparticles in aqueous medium.

Journal of Pharmaceutical Sciences. 2023;112:648-52. doi: 10.1016/j.xphs.2022.11.026

The efficacy of mRNA-lipid nanoparticles (mRNA-LNPs) depends on several factors, including their size

and morphology. This study presents a new technique to characterize mRNA-LNPs in an aqueous medium using atomic force microscopy (AFM). This method utilizes an anti-polyethylene glycol antibody to immobilize mRNA-LNPs onto a glass substrate without corruption, which cannot be avoided with conventional procedures using solid substrates such as mica and glass. The obtained AFM images showed spherical and bleb-like structures of mRNA-LNPs, consistent with previous observations made using cryo-transmission electron microscopy. The AFM method also revealed the predominant existence of nanoparticles with a diameter < 60 nm, which were not detectable by dynamic light scattering and nanoparticle tracking analysis. As mRNA-LNPs are usually not monodisperse, but rather polydisperse, the AFM method can provide useful complementary information about mRNA-LNPs in their development and quality assessment.

Keywords: lipid nanoparticle, quality assessment, atomic force microscopy

Aoyama M, Tada M, Ishii-Watabe A: FcγRIIIa-158V/F polymorphism affects the performance of FcγRIIIa-related bioassay.

Biochem Biophys Res Commun. 2022;608:149-155. doi: 10.1016/j.bbrc.2022.04.001

Bioassays are important for estimating biosimilarity between biological products. Comparability studies including bioassays are needed to demonstrate that a biosimilar product has no meaningful differences that affect safety and efficacy compared with the reference product. Among the most important biological characteristics of therapeutic mAbs are Fc-mediated functions, which induce immune-cell activation which can affect both efficacy and safety. Thus, when developing biosimilar products of therapeutic mAbs, it is necessary to compare the Fc-mediated functions by using various bioassays. Though it is reported that polymorphism of Fcγ receptors (FcγRs) affects Fc-mediated cellular activations of therapeutic mAbs, the impacts of the polymorphism of FcγRs on bioassay performance are still unclear.

In this study, we evaluated the impact of FcγRIIIa-158V/F polymorphism on assay performance in distinguishing differences in the biological activities of therapeutic mAbs. The results showed that different bioassay methods produced different assessments of biological activities of mAbs, and that the FcγRIIIa-158V/F polymorphism clearly affected the performance of the FcγRIIIa-binding assay using recombinant proteins and FcγRIIIa-expressing reporter assays. That is, the assays using the FcγRIIIa-158F variant were superior to those using the FcγRIIIa-158V variant in distinguishing the difference in FcγRIIIa-binding and -activation properties. These results indicate that we should evaluate the comparability of biosimilars by considering the impacts of FcγRIIIa-158V/F polymorphism on bioassay performance.

Keywords: assay performance, bioassay, FcγRIIIa-158V/F polymorphism

Shibata H, Terabe M[*1], Shibano Y[*2], Saitoh S[*1], Takasugi T[*3], Hayashi Y[*3], Okabe S[*4], Yamaguchi Y[*4], Yasukawa H[*4], Suetomo H[*5], Miyanabe K[*6], Ohbayashi N[*7], Akimaru M[*8], Saito S[*8], Ito D[*9], Nakano A[*9], Kojima S[*10], Miyahara Y[*11], Sasaki K[*11], Maruno T[*12], Noda M[*12], Kiyoshi M, Harazono A, Torisu T[*2], Uchiyama S[*2], Ishii-Watabe A: A Collaborative Study on the Classification of Silicone Oil Droplets and Protein Particles Using Flow Imaging Method.

J Pharm Sci. 2022;111(10):2745-2757. doi: 10.1016/j.xphs.2022.07.006

In this study, we conducted a collaborative study on the classification between silicone oil droplets and protein particles detected using the flow imaging (FI) method toward proposing a standardized classifier/model. We compared four approaches, including a classification filter composed of particle characteristic parameters, principal component analysis, decision tree, and convolutional neural network in the performance of the developed classifier/model. Finally, the points to be considered were summarized for measurement using the FI method, and for establishing the classifier/model using machine learning to differentiate silicone oil droplets and protein particles.

Keywords: flow imaging, silicone oil, protein particles

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柴田寛子，野村祐介，河上強志，山本栄一，安藤大介，内山奈穂子，徳本廣子，小出達夫，迫田秀行，吉田寛幸，阿部康弘，袴塚高志，五十嵐良明，酘島由二，石井明子，伊豆津健一，本間正充，合田幸広：モデルナ社の新型コロナワクチンに混入した異物に関する調査.

YAKUGAKU ZASSHI. 2022;142(8):867-874. doi: 10.1248/yakushi.22-00067

Particular batches of Moderna mRNA Coronavirus Disease 2019 (COVID-19) vaccine were recalled after foreign particles were found in some vaccine vials at the vaccination site in Japan in August 2021. We investigated the foreign particles at the request of the Ministry of Health, Labour and Welfare. Energy dispersive X-ray spectroscopy analysis suggested that the foreign particles found in the vials recalled from the vaccination sites were from stainless steel SUS 316L, which was in line with the findings of the root cause investigation by the manufacturer. The sizes of the observed particles ranged from <50 μm to 548 μm in the major axis. Similar foreign particles were also detected in 2 of the 5 vaccine vials of the same lot stored by the manufacturer, indicating that the foreign particles have already been administered to some people via vaccine. Observation of the vials of the same lot by digital microscope found smaller particles those were not detected by visual inspection, suggesting that more vials were affected. Contrarily, visual inspection and subvisible particulate matter test indicated no foreign particles in the vials of normal lots. Possible root cause and strategies to prevent such a deviation were discussed from technical and regulatory aspects.

Keywords: Coronavirus Disease 2019 vaccine, foreign particle, visual inspection

Kosuge H*1, Nagatoishi S*1, Kiyoshi M, Ishii-Watabe A, Terao Y*2, Ide T*2, Tsumoto K*1: Biophysical Characterization of the Contribution of the Fab Region to the IgG-FcγRIIIa Interaction.

Biochemistry. 2022;17;62(2):262-269. doi: 10.1021/acs.biochem.1c00832

The cell-surface receptor FcγRIIIa is crucial to the efficacy of therapeutic antibodies as well as the immune response. The interaction of the Fc region of IgG molecules with FcγRIIIa has been characterized, but until recently, it was thought that the Fab regions were not involved in the interaction. To evaluate the influence of the Fab regions in a biophysical context, we carried out surface plasmon resonance analyses using recombinant FcγRIIIa ligands. A van't Hoff analysis revealed that compared to the interaction of the papain-digested Fc fragment with FcγRIIIa, the interaction of commercially available, full-length rituximab with FcγRIIIa had a more favorable binding enthalpy, a less favorable binding entropy, and a slower off rate. Similar results were obtained from analyses of IgG1 molecules and an IgG1-Fc fragment produced by Expi293 cells. For further validation, we also prepared a maltose-binding protein-linked IgG1-Fc fragment (MBP-Fc). The binding enthalpy of MBP-Fc was nearly equal to that of the IgG1-Fc fragment for the interaction with FcγRIIIa, indicating that such alternatives to the Fab domains as MBP do not positively contribute to the IgG-FcγRIIIa interactions. Our investigation strongly suggests that the Fab region directly interacts with FcγRIIIa, resulting in an increase in the binding enthalpy and a decrease in the dissociation rate, at the expense of favorable binding entropy.

Keywords: biopolymer, enthalpy, immunology

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Huang X*1, Hyuga S*2, Amakura Y*3, Hyuga M, Uchiyama N, Hakamatsuka T, Goda Y, Odaguchi H*2, Hanawa T*2, Kobayashi Y*1: Overlooked switch from transient sedation to sustained excitement in the Biphasic effects of Ephedra Herb extract administered orally to mice.

J Ethnopharmacol. 2022;301:115827. doi: 10.1016/j.jep.2022.115827

We aimed to confirm whether Ephedra Herb extract (EHE) induces a switch from transient sedation to

sustained arousal, to investigate whether these effects of EHE are caused by the amount of ephedrine (Eph) and pseudoephedrine (Pse) in the herbal extract, and to clarify the molecular mechanism of the transient sedative effect. In this study, it was shown that EHE induces a diphasic effect by switching from transient sedation to sustained excitation. The transient sedation was induced by Eph and Pse in the herbal extract via activation of α2a adrenoceptor, and the sustained excitement was caused by Eph in the herbal extract.

Keywords: Ephedra Herb, ephedrine, ephedrine alkaloid-free

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森本和滋，日向昌司，石井明子：バイオ後続品の同等性／同質性評価技術の進歩，国内外における規制と承認の動向.
薬史学雑誌. 2022;57:128-137. doi: https://doi.org/10.34531/jjhp.57.2_128

バイオ後続品と先行バイオ医薬品の同等性／同質性評価技術の進歩，国内外における規制と承認の動向を調べた．欧州EMAは，2005年以降，総論的ガイドライン（GL）に加えて，品質GL，非臨床・臨床試験GL，および製品群別の非臨床・臨床GLを継続的に公表し，その起点になった．2009年には，我が国でも，バイオ後続品の品質・安全性・有効性確保のための指針（薬食審査発第0304007号）の通知が厚生労働省より発出された．米国では，2010年バイオ後続品の規制要件を定めた法律が成立し，2012年FDAからガイダンス案が公開された．先行品との同等性／同質性が承認のキーポイントとなっている．同等性／同質性評価においては，製法の影響で差異が生じやすいN-結合型糖鎖プロファイルや，抗体医薬品のADCC活性，FcγRⅢa結合活性などが重要となる場合が多く，NIHSでも関連する評価法の研究が行われている．抗体医薬品の重要な生物学的特性である免疫細胞活性のFc-介在機能の比較は重要である．

Keywords：バイオ後続品，同等性／同質性評価技術，規制動向

橋井則貴，蛭田葉子，鈴木琢雄，石井明子：日本薬局方グルカゴン（遺伝子組換え）各条確認試験で使用する試薬の規格に関する検討.
医薬品医療機器レギュラトリーサイエンス. 2023;54:69-81. doi: https://doi.org/10.51018/pmdrs.54.1_69

In Japan, two types of glucagon products, which contain recombinant glucagon or synthetic glucagon as an active ingredient, have been approved and marketed. On June 7th, 2021, the Glucagon (Genetical Recombination) monograph was newly listed in the eighteenth edition of the Japanese Pharmacopoeia (JP). In the monograph, peptide mapping with α-chymotrypsin was set as the identification test; however, the specification of theα-chymotrypsin reagent is unclear because there is no information about the substrate used in the activity assay, the definition of the enzyme unit or the purity. Therefore, selecting anα-chymotrypsin reagent can be an issue when performing the identification test. In this study, we measured the chymotrypsin activity and residual trypsin activity of severalα-chymotrypsins, including the United States Pharmacopeia and the European Pharmacopoeia chymotrypsin reference standards and commercially availableα-chymotrypsin reagents, and conducted an identification test according to the JP glucagon monograph; theseα-chymotrypsins were used to evaluate the effect of differences in the activity of the chymotrypsin and residual trypsin on the test results. As a result, it was found that the current specification for chymotryptic activity of the JPα-chymotrypsin reagent may be higher than necessary to obtain the intended peptide map, and slight tryptic activity is required to generate the reference peptide peaks. Based on these findings, we discuss the appropriate specification of the JPα-chymotrypsin reagent.

Keywords: Glucagon (Genetical Recombination), synthetic glucagon, α-chymotrypsin

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測定による分析性能評価と，試験法設定における留意点の考察.
医薬品医療機器レギュラトリーサイエンス. 2022;53:514-533. doi: 10.51018/pmdrs.53.6_514

Because protein aggregates may induce an immune response, the amount of aggregates in biopharmaceuticals is considered as a critical quality attribute. Protein aggregation could occur during manufacture and storage, so the amount of aggregates should be controlled appropriately. Many analytical techniques are used for the analysis of protein aggregates, depending on their size. Size exclusion chromatography (SEC) is often employed to evaluate small aggregates, such as dimers and multimers, due to its ease of use, high reproducibility and relatively high throughput. In this multi-laboratory study, a questionnaire survey of analytical conditions was conducted, and then analytical performance was evaluated using a therapeutic monoclonal antibody and forcibly degraded samples in order to clarify key points for consideration in developing SEC test procedures for small aggregates in biopharmaceuticals. The effects of operating parameters on analytical performance is discussed. Samples were analyzed using a TSKgel G3000SW$_{XL}$ column with a mobile phase of 0.3 mol/L sodium chloride in 100 mmol/L sodium phosphate buffer, pH 7.0. The results showed that the repeatability and reproducibility of percent area of high-molecular weight species were < 3% and < 10%, respectively. Furthermore, the values of percent area were consistent with results obtained by analytical ultracentrifugation. When the concentration of sodium chloride in the mobile phase was decreased to 0.2 mol/L, the percentage of larger aggregates was decreased due to adsorption, while the percentage of dimer was not changed. Considering that SEC is also used for stability testing, it is necessary that SEC can adequately evaluate the levels of larger and adhesive aggregates, which may not be present in the drug substance or drug products at the time of release testing. Evaluation using forcibly degraded samples is important during analytical validation.

Keywords: size exclusion chromatography, biopharmaceuticals, protein aggregate

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Inoue T[*1], Shinnakasu R[*1, 2, 3], Kawai C[*1], Yamamoto H[*1], Sakakibara S[*1], Ono C[*1], Itoh Y[*1], Terooatea T[*8], Yamashita K[*4], Okamoto T[*1], Hashii N, Ishii-Watabe A, Butler NS[*5], Matsuura Y[*1], Matsumoto H[*1], Otsuka S[*6], Hiraoka K[*6], Teshima T[*7, 8], Murakami M[*8, 9, 10], Kurosaki T[*1, 11] : Antibody feedback contributes to facilitating the development of Omicron-reactive memory B cells in SARS-CoV-2 mRNA vaccinees.
J Exp Med. 2023 Feb 6;220(2):e20221786. doi: 10.1084/jem.20221786. Epub 2022 Dec 13.

In contrast to a second dose of the SARS-CoV-2 mRNA vaccine, a third dose elicits potent neutralizing activity against the Omicron variant. To address the underlying mechanism for this differential antibody response, we examined spike receptor-binding domain (RBD)-specific memory B cells in vaccinated individuals. Frequency of Omicron-reactive memory B cells increased ~9 mo after the second vaccine dose. These memory B cells show an altered distribution of epitopes from pre-second memory B cells, presumably due to an antibody feedback mechanism. This hypothesis was tested using mouse models, showing that an addition or a depletion of RBD-induced serum antibodies results in a concomitant increase or decrease, respectively, of Omicron-reactive germinal center (GC) and memory B cells. Our data suggest that pre-generated antibodies modulate the selection of GC and subsequent memory B cells after the second vaccine dose, accumulating more Omicron-reactive memory B cells over time, which contributes to the

generation of Omicron-neutralizing antibodies elicited by the third vaccine dose.

Keywords: COVID-19, infectious disease and host defense

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生薬学雑誌 2022;76:37-44.

The origin of ISODONIS HERBA in the Japanese standards for non-Pharmacopoeial crude drugs 2018 is the aerial parts of *Isodon Japonicus* H. Hara (*Plectranthus japonicus* Koidzumi, *Rabdosia japonica* H. Hara) and *Isodon trichocarpus* Kudô (*Plectranthus trichocarpus* Maximowicz, *Rabdosia trichocarpa* H. Hara) (*Labiatae*). This herb is used as contains *ent*-kaurane diterpenoids such as enmein. Enmein is a characteristic compound in ISODONIS HERBA. In order to help better understanding and facilitate quality control of ISODONIS HERBA, methods for quantitative analyses of enmein in ISDONIS HERBA were elaborated.

Keywords: Isodonis herba, enmein, quantitative analysis

* 生薬品質集談会

Hirai M*, Ota Y*, Ito M: Diversity in principal constituents of plants with a lemony scent and the predominance of citral.

J. Nat. Med. 2022;76:254-258. doi: 10.1007/s11418-021-01553-7

In this study, we extracted essential oils from four species of plants with lemony scents (Melissa officinalis L., Aloysia citriodora Palau (= Lippia citriodora (Palau) Kunth), Thymus × citriodorus, Perilla citriodora (Makino) Nakai). We then examined the components of extracts using gas chromatography (GC) and GC-mass spectrometry (GC-MS). A comparison of components indicated that the largest proportions of essential oils were caryophyllene (25%) in M. officinalis, geraniol (50%) in T. citriodorus, and citral (61 and 82%) in A. citriodora and P. citriodora. Moreover, we used a sensory evaluation method using dilute aqueous solutions of extract components, citral, linalool, d-limonene, and geraniol, to select the mixture with a flavor that mostly resembled lemon. The participants in the study felt that an aqueous citral solution flavored more like lemon than aqueous d-limonene. Furthermore, an open field study of sedative effects of citral and d-limonene, when inhaled, on mice demonstrated that citral exhibited a sedative effect at a lower concentration than that of d-limonene.

Keywords: Aloysia citriodora, citral, d-limonene

* Kyoto University

Takamatsu S*, Ito M: Factors affecting 2-(2-phenylethyl)chromones in artificial agarwood.

J. Nat. Med. 2022;76:321-330. doi: 10.1007/s11418-021-01555-5

Recently, "artificial agarwood" manufactured by the artificial treatment on cultivated agarwood trees is popular in agarwood-producing countries. Although there are various treatment methods, they are not standardized. Moreover, factors that may affect the generated chemical compounds have not been investigated. In this research, the effects of different treatment methods and individual differences on the quantities and types of 2-(2-phenylethyl)chromone in agarwood were investigated to experimentally produce artificial agarwood using Aquilaria sinensis. Each solvent-extracted agarwood sample was analyzed using Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS), and peaks were identified by comparing ten types of 2-(2-phenylethyl)chromone with reference standards. The composition and 2-(2-phenylethyl)chromone content of each agarwood

sample were observed based on the type of chemical compound, and results indicated that when the treatment method was different, the accumulation pattern of the 2-(2-phenylethyl)chromones differed even when the number of resinification years was the same. Furthermore, the findings of this study showed that additional treatment on a single branch produced more 2-(2-phenylethyl)chromones. Moreover, market products composed of artificial agarwood pieces derived from different tree species and collected from different location were analyzed.

Keywords: agarotetrol, artificial agarwood, phytochemical

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Dougnon G*, Ito M: Essential oils from *Melia azedarach* L. (Meliaceae) leaves: chemical variability upon environmental factors.

J. Nat. Med. 2022;76:331-341. doi: 10.1007/s11418-021-01579-x

The chemical composition of the essential oils extracted from the leaves of Melia azedarach L. collected monthly from July 2019 to June 2020 was examined via gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques. Analysis of the essential oils identified about 17 compounds representing more than 85% of the oil. Oil yields were higher in the months of June and August, and the primary compounds identified were β-caryophyllene (3.50-63.41%), benzaldehyde (3.50-55.98%), and azulene (1.27-19.05%). A correlation analysis was performed to determine the relationship between yields and climatic conditions, and between constituent concentration and temperature and precipitation values during the study period. As per our findings, although not significant, a positive correlation was determined between yield and climatic parameters. However, the oil components were categorized into four groups based on their correlation with temperature and precipitation indices. Among the major components of the essential oils, only azulene and β-caryophyllene exhibited a negative correlation with both precipitation and temperature. The results show substantial differences in the chemical composition of M. azedarach essential oils and provide further insight into the phytochemical constituents that are sensitive to climate fluctuations. Furthermore, it provides an indication of the optimal time that the plant produces the important mono- and sesquiterpene components and the biological significance of their regulation.

Keywords: essential oil, Melia azedarach, seasonal variation

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Negishi H*, Ito M: Shorter and more efficient pretreatment for germination of perilla mericarps.

J. Nat. Med. 2022;76:509-518. doi: 10.1007/s11418-021-01600-3

Perilla (Perilla frutescens (L.) Britton) mericarps are known to undergo dormancy; however, this can be broken by sulfuric acid treatment and cold stratification. Cold stratification is thought to be the most effective treatment and is customarily performed for 2 weeks to induce germination of perilla mericarps. However, this procedure requires an additional 2 weeks before sowing and cultivation, thereby decreasing cultivation efficiency. To address this problem, germination experiments were conducted in this study in order to identify a shorter and more efficient pretreatment strategy for germination of perilla mericarps. Pretreatment with sulfuric acid (10 min versus 1 min) and gibberellin (8 h and 1 h versus 5 min, at a rate of 100 versus 10 ppm) were performed using mericarps from pure strains of perilla. As a result, sulfuric acid treatment tended to reduce the germination rate, while gibberellin treatment resulted in an equivalent or similar germination rate as cold stratification. Gibberellin treatment was also found to be effective in mericarps with a relatively old harvest date and low germination energy. Considering the convenience and safety of the treatment process as well as the results of the germination experiments, these findings suggest that a short period of gibberellin treatment could help shorten the process of perilla cultivation.

Keywords: cold stratification, germination rate, gibberellin

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Ito H*, Ito M: Comparison of phenolic compounds contained in Aquilaria leaves of different species.
J. Nat. Med. 2022;76(3):693-702. doi: 10.1007/s11418-022-01608-3

Leaves of Aquilaria plants contain a variety of phenolic compounds such as iriflophenone glycosides, mangiferin, and genkwanin. Previous studies showed that Aquilaria leaf extracts exhibit many pharmacological activities, including antidiabetic and laxative effects. However, a few studies have reported differences in the chemical content and compositions of Aquilaria species. Here, three Aquilaria species were identified using matK and trnL-trnF sequences and their leaves were analyzed by HPLC and LC/MS. Comparison of the chemical components and α-glucosidase inhibition activity of the three species showed that the level of iriflophenone glycosides in A. rugosa was higher than in A. sinensis and A. crassna. There was no difference in the α-glucosidase inhibition activity of leaf extracts of the three species, but the strength of the inhibition activity can possibly be explained by the total sum of active compounds in the leaf extracts.

Keywords: aquilaria leaves, DNA barcoding, α-glucosidase inhibition

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Takamatsu S*, Ito M: Agarotetrol as an index for evaluating agarwood in crude drug products.
J. Nat. Med. 2022;76(4):857-864. doi: 10.1007/s11418-022-01632-3

Agarotetrol in agarwood has been detected in water extracts or decoctions from medical use agarwood but the detection of agarotetrol has not been reported from other crude drugs. Agarwood generates the sedative benzylacetone upon heating. In this study, crude drug products containing many kinds of crude drugs in addition to agarwood were analyzed. Agarotetrol was detected and quantified, demonstrating that agarotetrol is useful for the quality evaluation of agarwood in complex prescriptions. High-performance liquid chromatography conditions to clearly separate agarotetrol from crude drug products were established and agarotetrol from Kampo decoctions was detected and quantified. Agarotetrol was also detected even from small crude drug product samples. These results suggest that agarotetrol is a useful component for the quality evaluation of agarwood in crude drug products.

Keywords: agarotetrol, agarwood, quality evaluation

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Dougnon G*, Ito M: Molecular descriptors and QSAR models for sedative activity of sesquiterpenes administered to mice via inhalation.
Planta Medica. 2022 doi: 10.1055/a-1770-7581

Essential oils are often utilized for therapeutic purposes and are composed of complex structural molecules, including sesquiterpenes, with high molecular weight and potential for stereochemistry. A detailed study on the properties of selected sesquiterpenes was conducted as part of a broader investigation on the effects of sesquiterpenes on the central nervous system. A set of 18 sesquiterpenes, rigorously selected from an original list of 114, was divided into 2 groups i.e., the training and test sets, with each containing 9 compounds. The training set was evaluated for the sedative activity in mice through inhalation, and all compounds were sedatives at any dose in the range of 4×10^{-4}-4×10^{-2} mg/cage, except for curzerene. Molecular determinants of the sedative activities of sesquiterpenes were evaluated using quantitative structure-activity relationship (QSAR) and structure-activity relationship (SAR) analyses. An additional test set of six compounds obtained from the literature was utilized for validating the QSAR model. The parental carbonyl cation and an oxygen-containing groups are possible determinants of sedative activity. The QSAR study using multiple regression models could reasonably predict the sedative activity of sesquiterpenes with statistical parameters such as the correlation coefficient r^2=0.82>0.6 and q^2_{LOO}=0.71>0.5 obtained using the leave-one-out cross-validation technique. Molar refractivity and the number of hydrogen bond acceptors were statistically important in predicting the activities. The present study could help predict the sedative activity of additional sesquiterpenes, thus accelerating the process of drug

development.

Keywords: essential oil, sesquiterpene, sedative

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Goto Y, Fujii T[*1], Takao Y[*2], Tsuchida T[*3], Sone M[*4], Kammoto T[*4], Matsuura T[*4], Yokokura T[*5], Minami M[*1], Komatsu K[*6], Kiuchi F[*7], Maruyama T: Genetic and chemical diversity of commercial Japanese Valerian.

Chem. Pharm. Bull. 2022;70:840-847. doi: 10.1248/cpb.c22-00105

In order to investigate the relationship between the chemical composition of essential oils and haplotypes of the *psb*A-*trn*H intergenic spacer region of chloroplast DNA (*psb*A-*trn*H) in Valerianae Fauriei Radix (Japanese Valerian; JV), we analyzed the DNA sequence and GC-MS metabolome of JV from Japanese markets and of herbal specimens from related species. DNA analysis revealed that JV products from Japan consisted of three haplotypes, namely AH-1, -2 and -5 reported in our previous study. The GC-MS metabolome revealed five chemotypes (J1, J2, C, K and O), of which J1, J2 and C were detected in the JV products from Japan. Chemotypes J1 and J2, with kessyl glycol diacetate (KGD) as the main volatile component, were found in the products of Japanese origin whereas chemotype C, with 1-*O*-acetyl-2,10-bisaboladiene-1,6-diol (ABD), was found in the products of Chinese and Korean origin. The haplotypes were correlated with the chemotypes: haplotype AH-1 for chemotype J1, AH-2 for chemotype J2 and AH-5 for chemotype C, suggesting that the chemical diversity of JV is not attributed to the environmental factors rather to the genetic factors. Since KGD and ABD were reported to have sedative effects and nerve growth factor (NGF)-potentiating effects, respectively, understanding the chemotypes and selecting an appropriate one would be important for the application of JV. The *psb*A-*trn*H haplotypes could be useful DNA markers for the quality control and standardization of JV.

Keywords: Japanese Valerian, GC-MS metabolome, *psb*A-*trn*H intergenic spacer

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Nose M[*1], Kobayashi R[*1], Tada M[*1], Hisaka S[*1], Masada S, Homma M[*2], Hakamatsuka T[*3]: Comparison of ephedrine and pseudoephedrine contents in 34 Kampo extracts containing Ephedrae Herba used clinically in Japan.

J. Nat. Med. 2023;77:476-488. doi: 10.1007/s11418-023-01687-w.

Ephedrae Herba is among the important crude drugs prescribed in Kampo medicine for the treatment of cold, flue, rhinitis, nasal congestion, cough, and asthma. The active ingredients of Ephedrae Herba, ephedrine (E) and pseudoephedrine (PE), are potent sympathomimetic compounds that stimulate α-, β1-, and β2-adrenoceptors resulting in dilatation and alleviation of nasal mucosal hyperemia. Hypertension, palpitations, insomnia, and dysuria are the main adverse effects of E and PE, which can be avoided by determining the actual contents of these alkaloids in Kampo extracts containing Ephedrae Herba. However, the extraction efficiencies of E and PE from Ephedrae Herba contained in Kampo formulas in combination with other crude drugs remain unknown. Therefore, we comprehensively determined the E and PE contents of 34 Kampo extracts containing Ephedrae Herba used clinically in Japan. In conclusion, these results show that the E and PE content of each Kampo formulation can be estimated from the compounding amount of Ephedrae Herba. Therefore, the amount of Ephedrae Herba should be carefully considered to ensure the safe use of Kampo formulations containing Ephedrae Herba.

Keywords: Ephedrae Herba, ephedrine, Kampo extracts

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Uchiyama N, Kiyota K*1, Hosoe J, Komatsu T*2, Sugimoto N, Ishizuki K, Koide T., Murabayashi M*3, Kobayashi K*4, Fujimine Y*5, Yokose T*5, Ofuji K*6, Shimizu H*6, Hasebe T*7, Asai Y*7, Ena E*7, Kikuchi J*1, Fujita K*1, Makino Y*8, Iwamoto Y*9, Miura T*9, Muto Y*9, Asakura *10, Suematsu T*2, Muto H*2, Kohama*11, Goto T*12, Yasuda M*12, Ueda T*13, Goda Y: Quantitative ^{31}P-NMR for purity determination of sofosbuvir and method validation.

Chem Pharm. Bull. 2022;70(12):892-900. doi: 10.1248/cpb.c22-00639

Quantitative ^{1}H-NMR (^{1}H-qNMR) is useful for determining the absolute purity of organic molecules; however, it is sometimes difficult to identify the target signal(s) for quantitation because of their overlap and complexity. Therefore, we focused on the ^{31}P nucleus because of the simplicity of its signals and previously reported ^{31}P-qNMR in D_2O. Here we report ^{31}P-qNMR of an organophosphorus compound, sofosbuvir (SOF), which is soluble in organic solvents. Phosphonoacetic acid (PAA) and 1,4-BTMSB-d_4 were used as reference standards for ^{31}P-qNMR and ^{1}H-qNMR, respectively, in methanol-d_4. The purity of SOF determined by ^{31}P-qNMR was 100.63 ± 0.95%, whereas that determined by ^{1}H-qNMR was 99.07 ± 0.50%. The average half bandwidths of the ^{31}P signal of PAA and SOF were 3.38 ± 2.39 Hz and 2.22 ± 0.19 Hz, respectively, suggesting that the T_2 relaxation time of the PAA signal was shorter than that of SOF and varied among test laboratories. This difference most likely arose from the instability in the chemical shift due to the deuterium exchange of the acidic protons of PAA, which decreased the integrated intensity of the PAA signal. Next, an aprotic solvent, DMSO-d_6, was used as the dissolving solvent with PAA and DSS-d_6 as reference standards for ^{31}P-qNMR and ^{1}H-qNMR, respectively. SOF purities determined by ^{31}P-qNMR and ^{1}H-qNMR were 99.10 ± 0.30% and 99.44 ± 0.29%, respectively. SOF purities determined by ^{31}P-qNMR agreed with the established ^{1}H-qNMR values, suggesting that an aprotic solvent is preferable for ^{31}P-qNMR because it is unnecessary to consider the effect of deuterium exchange.

Keywords: quantitative ^{31}P-NMR, sofosbuvir, absolute purity

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Tokumoto H, Yamamoto E, Hakamatsuka T, Uchiyama N: A new method to visualize the internal morphology of crude drugs using high-resolution X-Ray computed tomography.

Biol. Pharm. Bull. 2022;45:919-925. doi: 10.1248/bpb.b22-00183

In recent years, high-resolution X-ray computed tomography (HRXCT) has been used to visualize the internal structure of plants. HRXCT scans an object using X-rays and enables visualization of the internal structure of the crude drug using a computer. Therefore, in this report, HRXCT was used to easily observe the internal morphology of crude drugs using the Ephedra Herb as an example. The same internal morphological characteristics were observed using both, microscopic examination and HRXCT methods. Image analysis using HRXCT did not require specific techniques, such as sectioning, and the same tissue could be observed from any orientation using a single scan. Therefore, image analysis using HRXCT is a useful method for crude drug identifications.

Keywords: high-resolution X-ray computed tomography, internal morphology, Ephedra Herb

Mizuno T*1, Uchiyama N, Tanaka S, Nakane T*2, Devkota H P*3, Fujikawa K*4, Kawahara N*4, Iwashina T*1: Flavonoids from *Sedum japonicum* subsp. *oryzifolium* (Crassulaceae).

Molecules. 2022;27:7632. doi: 10.3390/molecules27217632

Twenty-two flavonoids were isolated from the leaves

and stems of *Sedum japonicum* subsp. *oryzifolium* (Crassulaceae). Of these compounds, five flavonoids were reported in nature for the first time, and identified as herbacetin 3-*O*-xyloside-8-*O*-glucoside, herbacetin 3-*O*-glucoside-8-*O*-(2′′′-acetylxyloside), gossypetin 3-*O*-glucoside-8-*O*-arabinoside, gossypetin 3-*O*-glucoside-8-*O*-(2′′′-acetylxyloside) and hibiscetin 3-*O*-glucoside-8-*O*-arabinoside via UV, HR-MS, LC-MS, acid hydrolysis and NMR. Other seventeen known flavonoids and some flavonol were found in *Sedum japonicum* subsp. *oryzifolium* as major flavonoids in this survey. They were presumed to be the diagnostic flavonoids in the species. Flavonoids were reported from *S. japonicum* for the first time.

Keywords: *Sedum japonicum* subsp. *oryzifolium*, flavonoid

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田中誠司，辻本恭*，小関良宏*，袴塚高志，内山奈穂子：トウゲシバ（*Huperzia serrata*）関連製品の流通実態調査

日本食品化学学会誌 2022;29:184-188. doi: 10.18891/jjfcs.29.3_184

Huperzine A, an alkaloid isolated from *Huperzia serrata*, inhibits acetylcholinesterase and is expected to be a therapeutic agent for Alzheimer's disease. However, there have been reports of health problems caused by foods containing Huperzine A. This study conducted qualitative and quantitative analyses using UHPLC-PDA-MS on 13 health food products related to toothed clubmoss. Qualitative analysis showed that Huperzine A was detected in 10 of the 13 samples. The maximum daily intake of Huperzine A calculated from the results of the quantitative study was between 7.83 and 691.68 μg/day. These products derived from toothed clubmoss may have health risks because the acceptable daily intake of Huperzine A calculated from a previous toxicity study was 3.03 μg/day.

Keywords: toothed clubmoss, health food product, Huperzine A

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田中理恵，河村麻衣子，水谷佐久美，袴塚高志，花尻（木倉）瑠理：危険ドラッグ製品中のarylcyclohexylamine誘導体, MXPr, MXiPr, DMXEの同定.

薬学雑誌 2022;142:675-681

Arylcyclohexylamines is a category of substances to which the anesthetic ketamine belongs. The arylcyclohexylamines have been reported to act as antagonists of the NMDA receptor. An analog of ketamine, 2-(ethylamino)-2-(3-methoxyphenyl)-cyclohexanone (Methoxetamine, MXE), has been controlled as a narcotic in Japan and overdoses of MXE have been reported to cause health problems. In recent years, MXE derivatives have been detected in illegal products in Japan. In this study, we describe the identification of three MXE derivatives, 2-(3-methoxyphenyl)-2-(propylamino)cyclohexan-1-one (Methoxpropamine, MXPr), 2-(isopropylamino)-2-(3-methoxyphenyl)cyclohexan-1-one (Methoxisopropamine, MXiPr) and 2-(3-methoxyphenyl)-2-(propylamino)cyclohexan-1-one (Deoxymethoxetamine, DMXE), from illegal products.

Keywords: Arylcyclohexylamine, methoxetamine, methoxpropamine

Morita I*1, Kiguchi Y*1, Oyama H*1, Yamaki K*1, Sakio N*1, Kashiwabara K*1, Kuroda Y*1, Ito A*1, Yokota A*1, Ikeda N*1, Kikura-Hanajiri R, Ueda H*2, Numazawa S*3, Yoshidade T*3 and Kobayashi N*1:

Derivatization-assisted immunoassays: application for group-specific detection of potent methamphetamine and amphetamine enantiomers.

Anal.Methods 2022;14:2745-2753.

Reliable and feasible tools for detecting (*S*)-methamphetamine [(*S*)-MAP] and (*S*)-amphetamine [(*S*)-AP] are required for regulating their illicit circulation. Antibodies that react equally to these stimulants are desirable for this purpose but have been difficult to generate because of the crucial difference between their characteristic structures: *i.e.*, *N*-methylamino (MAP) and amino (AP) groups. Furthermore, their small molecular masses (M_r < 150) have hampered the generation of high-affinity antibodies. To overcome these problems, we converted (*S*)-MAP and -AP into their 2-(trimethylsilyl)ethyl carbamate forms, Teoc-(*S*)-MAP and -AP, respectively, as surrogate analytes. The Teoc-derivatization not only

increases their molecular masses, but also masks their structural differences. We generated a novel monoclonal antibody that showed a satisfactory affinity to Teoc-(*S*)-MAP residues (K_d = 13 nM as the IgG form) and developed a competitive enzyme-linked immunosorbent assay (ELISA) using microplates containing immobilized Teoc-(*S*)-MAP residues. Almost overlapping dose-response curves were obtained for Teoc-(*S*)-MAP and -AP, with the limit of detection of 0.078 and 0.10 ng per assay, respectively. A fixed amount of test powder sample (1 mg) was derivatized with Teoc-*O*-succinimidyl for 5 min and subjected to ELISA using Teoc-(*S*)-MAP as the calibration standard. Under this protocol, (*S*)-MAP and -AP were converted to their Teoc derivatives with 30% and 34% yield, respectively, determined using ELISA as "Teoc-(*S*)-MAP equivalent," being distinguished from the derivatization products of (*R*)-MAP, (*R*)-AP, ephedrine, (*S*)-methylenedioxymethamphetamine, tyramine, dopamine, and β-alanine. This ELISA detected as little as 10 μg of (*S*)-MAP and -AP, and (*S*)-MAP in urine obtained from (*S*)-MAP-administered rats. Immunochromatography devices were also developed using gold nanoparticles coated with the monoclonal antibody, with which 0.10 mg of (*S*)-MAP and -AP was detected by the naked eye. We conclude that the present derivatization-assisted immunoassays may be useful for the detection of (*S*)-MAP and/or -AP in early-stage screening of suspicious substances.

Keywords: enantiomer, methamphetamine, derivatization-assisted immunoassays

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Irie T, Yanase Y, Demizu Y, Usami M*, Kikura-Hanajiri R: Derivatives of methoxetamine and major methoxetamine metabolites potently block NMDA receptors.

Journal of Pharmacological Sciences 2022;150:233-243.

N-Methyl-D-aspartate receptors (NMDARs) in the brain are influenced by psychoactive drugs such as 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one (ketamine) and its analog 2-(ethylamino)-2-(3-methoxyphenyl)-cyclohexanone (methoxetamine). The recreational methoxetamine use can cause several toxicities and methoxetamine-related deaths have also been reported. Therefore, it has been banned in many countries. Since 2020, methoxetamine derivatives, 2-(ethylamino)-2-(*m*-tolyl)cyclohexan-1-one (deoxymethoxetamine) and 2-(isopropylamino)-2-(3-methoxyphenyl)cyclohexan-1-one (methoxisopropamine), have been sold online as designer drugs. However, how deoxymethoxetamine and methoxisopropamine act on NMDARs remains unknown. In this study, we first performed *in silico* docking studies of NMDARs, and deoxymethoxetamine and methoxisopropamine in addition to the major methoxetamine metabolites, 2-amino-2-(3-methoxyphenyl)-cyclohexanone (*N*-desethyl methoxetamine) and 2-(ethylamino)-2-(3-hydroxyphenyl)-cyclohexanone (*O*-desmethyl methoxetamine). The docking study suggested each compound interacts with NMDARs. We also determined the half-maximal inhibitory concentration (IC_{50}s) of the methoxetamine-related compounds for NMDARs using NMDAR-expressing cartwheel interneurons of mice and patch-clamp recordings. We found that the IC_{50}s of methoxetamine, deoxymethoxetamine, methoxisopropamine, *N*-desethyl methoxetamine, and *O*-desmethyl methoxetamine for NMDARs were 0.524, 0.679, 0.661, 1.649, and 0.227 μM, respectively. These results indicate that the methoxetamine-related compounds act as potent NMDAR blockers. Thus, deoxymethoxetamine and methoxisopropamine, both of which may cause damage by blocking NMDARs, are serious concerns. *N*-Desethyl methoxetamine and *O*-desmethyl methoxetamine may cause several adverse effects when methoxetamine is metabolized.

Keywords: *N*-Methyl-D-aspartate receptors, deoxymethoxetamine, methoxisopropamine

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吉見嵩志*，張紅燕*，堀井周文*，小此木明*，高橋隆二*，鎌倉浩之，袴塚高志，合田幸広：葛根湯エキス製剤及び湯剤の同等性に関する研究（Ⅲ）

生薬学雑誌 2023;77:1-6.

As previously reported (Horii, C., *et al.*, *Shoyakugaku*

Zasshi, **68** (1), 9-12 (2014), *Shoyakugaku Zasshi*, **69** (2), 59-65 (2015), *Shoyakugaku Zasshi*, **68** (2), 65-69 (2014), *Shoyakugaku Zasshi*, **73** (2), 73-83 (2019), and *Shoyakugaku Zasshi*, **74** (1), 46-57 (2020)), we investigated the bioequivalence of the decoctions and extract preparations of Kakkonto, Shoseiryuto and Hachimijiogan, demonstrating that some components may serve as marker compounds for the evaluation of bioequivalence. Of them, ephedrine and pseudephedrine contained in the Ephedra Herba might serveas the most effective marker compounds for examining the bioequivalence between the decoction and the extract preparation in a increased number of subjects. The present study examined the bioequivalence between the decoction and the extract preparation of Kakkonto by comparing the plasma concentration s of the components of the decoction of Kakkonto and Kracie Kakkonto extract fine granules, prepared with the same crude drug, as test and control drugs, respectively, after administration to 20 human subjects. As a result, the decoction and the extract preparation showec no significant difference in the plasma concentrations, maximum plasma concentrations (C_{max}), or area under the plasma concentration versus time curve (AUC_{24h}) at any blood sampling time. As the 90% confidence interval of the difference in the mean logarithmic values of the C_{max} and AUC_{24h} of ephedrine and pseudoephedrine between the decoction and the extract preparation were within the range of log (0.80) to l0g (1.25) (criterion for bioequivalence), a statistical power (1-β) was employed. As a result, the C_{max} and AUC_{24h} in ephedrine and pseudoephedrine has sufficient power (more than 80%), suggesting that the bioequivalence of the decoction and the extract preparation of Kakkonto can be evaluated using ephedrine and pseudoephedrine as marker compounds.

Keywords: bioequivqlence, Kakkonto, ephedrine and pseudoephedrine plasma concentrations

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生薬学雑誌 2023;77:7-23.

In this study, we report the designed and verified methods for the identification tests and assay of six single crude drug extracts, *i.e.* Ginseng Extract (**1**), Red Ginseng Extract (**2**), Polygala Root Extract (**3**), Bupleurum Root Extract (**4**), Peony Root Extract (**5**), and Citrus Unshiu Peel Extract (**6**), which were newly listed in Non-JPS 2022, published in March 2022. Ginsenoside Rg_1 was selected as the marker compound (MC) of **1** and **2** for the identification test by TLC, and ginsenoside Rg_1 and ginsenoside Rb_1 were selected as the MCs of **1** and **2** for the assay by HPLC. Tenuifolin and 3,6'-di-*O*-sinapoylsucrose were selected as the MCs of **3** for the identification test by TLC and the assay by HPLC, respectively. Saikosaponins a and c were selected as the MCs of **4** for the identification test by TLC. Saikosaponin b_2 was selected as the MC of **4** for the assay using HPLC. Paeoniflorin was selected as the MC of **5** for the identification test using TLC and the assay using HPLC. Hesperidin and narirutin were selected as the MCs of **6** for the identification test using HPLC and hesperidin was selected as the MC of **6** for the assay using HPLC.

Keywords: single crude drug extract, Non-JP Crude Drug Standards

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Kamata K*1,2, Tatsuzaki J*1, Ishikawa T*3, Arai R, Hakamatsuka T*4, Uchiyama N: HPLC analysis of ammonium glycyrrhizate listed in the European, United States, and Japanese Pharmacopoeias under reported and modified conditions: revision of the peak assignment for 18*α*-glycyrrhizin in the European and United States Pharmacopoeias.

J. Nat. Med. 2023;77:202-206. doi: 10.1007/s11418-022-01649-8.

We examined ammonium glycyrrhizate listed in the monographs of the European Pharmacopoeia (EP) and United States Pharmacopoeia (USP) as well as in the reagents and solutions used in the general test of the Japanese Pharmacopoeia by performing HPLC on their sample standards or reference reagents under reported and modified conditions. Comparative experiments involving five authentic samples, namely, 18*β*-glycyrrhizin (**1**), 18*α*-glycyrrhizin (**2**), licorice-saponin G2 (**3**), licorice-saponin H2 (**4**), and galacturonic acid-replaced glycyrrhizin (the 4″ -epimer of 18*β*-glycyrrhizin) (**5**), led us to propose the revision of the peak assignment of 18*α*-glycyrrhizin (**2**) and postscript a possible co-existence of galacturonic acid-replaced glycyrrhizin (**5**) as a hidden component in the EP and USP. We also proposed that the *α*-configuration used in the nomenclature of the glycosidic bond between aglycone and the sugar units of ammonium glycyrrhizate and impurities in the EP and USP should be revised to the *β*-configuration.

Keywords: ammonium glycyrrhizate, 18*α*-glycyrrhizin, European Pharmacopoeia

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Yokoo H, Tanaka S, Yamamoto E, Tsuji G, Demizu Y, Uchiyama N: Advanced solid-state NMR analysis of two crystal forms of ranitidine hydrochloride: Detection of 1H-14N intra-/intermolecular correlations.

Chem. Pharm. Bull. 2023;71:58-63. doi: 10.1248/cpb.c22-00628

In this study, we selected ranitidine hydrochloride, which is known to exist in two forms, 1 and 2, as the model drug and investigated each form using solid-state NMR. The ^{1}H-^{14}N dipolar-based heteronuclear multiple quantum coherence (D-HMQC) analysis revealed the intermolecular correlation of Form 1 between the N atom of the nitro group and a proton of the furan moiety, which were closer than those of the intramolecular correlation reported using single X-ray crystal analysis. Thus, ^{1}H-^{14}N D-HMQC analysis could be useful for characterizing intermolecular interaction in ranitidine hydrochloride crystals. In addition, we reassigned the ^{13}C solid-state NMR signals of ranitidine hydrochloride according to the liquid-state and multiple solid-state NMR experiments.

Keywords: solid-state NMR, intermolecular correlation, ranitidine hydrochloride

Hirai T, Kono K, Kusakawa S, Yasuda S, Sawada R, Morishita A*1, Hata S*1, Wakita A*2, Kageyama T*2, Takahashi R*2, Watanabe S*3, Shiraishi N*4, Sato Y: Evaluation of the reproducibility and positive controls of cellular immortality test for the detection of immortalized cellular impurities in human cell-processed therapeutic products.

Regen Ther. 2022;21:540-46. doi: 10.1016/j.reth.2022.10.009.

Introduction: Contamination of human cell-processed therapeutic products (hCTPs) with tumorigenic/immortalized cellular impurities is a major concern in the manufacturing and quality control of hCTPs. The cellular immortality test based on cell growth analysis is a method for detecting tumorigenic/immortalized cellular impurities in hCTPs. However, the performance of the cellular immortality test has not yet been well characterized. In this study, we examined the reproducibility of the cellular immortality test in detecting HeLa cells as a model of

tumorigenic cellular impurities, as well as the applicability of other models of cellular impurities with different tumorigenicity to the cellular immortality test. Methods: Using HeLa cells as a model for cellular impurities, we measured the growth rate of human mesenchymal stem cells (hMSCs) supplemented with HeLa cells at concentrations ranging from 0.01 to 0.0001% at each passage in three laboratories and evaluated the reproducibility of the detection of immortalized cellular impurities. In addition, HEK293 cells (another immortalized cell line) and MRC-5 cells (a non-immortalized cell line) were employed as cellular impurity models that exhibit different growth characteristics from HeLa cells, and the ability of the cellular immortality test to detect these different impurities when mixed with hMSCs was examined. Results: In the multisite study, the growth rate of hMSCs supplemented with 1 and 10 HeLa cells (0.0001% and 0.001%) significantly increased and reached a plateau in all three laboratories, whereas those of hMSCs alone eventually decreased. Moreover, when hMSCs were supplemented with 10 and 100 HEK293 and MRC-5 cells (0.001% and 0.01%), the growth rate significantly increased. The growth rate of hMSCs supplemented with HEK293 cells increased with passage and remained high, whereas that of hMSCs supplemented with MRC-5 cells eventually decreased, as in the case of hMSCs alone. Conclusions: These results indicate that the cellular immortality test is reproducible and can detect immortalized (*i.e.*, potentially tumorigenic) cells such as HEK293 cells with a lower growth rate than HeLa cells by discriminating against normal cells, which could contribute to ensuring the safety and quality of hCTPs.

Keywords: cellular immortality test, tumorigenicity, human cell-processed therapeutic products

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Hirasawa R*1, Takakura M*2, Hirai T, Kono K, Sato Y: Attitude and perception survey for the Japanese pharmaceutical industry to utilize next-generation sequencing for virus safety assessment of biologics. *Translat Regulat Sci*. 2022;4:61-7. doi: 10.33611/trs.2022-004.

In the past decade, broad virus detection methods, as represented by next-generation sequencing (NGS) technology, have gained more recognition as an effective approach to assessing the virus safety of biologics such as antibody drugs, vaccines, and gene/cell therapy products. A global group was organized as a joint effort of regulatory and industry scientists from the United States and Europe to further discuss this regulatory issue and to facilitate the implementation of NGS testing in the virus safety assessment of biologics. This global activity has proactively promoted practical studies for performance evaluations of virus detection by NGS, as well as the development of analytical tools such as reference viral reagents and a virus database. However, in Japan, it is ambiguous whether this regulatory issue concerns domestic pharmaceutical companies or the regulatory body. Therefore, we conducted a questionnaire survey to gain a picture of the Japanese pharmaceutical industry's views regarding the utilization of NGS for virus safety assessment for biologics. The survey results indicate that most respondents have little or limited experience with NGS and a passive attitude towards NGS utilization for virus safety assessments. With the ongoing revision of the relevant guideline, ICH Q5A, concerned parties in Japanese industry and regulatory body must urgently catch up with global discussions on NGS utilization for the virus safety assessment of biologics and join the international movement.

Keywords: virus safety, next-generation sequencing, biologics

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Watanabe T*1, Yasuda S, Chen CL*2, Delsing L*3, Fellows MD*3, Foldes G*4, Kusakawa S, Mouries LP*2, Sato Y: International evaluation study of a highly efficient culture assay for detection of residual human pluripotent stem cells in cell therapies. *Regenerative Medicine*. 2022;18:219-27. doi: 10.2217/rme-2022-0207

Aim & methods: The Health and Environmental Sciences Institute Cell Therapy-TRAcking, Circulation

& Safety Technical Committee launched an international, multisite study to evaluate the sensitivity and reproducibility of the highly efficient culture (HEC) assay, an *in vitro* assay to detect residual undifferentiated human pluripotent stem cells (hPSCs) in cell therapy products. *Results:* All facilities detected colonies of human induced pluripotent stem cells (hiPSCs) when five hiPSCs were spiked into 1 million hiPSC-derived cardiomyocytes. Spiking with a trace amount of hiPSCs revealed that repeatability accounts for the majority of reproducibility while the true positive rate was high. *Conclusions:* The results indicate that the HEC assay is highly sensitive and robust and can be generally applicable for tumorigenicity evaluation of hPSC-derived cell therapy products.

Keywords: *in vitro* assay, multisite study, pluripotent stem cells

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Oda S*1,2, Nishiyama K*3, Furumoto Y*3, Yamaguchi Y*4, Nishimura A*1,2, Tang X*1,2, Kato Y*3, Numaga-Tomita T*1,5, Kaneko T*4, Mangmool S*6, Kuroda T, Okubo R*3, Sanbo M*1, Hirabayashi M*1, Sato Y, Nakagawa Y*7, Kuwahara K*5, Nagata R*8, Iribe G*4, Mori Y*7, Nishida M*1,2,3: Myocardial TRPC6-mediated Zn^{2+} influx induces beneficial positive inotropy through β-adrenoceptors.

Nat Commun, 2022;13:6374. doi: 10.1038/s41467-022-34194-9.

Baroreflex control of cardiac contraction (positive inotropy) through sympathetic nerve activation is important for cardiocirculatory homeostasis. Transient receptor potential canonical subfamily (TRPC) channels are responsible for α_1-adrenoceptor (α_1AR)-stimulated cation entry and their upregulation is associated with pathological cardiac remodeling. Whether TRPC channels participate in physiological pump functions remains unclear. We demonstrate that TRPC6-specific Zn^{2+} influx potentiates β-adrenoceptor (βAR)-stimulated positive inotropy in rodent cardiomyocytes. Deletion of trpc6 impairs sympathetic nerve-activated positive inotropy but not chronotropy in mice. TRPC6-mediated Zn^{2+} influx boosts α_1AR-stimulated βAR/G_s-dependent signaling in rat cardiomyocytes by inhibiting β-arrestin-mediated βAR internalization. Replacing two TRPC6-specific amino acids in the pore region with TRPC3 residues diminishes the α_1AR-stimulated Zn^{2+} influx and positive inotropic response. Pharmacological enhancement of TRPC6-mediated Zn^{2+} influx prevents chronic heart failure progression in mice. Our data demonstrate that TRPC6-mediated Zn^{2+} influx with α_1AR stimulation enhances baroreflex-induced positive inotropy, which may be a new therapeutic strategy for chronic heart failure.

Keywords: chronic heart failure, transient receptor potential canonical 6, β-adrenoceptor

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Shirasago Y*1, Fukazawa H*1, Nagase S*2, Shimizu Y*1,3, Mizukami T*1, Wakita T*1, Suzuki T*4, Tani H*5, Kondoh M*2, Kuroda T, Yasuda S, Sato Y, Hanada K*1, Fukasawa M*1: A single mutation in the E2 glycoprotein of hepatitis C virus broadens the claudin specificity for its infection.

Sci Rep, 2022;24:20243. doi: 10.1038/s41598-022-23824-3.

Entry of the hepatitis C virus (HCV) into host cells is a multistep process mediated by several host factors, including a tight junction protein claudin-1 (CLDN1). We repeatedly passaged HCV-JFH1-tau, an HCV substrain with higher infectivity, on Huh7.5.1-8 cells. A multi-passaged HCV-JFH1-tau lot was infectious to CLDN1-defective S7-A cells, non-permissive to original HCV-JFH1-tau infection. We identified a single mutation, M706L, in the E2 glycoprotein of the HCV-JFH1-tau lot as an essential mutation for infectivity to S7-A cells. The pseudovirus JFH1/M706L mutant could not infect human embryonic kidney 293 T (HEK293T) cells lacking CLDN family but infected HEK293T cells expressing CLDN1, CLDN6, or CLDN9.

Thus, this mutant virus could utilize CLDN1, and other CLDN6 and CLDN9, making HCV possible to infect cells other than hepatocytes. iPS cells, one of the stem cells, do not express CLDN1 but express CLDN6 and other host factors required for HCV infection. We confirmed that the HCV-JFH1-tau-derived mutant with an M706L mutation infected iPS cells in a CLDN6-dependent manner. These results demonstrated that a missense mutation in E2 could broaden the CLDN member specificity for HCV infection. HCV may change its receptor requirement through a single amino acid mutation and infect non-hepatic cells.

Keywords: hepatitis C virus, infection to non-hepatocytes, claudin

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Yamamoto T*1, Sato Y, Yasuda S, Shikamura M*1, Tamura T*1, Takenaka C*1, Takasu N*2, Nomura M*2, Dohi H*2, Takahashi M*3,4, Mandai M*3, Kanemura Y*5, Nakamura M*6, Okano H*6, Kawamata S*1,3: Correlation Between Genetic Abnormalities in Induced Pluripotent Stem Cell-Derivatives and Abnormal Tissue Formation in Tumorigenicity Tests.

Stem Cells Transl Med. 2022;11:527-538. doi: 10.1093/stcltm/szac014.

Cell therapy using induced pluripotent stem cell (iPSC) derivatives may result in abnormal tissue generation because the cells undergo numerous cycles of mitosis before clinical application, potentially increasing the accumulation of genetic abnormalities. Therefore, genetic tests may predict abnormal tissue formation after transplantation. Here, we administered iPSC derivatives with or without single-nucleotide variants (SNVs) and deletions in cancer-related genes with various genomic copy number variant (CNV) profiles into immunodeficient mice and examined the relationships between mutations and abnormal tissue formation after transplantation. No positive correlations were found between the presence of SNVs/deletions and the formation of abnormal tissues; the overall predictivity was 29%. However, a copy number higher than 3 was correlated, with an overall predictivity of 86%. Furthermore, we found CNV hotspots at 14q32.33 and 17q12 loci. Thus, CNV analysis may predict abnormal tissue formation after transplantation of iPSC derivatives and reduce the number of tumorigenicity tests.

Keywords: Shibata List, single-nucleotide variants, tumorigenicity test

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Yoshida T, Morihiro K*1,2, Naito Y*3,4, Mikami A*1, Kasahara Y*1,2, Inoue T, Obika S*1,2: Identification of nucleobase chemical modifications that reduce the hepatotoxicity of gapmer antisense oligonucleotides.

Nucleic Acids Research. 2022;50:7224-34. doi: 10.1093/nar/gkac562

Currently, gapmer antisense oligonucleotide (ASO) therapeutics are under clinical development for the treatment of various diseases, including previously intractable human disorders; however, they have the potential to induce hepatotoxicity. Although several groups have reported the reduced hepatotoxicity of gapmer ASOs following chemical modifications of sugar residues or internucleotide linkages, only few studies have described nucleobase modifications to reduce hepatotoxicity. In this study, we introduced single or multiple combinations of 17 nucleobase derivatives, including four novel derivatives, into hepatotoxic locked nucleic acid gapmer ASOs and examined their effects on hepatotoxicity. The results demonstrated successful identification of chemical modifications that strongly reduced the hepatotoxicity of gapmer ASOs. This approach expands the ability to design gapmer ASOs with optimal therapeutic profiles.

Keywords: gapmer, hepatotoxicity, chemical modification

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Sakurai Y*, Yamaguchi T*, Yoshida T, Horiba M*, Inoue T, Obika S*: Synthesis and Properties of Nucleobase-Sugar Dual Modified Nucleic Acids: 2'-OMe-RNA and scpBNA Bearing a 5-Hydroxycytosine Nucleobase.
J. Org. Chem. 2023;88:154-62. doi: 10.1021/acs.joc.2c02038

Naturally occurring 5-hydroxycytosine ($^{5\text{-OH}}$Cyt), which is associated with DNA damage, was recently found to reduce the hepatotoxicity of antisense oligonucleotides (ASOs) without compromising its antisense activity when used as a replacement for cytosine (Cyt). Additionally, sugar-modified nucleic acids, such as 2'-O-methylribonucleic acid (2'-OMe-RNA) and 2'-O,4'-C-spirocyclopropylene-bridged nucleic acid (scpBNA), have emerged as useful antisense materials. Herein, we aimed to combine these two advantages by designing dual modified nucleic acids 2'-OMe-RNA-$^{5\text{-OH}}$Cyt and scpBNA-$^{5\text{-OH}}$Cyt bearing the $^{5\text{-OH}}$Cyt nucleobase to develop efficient and safe ASOs. We describe the synthesis of 2'-OMe-RNA-$^{5\text{-OH}}$Cyt and scpBNA-$^{5\text{-OH}}$Cyt phosphoramidites and their incorporation into oligonucleotides (ONs). The duplex-forming ability and base discrimination properties of 2'-OMe-RNA-$^{5\text{-OH}}$Cyt- and scpBNA-$^{5\text{-OH}}$Cyt-modified ONs were similar to those of 2'-OMe-RNA-Cyt- and scpBNA-mCyt-modified ONs, respectively. We also synthesized two 2'-OMe-RNA-$^{5\text{-OH}}$Cyt-modified ASOs, and one of the two was found to exhibit reduced hepatotoxicity while retaining target mRNA knockdown activity in *in vivo* experiments.
Keywords: antisense oligonucleotide, hepatotoxicity, dual modified nucleic acid

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Ohoka N, Suzuki M*, Uchida T*, Tsukumo Y, Yoshida M*, Inoue T, Ohki H*, Naito M: Development of a potent small-molecule degrader against oncogenic BRAF^{V600E} protein that evades paradoxical MAPK activation.
Cancer Science. 2022;113:2828-38. doi: 10.1111/cas.15401

BRAF mutations are frequently observed in melanoma and hairy-cell leukemia. Currently approved rapidly accelerated fibrosarcoma (RAF) kinase inhibitors targeting oncogenic BRAF V600 mutations have shown remarkable efficacy in the clinic, but their therapeutic benefits are occasionally hampered by acquired resistance due to RAF dimerization-dependent reactivation of the downstream MAPK pathway, which is known as paradoxical activation. There is also a concern that paradoxical activation of the MAPK pathway may trigger secondary cancer progression. In this study, we developed chimeric compounds, proteolysis targeting chimeras (PROTACs), that target BRAF^{V600E} protein for degradation. CRBN(BRAF)-24, the most effective chimera, potently degraded BRAF^{V600E} in a ubiquitin-proteasome system (UPS)-dependent manner and inhibited the proliferation of BRAF^{V600E} -driven cancer cells. In BRAF wild-type cells, CRBN(BRAF)-24 induced neither BRAF^{WT} degradation nor paradoxical activation of the MAPK pathway. Biochemical analysis revealed that CRBN(BRAF)-24 showed more potent and sustained suppression of MAPK signaling than a BRAF^{V600E} inhibitor, PLX-8394, in BRAF^{V600E} -driven cancer cells. Targeted degradation of BRAF^{V600E} by CRBN(BRAF)-24 could be a promising strategy to evade paradoxical activation of the RAF-MAPK pathway.
Keywords: BRAF, MAPK, PROTAC

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Ohoka N, Suzuki M*, Uchida T*, Tsuji G, Tsukumo Y, Yoshida M*, Inoue T, Demizu Y, Ohki H*, Naito M: Development of Gilteritinib-Based Chimeric Small Molecules that Small Molecules that Potently Induce Degradation of FLT3-ITD Protein.
ACS Medicinal Chemistry Letters. 2022;13:1885-91. doi: 10.1021/acsmedchemlett.2c00402

Internal tandem duplication (ITD) in the gene encoding FMS-like tyrosine kinase 3 (*FLT3*) (FLT3-ITD) is the most frequently observed mutation in acute myeloid leukemia (AML). Currently approved FLT3 kinase inhibitors have high efficacy, but drug resistance caused by reactivation of FLT3 kinase activity is often clinically observed. In this study, we

developed novel FLT3 degraders by introducing gilteritinib, an FDA-approved FLT3 inhibitor, into targeted protein degradation technology. The most active compound, CRBN(FLT3)-8, potently degraded FLT3-ITD via the ubiquitin-proteasome system and inhibited the proliferation of FLT3-ITD mutant AML cells more effectively than gilteritinib. These findings provide a new lead compound for degradation-based drugs targeting FLT3-ITD-positive cancers.

Keywords: FLT3, gilteritinib, degradation

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Hashimoto D*1, Okamoto Y, Onishi S*2, Higashi K*2, Wada T*3, Toida T*1: Quality control of proteoglycan obtained from salmon nasal cartilage in dietary supplements.

Jpn. J. Food Chem. Safety 2022;29:2:104-113. doi: 10.18891/jjfcs.29.2_104

Aggrecan, which is a chondroitin sulfate proteoglycan (CSPG), has been considered as a superior functional nutraceutical for the treatment of joint diseases and other immune system diseases when compared to chondroitin sulfate (CS). The industrial production of CSPG generally employs salmon nasal cartilage, and the quality control of CSPG is required to meet the regulations for nutraceutical products prepared from natural resources. Although there are several commercially available nutraceuticals that contain CSPG as a major component, the quality and quantity of CSPG in each supplement are not guaranteed. This paper presents a simple, rapid, and reliable analytical approach for the quality control of CSPG during production, where electrophoresis, gel filtration HPLC, and CS unsaturated disaccharide analysis with CS degradation enzymes were employed. Finally, the quality of CSPG obtained from different extraction and purification processes were confirmed using these newly developed analytical procedures.

Keywords: chondroitin sulfate proteoglycan, salmon nasal cartilage, osteoarthritis, quality control, nutraceuticals

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Yamamoto E, Hosogi N*, Takechi-Haraya Y, Izutsu K, Uchiyama N, Goda Y: Folded, undulating, and fibrous doxorubicin sulfate crystals in liposomes. Nanomedicine: Nanotechnology.

Biology and Medicine. 2023;47:102631. doi: https://doi.org/10.1016/j.nano.2022.102631.

High-resolution cryogenic transmission electron microscopy (cryo-TEM) evidenced that doxorubicin sulfate crystals in liposomes (prepared by remote loading with ammonium sulfate) form folded, undulating, and fibrous crystals with a diameter of approximately 2.4 nm. An undulating, fibrous crystal considered to be undergrowth, in addition to bundles of fibrous crystals, was also observed in doxorubicin-loaded liposomes. This explains the validity of the formation of doxorubicin sulfate crystals of various shapes, e.g., curved, U-shaped, or circular, in addition to cylinder and/or rod-like crystals reported in the literature. Liposomes that do not contain crystals have inner aqueous phases with high electron density, suggesting that the doxorubicin is remotely loaded and remains as a solute without precipitation.

Keywords: Liposomes, Doxorubicin sulfate, Nanocrystal, Morphology, Equivalency

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* JEOL Ltd

Amano Y*, Misawa T, Miyazaki T, Ando D, Koide T, Izutsu K, Kanazawa H*, Hanaoka K*, Yamamoto E: Real-time in situ X-ray micro-computed tomography study of the effect of impurities on the crystallization of amorphous nifedipine.

J Pharm Biomed Anal. 2023;226:115248. doi: https://doi.org/10.1016/j.jpba.2023.115248.

Controlling the physical stability of noncrystalline active pharmaceutical ingredients remains a major challenge in the development of amorphous formulations such as amorphous solid-dispersion (ASD) formulations. To establish new evaluation and formulation strategies, the spatial distribution of the crystal phase in bulk amorphous nifedipine (NFD) was investigated as a model. The crystallization of amorphous NFD and the effect of a deliberately added impurity were investigated using powder X-ray diffraction (PXRD), differential scanning calorimetry and real-time in situ X-ray micro-computed tomography (X-ray CT). The stability data of

amorphous samples, i.e., NFD and a mixture of NFD with an oxidative degradation product of NFD, impurity A (Imp A), at a weight ratio of 90:10, presented as percent amorphous remaining, suggests that Imp A accelerates the bulk crystal growth of NFD. Real-time in situ X-ray CT results showed surface-enhanced crystal growth and cavity formation in solid NFD samples. Moreover, the crystals were heterogeneous in density. These results suggest that Imp A affects the physical stability of the amorphous NFD. X-ray CT equipped with a heating unit can aid in-situ evaluation and assessment of physicochemical properties and physical stability of amorphous samples and formulations.

Keywords: Image analysis, crystallization, crystal growth, degradation product, forced condition, stability

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Hamahashi K[*1], Toyoda E[*1], Ishihara M[*2], Mitani G[*1], Takagaki T[*1], Kaneshiro N[*1], Maehara M[*1], Takahashi T[*1], Okada E[*1], Watanabe A[*1], Nakamura Y[*1], Kato R, Matoba R[*3], Takagi T[*4], Akutsu H[*4], Umezawa A[*4], Kobayashi H[*1], Akamatsu T[*1], Yamato M[*5], Okano T[*6], Watanabe M[*1] and Sato M[*1]: Polydactyly-derived allogeneic chondrocyte cell-sheet transplantation with high tibial osteotomy as regenerative therapy for knee osteoarthritis.

NPJ Regen Med. 2022;7:71, 2022. doi: 10.1038/s41536-022-00272-1.

Allogeneic cell therapies are not fully effective in treating osteoarthritis of the knee (OAK). We recently reported that transplantation of autologous chondrocyte cell-sheets along with open-wedge high tibial osteotomy promoted hyaline cartilage repair in humans. Here we describe our regenerative therapy for OAK using polydactyly-derived allogeneic chondrocyte cell-sheets (PD sheets) and temperature-responsive culture inserts. Ten patients with OAK and cartilage defects categorized arthroscopically as Outerbridge grade III or IV received the therapy. Cartilage viscoelasticity and thickness were assessed before and after transplantation. Arthroscopic biopsies obtained 12 months after transplantation were analyzed histologically. Gene expression was analyzed to evaluate the PD sheets. In this small initial longitudinal series, PD sheet transplantation was effective in treating OAK, as indicated by changes in cartilage properties. Gene marker sets in PD sheets may predict outcomes after therapy and provide markers for the selection of donor cells. This combined surgery may be an ideal regenerative therapy with disease-modifying effects in OAK patients.

Keywords: cell-sheet transplantation, regenerative therapy, allogeneic chondrocyte

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Uematsu M, Miyamoto Y, Ito Y*, Naito T*, Fujii S*, Takahashi Y*, Sakoda H, Okamoto Y, Nakaoka R, Haishima Y: Novel method to recover and quantify residual proteins for cleanliness evaluation of reusable and reprocessed medical devices.

The Jpn J Med Instrum. 2022;92:400-414.

There is no standard cleanliness evaluation procedure for reusable medical devices, only recommended guidelines. Our aim was to develop an efficient and reproducible method to recover proteins in greater amounts than conventional procedures. In addition to the solvents indicated in the guidelines, sample buffers (SB1 and SB2) containing the detergent sodium dodecyl sulfate (SDS) were tested. Protein recovery was compared using pseudo-blood-contaminated samples heat-treated under dry and wet conditions. Protein recovery from dry-heated samples did not change significantly regardless of extraction conditions, whereas the recovery rate from wet-heated samples using Milli-Q water or 1% SDS decreased proportionately to the increase in temperature. SB1 and SB2 extraction showed excellent protein recovery up to 95℃. In quantifying the protein using bicinchoninic acid (BCA), the interference of tris (2-carboxyethyl) phosphine in SB2, but not dithiothreitol in SB1, was quenched by pretreatment with iodoacetamide (IAM). After cleaning samples wet-heated at 95℃ for 10 min with a washer-disinfector, the protein recovery rate per sample was 8.67 ± 3.0 μg using SB2 vs. 6.37 ± 2.5 μg using 1%

SDS. These results indicate that residual proteins binding strongly to the surface of medical devices by thermal coagulation can be almost completely recovered and quantified by combining SB2 extraction, pretreatment with IAM, and BCA quantification.

Keywords: cleanliness evaluation, residual protein, reusable medical device

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Shimada K*[1], Daisaki H*[2], Higashiyama S*[3], Kawabe J*[3], Nakaoka R, Shimizu A*[1]: Simulation of postmarket fine-tuning of a computer-aided detection system from bone scintigrams and its performance analysis

Adv Biomed Eng. 2023;12:51-63.

In this study, we performed simulations for bone scintigrams before and after a hotspot detection support system was fine-tuned using a postmarket dataset, and statistically identified the factors that affected the performance changes. Datasets from five hospitals were used to train the premarket system, and another postmarket hospital dataset was added to fine-tune the system. We applied pre- and postmarket fine-tuned systems to postmarket test data and computed the difference in the number of pixels of false positives and false negatives before and after fine-tuning. Structural equation modeling was used to analyze the relationship between the four possible factors and performance changes. The experimental results indicated that the image contrast and number of pixels of hot spots per image were the main factors affecting the performance. In addition, we identified the conditions for determining whether fine-tuning the system using postmarket datasets is appropriate. The experimental findings from this study will be useful for deriving an effective design scheme for continuous learning in artificial intelligence systems.

Keywords: computer-aided detection, postmarket learning, regulatory science

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Inuzuka, R.*[1], Tachimori, H.*[2,3,4], Kim, S. H.*[5], Matsui, H.*[1], Kobayashi, T.*[6], Kato, A.*[7], Fujii, T.*[8], Ho, M.*[9], Morikawa, H.*[10], Takahashi, S*[11], Shirato, H.*[11], Haishima, Y., Okamoto, Y., Sakoda, H., Tomita, H.*[8]: Practice and safety of static balloon atrial septostomy based on a nationwide registry data.

Circulation Journal 2022;86:1990-1997. doi: 10.1253/circj.CJ-22-0185

Background: Balloon atrial septostomy (BAS) is an essential catheterization procedure for congenital heart lesions. Recently, a balloon catheter for static BAS was approved for the first time in Japan as an alternative to the conventional pull-through BAS. Despite the expected increase in the use of static BAS, reports on its safety are scarce worldwide.

Methods and Results: Data on static and pull-through BAS registered in a national registry between 2016 and 2018 were collected. During the study period, 247 sessions of static BAS and 588 sessions of pull-through BAS were performed on a total of 674 patients. Patients who underwent static BAS were older (P<0.001). The incidence of serious adverse events (4.3% vs. 0.9%, P=0.03) and the overall incidence of adverse events (8.1% vs. 3.2%, P=0.03) were higher in static BAS than in pull-through BAS. Among patients who underwent static BAS, the risk factor for adverse events was a body weight < 3kg at the time of the procedure (odds ratio: 4.3 [confidence interval: 1.7-11], P=0.003).

Conclusions: This nationwide study revealed differences in patient background between static and pull-through BAS, as well as a higher incidence of adverse events related to static BAS. Patients weighing <3 kg are at high risk for adverse events after static BAS and may require surgical and circulatory support backup.

Keywords: children, congenital heart disease, risk factors

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迫田秀行，岡本吉弘：デラミネーション試験法の検出感度比較.
臨床バイオメカニクス 2022;43:149-153.

人工関節の超高分子量ポリエチレン部材におけるデラミネーションの発生リスクは，その材料強度に大きく依存する．しかし，既存の単純往復動を用いる材料試験法は検出感度が低く，限られた材料の評価にしか応用できない．我々は，コの字型摺動を用いる新規デラミネーション試験法を提案し，様々な材料に応用すると共に，その妥当性を示してきた．そこで本研究では，規格化されている既存法と新規法の検出感度を直接比較した．

臨床上デラミネーションの発生リスクがある製品を模擬した，２種の試験材料を両試験法で評価した．既存法では一方でデラミネーションが再現されず，臨床上のリスクを見逃す可能性があると共に，この材料と耐デラミネーション強度が同等以上の材料は評価できないことがわかった．一方，新規法ではいずれの材料でもデラミネーションが再現された．新規法の検出感度は高く，本法により幅広い材料の耐デラミネーション強度を評価可能である.

Keywords: artificial joint, ASTM, *in vitro* test

Oshima N, Takagi M, Sakai S, Ikarashi Y: Comparison of helium-alternative carrier gases for gas chromatography/mass spectrometry of standard test methods for indoor air quality guidelines in Japan.
BPB Reports 2022;5:84-7. doi: 10.1248/bpbreports.5.4_84

Helium is the most frequently used carrier gas for GC/MS, which is the official standardized test method in Japan to assess chemical substances in indoor air. However, recent global challenges in the supply chain for helium have led to a need to validate GC/MS using alternative carrier gases. In this study, we examined the applicability of hydrogen and nitrogen as helium-alternative carrier gases in the standardized GC/MS analytical test method for volatile organic compounds (VOC) and phthalate esters in indoor air. Comparison of the signal-to-noise ratios of standard solutions showed that detection sensitivities of hydrogen and nitrogen analysis were enough for the standard test method, although these gases, especially nitrogen, were less sensitive than helium. Measurements using these alternative carrier gases showed good linearity and could quantify around 1/100th of Japanese guideline values for indoor air concentrations. Therefore, hydrogen and nitrogen gases can be applied to the standard GC/MS analysis test method for VOC and phthalate esters in indoor air as alternative carrier gases to helium.

Keywords: indoor air, helium, alternative carrier gas

Oshima N, Tahara M, Sakai S, Ikarashi Y: A nationwide survey on indoor air concentrations of benzene and naphthalene in general residential housings.
Indoor Environment 2022;25:177-184.

The World Health Organization (WHO) Regional Office for Europe has established indoor air quality (IAQ) guidelines for benzene and naphthalene. However, these chemicals are not subjected to the guideline values for indoor air concentrations in Japan (Japanese IAQ guideline). Therefore, this survey was investigated the pollution level of benzene and naphthalene in houses. We measured the indoor and outdoor air at 28 houses in Japan four times a year and assessed their seasonal variations. The results showed that benzene had high indoor air concentrations from December to March. Also, indoor benzene concentrations in many houses exceeded the WHO IAQ guideline values. The naphthalene concentration in one house exceeded the WHO IAQ guideline values throughout the year although overall concentrations of other houses measured were low. Further, the indoor concentrations of benzene and naphthalene were higher than the outdoor concentrations in most houses, indicating that these pollution sources were originated

from indoors. The results obtained from this survey will be useful for the considering to set of Japanese IAQ guideline for benzene and naphthalene in the future.

Keywords: indoor air quality, sick house syndrome, nationwide survey

Sakai S, Tahara M, Kubota R, Kawakami T, Inoue K, Ikarashi Y: Characterization of synthetic turf rubber granule infill in Japan: Volatile organic compounds. *Sci Total Environ* 2022;838:156400. doi: 10.1016/j.scitotenv2022.156400

There has been extensive studies on the composition of tires and industrial rubber. However, there is insufficient information on volatile organic compounds (VOCs) emitted from rubber granule products used to fill synthetic turf fields. In this study, we applied a passive sampling method for assessing the VOCs emitted from rubber granule products used for filling synthetic turf fields. We also performed a quantitative component analysis using a gas chromatography-mass spectrometer (GC-MS). The component analysis results of 46 rubber granule-based products showed the predominant presence of benzothiazole and methyl isobutyl ketone. The level of benzene, which the International Agency for Research on Cancer classifies as a substance with sufficient evidence for carcinogenicity to humans, was below the lower quantification limit in the products tested in this study. Our study included most of the rubber granule products used for synthetic turf fields in Japan (>95% of the products in the current domestic market of Japan). Therefore, we obtained a comprehensive overview of the VOCs emitted from the rubber granule-based products used in Japan's synthetic turf fields. Estimating the exposure to these airborne VOCs is essential to evaluate the adverse health effects of the VOCs emitted from these rubber granule-based products. Our sampling method and results can help provide key data for such risk assessment studies in the future.

Keywords: GC-MS, monolithic material sorptive extraction, rubber granule

Mori Y[*1], Tanaka-Kagawa T[*2], Tahara M, Kawakami T, Aoki A[*1], Okamoto Y[*1], Isobe T[*2], Ohkawara S[*2], Hanioka N[*2], Azuma K[*3], Sakai S, Jinno H[*1]: Species differences in activation of TRPA1 by resin additive-related chemicals relevant to indoor air quality.

J Toxicol Sci. 2023;48(1):37-45. doi: 10.2131/jts.48.37

Transient Receptor Potential Ankyrin 1 (TRPA1), which is expressed in the airways, has causative and exacerbating roles in respiratory diseases. TRPA1 is known as a target of sick building syndrome-related air pollutants, such as formaldehyde. Thus, an *in vitro* TRPA1 activation assay would be useful for predicting the potential risk of air pollution. In this study, we used human TRPA1 (hTRPA1)- and mouse TRPA1 (mTRPA1)-expressing cell lines to measure TRPA1 activation by the emerging indoor air pollutants 2-ethyl-1-hexanol (2-EH), a mixture of 2,2,4-trimethyl-1,3-pentanediol 1- and 3-monoisobutyrate (Texanol), and 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB). The results indicated that 2-EH activated both hTRPA1 and mTRPA1 in a concentration-dependent manner, whereas TXIB did not activate hTRPA1 or mTRPA1. Texanol also activated hTRPA1 in a concentration-dependent manner. In contrast, a bell-shaped concentration-dependent curve was observed for mouse TRPA1 activation by Texanol, indicating inhibitory effects at a higher concentration range, which was also reported for menthol, a typical TRPA1 modulator. To further elucidate the mechanism underlying the species difference in TRPA1 activation by Texanol, V875G and G878V mutations were introduced into hTRPA1 and mTRPA1, respectively, which were reported to be key mutations for the inhibitory effect of menthol. These mutations switched the inhibitory effects of Texanol; thus, hTRPA1/V875G, but not mTRPA1/G878V, was inhibited at higher concentrations of Texanol. These results indicate that Texanol shares an interaction site with menthol. Overall, these findings suggest that careful interpretation is necessary when extrapolating rodent TRPA1-dependent toxicological effects to humans, especially with respect to the risk assessment of indoor air pollutants.

Keywords: Indoor air quality guideline, Nociceptive receptor, Transient Receptor Potential Ankyrin 1

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Hayazaki M[*1], Hanano O[*2], Shimabayashi S[*1], Akiyama T, Takemori H[*1], Hamamoto A[*1]: Zebrafish as a new model for rhododendrol-induced leukoderma.

Pigment Cell Melanoma Res 2021;34:1029-38. doi: 10.1111/pcmr.13005

Idiopathic leukoderma is a skin disorder characterized by patchy loss of skin pigmentation due to melanocyte dysfunction or deficiency. Rhododendrol (RD) was approved as a cosmetic ingredient in Japan in 2008. However, it was shown to induce leukoderma in approximately 20,000 customers. The prediction of cytotoxicity, especially to melanocytes *in vivo*, is required to avoid such adverse effects. Since the use of higher vertebrates is prohibited for medicinal and toxicological assays, we used zebrafish, whose melanocytes were regulated by mechanisms similar to mammals. Zebrafish larvae were treated with RD in breeding water for 3 days, which caused body lightening accompanied by a decrease in the number of melanophores. Interestingly, black particles were found at the bottom of culture dishes, suggesting that the melanophores peeled off from the body. In addition, RT-PCR analysis suggested that the mRNA levels of melanophore-specific genes were significantly low. An increase in the production of reactive oxygen species was found in larvae treated with RD. The treatments of the fish with other phenol compounds, which have been reported to cause leukoderma, also induced depigmentation and melanophore loss. These results suggest that zebrafish larvae could be used for the evaluation of leukoderma caused by chemicals, including RD.

Keywords: rhododendrol, vitiligo, zebrafish

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Maeda N[*1], Shimizu S[*1], Takahashi Y[*1,2], Kubota R, Uomoto S[*1], Takesue K[*1], Takashima K[*1,2], Okano H[*1,2], Ojiro R[*1,2], Ozawa S[*1,2], Tang Q[*1,2], Jin M[*3], Ikarashi Y, Yoshida T[*1,2], Shibutani M[*1,2,4]: Oral Exposure to Lead Acetate for 28 Days Reduces the Number of Neural Progenitor Cells but Increases the Number and Synaptic Plasticity of Newborn Granule Cells in Adult Hippocampal Neurogenesis of Young-Adult Rats.

Neurotox Res 2022; Dec;40(6):2203-20. doi: 10.1007/s12640-022-00577-5

Lead (Pb) causes developmental neurotoxicity. Developmental exposure to Pb acetate (PbAc) induces aberrant hippocampal neurogenesis by increasing or decreasing neural progenitor cell (NPC) subpopulations in the dentate gyrus (DG) of rats. To investigate whether hippocampal neurogenesis is similarly affected by PbAc exposure in a general toxicity study, 5-week-old Sprague-Dawley rats were orally administered PbAc at 0, 4000, and 8000 ppm (w/v) in drinking water for 28 days. After exposure to 4000 or 8000 ppm PbAc, Pb had accumulated in the brains. Neurogenesis was suppressed by 8000 ppm PbAc, which was related to decreased number of type-2b NPCs, although number of mature granule cells were increased by both PbAc doses. Gene expression in the 8000 ppm PbAc group suggested suppressed NPC proliferation and increased apoptosis resulting in suppressed neurogenesis. PbAc exposure increased numbers of metallothionein-I/II$^+$ cells and GFAP$^+$ astrocytes in the DG hilus, and upregulated *Mt1*, antioxidant genes (*Hmox1* and *Gsta5*), and *Il6* in the DG, suggesting the induction of oxidative stress and neuroinflammation related to Pb accumulation resulting in suppressed neurogenesis. PbAc at 8000 ppm also upregulated *Ntrk2* and increased the number of CALB2$^+$ interneurons, suggesting the activation of BDNF-TrkB signaling and CALB2+ interneuron-mediated signals to ameliorate suppressed neurogenesis resulting in increased number of newborn granule cells. PbAc at both doses increased the number of ARC$^+$ granule cells, suggesting the facilitation of synaptic plasticity of newborn granule cells through the activation of BDNF-TrkB signaling. These results suggest that PbAc exposure during the young-adult stage disrupted hippocampal neurogenesis, which had a different pattern from developmental exposure to PbAc. However, the induction of oxidative stress/neuroinflammation and activation of identical cellular signals occurred irrespective of the life stage at PbAc exposure.

Keywords: hippocampal neurogenesis, lead acetate, neuroinflammation

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Kubota R, Obama T, Kawakami T, Sakai S, Inoue K, Ikarashi Y: Characterization of synthetic turf rubber granule infill in Japan: Total content and migration of metals.
Sci Total Environ 2022;842:156705. doi: 10.1016/j.scitotenv.2022.156705

We evaluated the total content of 28 metals in synthetic turf rubber granule infill and performed extraction tests using four types of simulated biofluids to assess the health effects of synthetic turf crumb rubbers used in Japan. The highest median metal concentration was obtained for Zn, with median concentrations above 100 μg/g, followed by Al, Fe, and Mn. The highest median Pb concentration was 19.9 μg/g. The metal concentrations of the samples were different depending on the origin/material. Among high-concentration metals, Al, Fe, and Mn were higher in ethylene propylene diene monomer rubber, and Zn was higher in tires. Significantly higher Sb and Sr concentrations were observed in other materials, including industrial rubber, synthetic rubber, and thermoplastic elastomer, compared with tires. However, significantly higher Sn, Co, Pb, and Cd concentrations were detected in tires compared with other materials. Metals with high concentrations independent of the origin/material were considered derived from materials added during the manufacturing process. To evaluate the bioaccessibility, extraction tests were conducted using simulated biofluids. In gastric fluid, many metals were detected in higher concentrations than in other biofluids, intestinal fluid, saliva, and sweat, and the extraction rate of most metals exceeded 10% in artificial gastric fluid. Because the amount of metals leached into the simulated biofluids was much lower than several standards on the amount of certain metals that have the potential to be extracted from the object if ingested, the risk related to the exposure to metals from synthetic turf rubber granule infill is considered low.
Keywords: health risk, metals, rubber granule

飯島茂子*1，村山佳代*1，髙山典子*1，秋山卓美，杉山真理子*2,3，松永佳世子*2,3，コカミドプロピルベタイン含有洗浄剤によるアレルギー性接触皮膚炎の１例―洗浄剤に含まれる不純物が原因抗原と考えられた例―

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アレルギー 2022;71:1136-42. doi: 10.15036/arerugi.71.1136

コカミドプロピルベタイン（CAPB）は両性界面活性剤の１つで，起泡・洗浄などの作用を有し，シャンプー・ボディソープなどの多数の製品に含有されている．CAPBによるアレルギー性接触皮膚炎（ACD）は，近年その中に微量に混入する不純物が感作物質であると考えられている．今回，CAPBによるACDを経験し，不純物を含めたパッチテストを施行した．症例は64歳女性．初診の１カ月前から，額と毛の生え際に発疹が出現し，その後，顔面，頸部，背部，胸部に拡大した．パッチテストにて持参のシャンプー・ボディソープ1%水溶液（aq.）およびその成分CAPB 1% aq.，ラウラミドプロピルベタイン（LAPB）1% aq.に陽性，さらに不純物であるラウラミドプロピルジメチルアミン（LAPDMA）0.05% aq.に陽性であった．以上より自験例をシャンプー・ボディソープによるACD，感作物質はLAPDMAの可能性を強く考えた．これらの界面活性剤を含有しない製品に変更後，皮疹は急速に改善した．界面活性剤には化粧品成分名称と医薬部外品成分名称が異なるものがあるので，十分な知識が必要である．
Keywords: allergic contact dermatitis, cocamidopropyl betaine, dimethylaminopropylamine

Kobayashi N, Takagi S*1, Kinoshita T*2, Sakata O*3, Nakano F*4, Watanabe N*5, Nomura A*6, Kawai N*7, Hiraiwa T*8, Okumura M*9, Furukawa K*10, Kasuya T*11, Iwama N*12, Yonekubo J*13, Takahara R*14, Tanaka S*15, Tsuchiya Y, Ikarashi Y: Development and validation of an analytical method for simultaneous determination of perfluoroalkyl acids in drinking water by liquid chromatography/tandem mass spectrometry.

Journal of Water and Environment Technology
2022;20(6):219-37. doi: 10.2965/jwet.22-058

The environmental presence and drinking water contamination of per- and polyfluoroalkyl substances (PFAS) have been reported since the early 2000s. This study seeks to develop a liquid chromatography/tandem mass spectrometry analytical method for the simultaneous determination of 21 perfluoroalkyl acids (PFAAs) in drinking water to support future regulations in Japan. Inter-laboratory tests were conducted in 16 laboratories using different instrument to verify the applicability of the developed method for a wide range of drinking water samples in Japan. Recovery tests of PFAA-fortified tap water samples obtained in each laboratory were conducted at set points of 1 and 10 ng/L. Calibration curve linearity, trueness (recovery), repeatability, and reproducibility at these analyte concentrations were calculated using data obtained from the recovery tests. The trueness, repeatability (RSD_r), and reproducibility (RSD_R) of most PFAA analytes were satisfactory when the recoveries were corrected by ^{13}C-PFAA extraction standards with similar recovery to the corresponding PFAS analytes. The developed analytical method is valid for the quantification of the target PFAAs in drinking water. However, satisfactory PFAA quantification requires recovery adjustment using surrogates with similar recovery characteristics to the PFAA analytes.

Keywords: per- and polyfluoroalkyl substances (PFAS), drinking water, LC/MS/MS

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小林憲弘，土屋裕子，高木総吉*1，吉田仁*1，大窪かおり*2，北原健一*2，坂本晃子*2，木下輝昭*3，仲野富美*4，橋本博之*5，古川浩司*6，粕谷智浩*7，岩間紀知*8，平林達也*9，小嶋隼*10，林幸範*11，古口健太郎*12，五十嵐良明：水道水中農薬のGC/MSターゲットスクリーニング分析のデータ解析における誤差要因の分析．

環境化学 2023;33:26-40. doi: 10.5985/jec.33.26

水道水質検査において，検査時に標準品を測定せずにデータベースの登録情報を基に定性・定量を行う「スクリーニング分析」を実運用するためには，様々な機関や検査員が解析しても，一致した分析結果が得られる必要がある．本研究では，クロマトグラムのデータ解析者による定性・定量結果の違いおよびその要因を明らかにするため，16人の解析者が，同一のデータベース，ソフトウェア，試料クロマトグラムを用いたデータ解析のバリデーション試験を実施した．解析者により検出農薬に大きな違いが見られ，その原因は主に２つあった．１つは定量下限付近の低濃度ではピーク検出・不検出の判断が解析者によって異なることが多く，特に定量下限の３倍未満の濃度では解析者の判断が分かれたためであった．もう１つは解析者によっては明らかな誤同定があり，定量・定性イオンの存在比（QT比）やマススペクトルのデータベースとの一致度が低いにも関わらず，予想保持時間の近くのピークを同定したり，分解物・代謝物等のピークを誤同定した解析者もいた．水質検査機関においてスクリーニング分析を実運用するためには，本研究で実施したバリデーション試験の方法を参考として，実試料のデータ解析において実用的な定量下限の設定や，解析者間でのピーク同定・定量方法の擦り合わせを行うことが重要である．

Keywords：農薬，スクリーニング分析，GC/MS

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Kawakami T, Miyajima A, Komoriya K, Kato R,

Isama K: Effect of secondary particle size of nickel oxide nanoparticles on cytotoxicity in A549 cells.
J Toxicol Sci 2022;47:151-7. doi: 10.2131/jts.47.151

The effect of nanoparticle type, shape, as well as primary and secondary particle size on toxicity remains poorly characterized. In this study, suspensions of nickel oxide (NiO) nanoparticles with the same primary particle size (< 50 nm) but different secondary particle sizes were prepared, and their cytotoxicity was investigated. A planetary ball mill wet nanopulverizer with zirconium milling balls of decreasing sizes (ϕ: 0.5, 0.1, and 0.05 mm) yielded NiO nanoparticles of decreasing mean particle size (310.4 ± 6.7, 172.0 ± 2.8, and 102.0 ± 0.5 nm). Stock solutions were diluted to various concentrations in 10% heat-inactivated fetal bovine serum containing minimum essential medium, and shown to have the same primary particle size, but different secondary particle sizes. Tests with A549 cells revealed that cytotoxicity increased with increasing secondary particle size: milling ball diameter ϕ 0.05 mm (IC_{50}: 148 μg/mL) < ϕ 0.1 mm (IC_{50}: 83.5 μg/mL) < ϕ 0.5 mm (IC_{50}: 33.4 μg/mL). Uptake experiments indicated that the intracellular amount of Ni increased with increasing secondary particle size. In summary, the present findings show that differences in secondary particle size affected the cytotoxicity of NiO suspensions, which could be ascribed at least in part to differences in the amount of NiO taken up by the cells.
Keywords: NiO nanoparticles, secondary particle, cytotoxicity

Yamamoto Y[*1], Fujita M[*1], Watanabe S[*2], Yamaga H[*2], Wakabayashi K[*3], Tahara Y[*3], Horie N[*4], Fujimoto K[*4], Takeuchi K[*5], Kamiya K[*5], Kawakami T, Kojima K[*6], Sozu T[*7], Kojima H, Kasahara T[*1], Ono A[*8]: Within- and between-laboratory reproducibility and predictive capacity of amino acid derivative reactivity assay (ADRA) using a 0.5 mg/ml test chemical solution: Results of the study for reproducibility confirmation implemented in five participating laboratories.
J Appl Toxicol 2022;42:1078-90. doi: 10.1002/jat.4279

The amino acid derivative reactivity assay (ADRA) is an *in chemico* alternative assay for skin sensitization listed in OECD test guideline 442C. ADRA evaluates the reactivity of sensitizers to proteins, which is key event 1 in the skin sensitization adverse outcome pathway. Although the current key event 1 evaluation method is a simple assay that evaluates nucleophile and test chemical reactivity, mixtures of unknown molecular weights cannot be evaluated because a constant molar ratio between the nucleophile and test chemical is necessary. In addition, because the nucleophile is quantified by HPLC, the frequency of co-eluting the test chemical and nucleophile increases when measuring multi-component mixtures. To solve these issues, test conditions have been developed using a 0.5 mg/mL test chemical solution and fluorescence-based detection. Since the practicality of these methods has not been substantiated, a validation test to confirm reproducibility was conducted in this study. The 10 proficiency substances listed in the ADRA guidelines were tested three times at five different laboratories. The results of both within- and between-laboratory reproducibility were 100%, and the results of ultraviolet- and fluorescence-based measurements were also consistent. In addition to the proficiency substances, a new positive control, squaric acid diethyl ester, was tested three times at the five laboratories. The results showed high reproducibility with *N*-(2-(1-naphthyl)acetyl)-L-cysteine depletion of 37%-52% and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine depletion of 99%-100%.Thus, high reproducibility was confirmed in both evaluations of the 0.5 mg/mL test chemical and the fluorescence-based measurements, validating the practicability of these methods.
Keywords: ADRA (0.5 mg/mL), amino acid derivative reactivity assay, between-laboratory reproducibility

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Kawakami T, Obama T, Sakai S, Takagi M, Takahashi N, Oshima N, Tahara M, Ikarashi Y: Free formaldehyde in non-medical face masks purchased

from Japanese market since the COVID-19 outbreak. *J Environ Sci Health Part A* 2022;57:193-7. doi: 10.1080/10934529.2022.2047560

Since the Coronavirus Disease 2019 (COVID-19) pandemic began, people have been wearing face masks for many hours every day. As these face masks are in contact with the skin, it is important to pay more attention to their quality and safety. This study examined the concentration of free formaldehyde in 90 non-medical face masks and related products (33 nonwoven, 30 woven cloth, 12 polyurethane, and 15 related products) because formaldehyde is a common contact allergen in textile products. For products consisting of mixed materials, each material was sampled, resulting in 103 samples for analysis. Free formaldehyde (34-239 μg/g) was found in three cloth masks, which consisted of cotton and polyester, with antibacterial and antiviral labelling. It was confirmed that the detected formaldehyde originated from the mask-finishing treatment by a hydrochloric acid extraction discrimination test. These masks may elicit contact dermatitis if the consumers have already been sensitized to formaldehyde. However, the risk of contact dermatitis caused by formaldehyde in masks may be considered low since the frequency of formaldehyde detection in masks in Japan is low.

Keywords: mask, formaldehyde, allergic contact dermatitis

Seo T*, Miyauchi T*, Kawakami T, Ujiie H*: Human figure-shaped contact dermatitis due to the illustration on the inner surface of compression sleeves.

J Dermatol 2022;49:e241-2. doi: 10.1111/1346-8138.16363

Venous thromboprophylaxis is a fundamental procedure in perioperative management that is conducted worldwide. An intermittent pneumatic compression device that uses compression sleeves. Here, we report a case of contact dermatitis in which the illustration on the inner surface of the sleeves was clearly imprinted on the skin. The manufacture disclosed that the illustration contains hydrogenated colophony, there was no information about other ingredients. To identify the other components, we performed chemical analysis using gas and liquid chromatograph mass spectrometer using a piece of the sleeve, and we detected diisononyl phthalate and Solvent Blue 104, in addition to the colophony-related compounds from the illustration. Colophony-related compounds included methyl dihydroabietate, dihydroabietic acid and dehydroabietic acid. We made the final diagnosis of allergic contact dermatitis caused by colophony contained in the illustration. This illustration is printed as a safety precaution to ensure that the device is not misused. In practice, stockings or cylindrical bandages are often worn inside the sleeve, which may have prevented this issue in many cases, but the problem is that usage varies from hospital to hospital. In response to this case and a similar case, a specification change to move the illustration to the outer surface was conducted worldwide in 2021. We believe that our case will be helpful in preventing similar cases in the future and will improve important medical devices.

Keywords: intermittent pneumatic compression device, colophony, contact dermatitis

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Kawakami T, Sakai S, Obama T, Kubota R, Inoue K, Ikarashi Y: Characterization of synthetic turf rubber granule infill in Japan: Rubber additives and related compounds.

Sci Total Environ 2022;840:156716. doi: 10.1016/j.scitotenv.2022.156716

We have conducted several studies with an overall goal of assessing the effects of rubber granules in synthetic turf on the health of athletes, other players, and children in Japan. As part of these studies, the investigation reported herein was aimed at analyzing the concentrations of rubber additives (vulcanization accelerators, antioxidants, and cross-linking agents) and related chemicals in 46 rubber infills prior to their use in synthetic turf fields in Japan. Of the 36 chemicals selected for targeted analysis, 26 were detected and quantified. Nontargeted analyses further identified and quantified 16 compounds derived from vulcanization accelerators, plasticizers, and other additives. The types and concentrations of the detected compounds varied both between products and within the same product; in the case of rubber infill products made from recycled rubber, this

variation was caused by the different types of rubber products recycled as raw materials. Elution tests with four simulated biofluids (gastric juice, intestinal juice, saliva, and perspiration) revealed that the elution rates varied between compounds and were affected by the presence of coatings. Most compounds had low elution rates in all the simulated biofluids, with many at or below the limit of quantification. The data reported herein will be utilized in the risk characterization part of our subsequent study on the health risk assessment of rubber infill.

Keywords: synthetic turf, rubber additive, elution test

Nishi I*, Kawakami T, Sakai S, Obama T, Kubota R, Inoue K, Ikarashi Y: Characterization of synthetic turf rubber granule infill in Japan: Polyaromatic hydrocarbons and related compounds.

Sci Total Environ 2022;842:156684. doi: 10.1016/j.scitotenv.2022.156684

Although the health effects of artificial turf fillings have been investigated in Europe and the United States, the actual situation in Japan is unclear. To address this issue, the concentrations of 46 polyaromatic hydrocarbons (PAHs) and related compounds in rubber infills were analyzed prior to their use in synthetic turf fields in Japan. Based on information obtained from the sample suppliers, the investigated samples were divided into five categories: discarded tires, industrial rubber, combinations of these products or unidentified components (mixture/unknown), synthetic rubber specifically manufactured for synthetic turf, and special-purpose thermoplastic elastomers (TPEs). The industrial rubber samples were mixtures of styrene butadiene rubber, natural rubber, and ethylene propylene diene rubber (EPDM). The synthetic rubber samples consisted only of EPDM. A few or none of the PAHs were detected in the synthetic rubber and TPE samples. However, in the discarded tire and industrial rubber samples, benzo[*a*]pyrene, cyclopenta[*cd*]pyrene, and 30 other compounds were detected. A comparison between these two categories indicated that the discarded tire samples exhibited higher concentrations of the target compounds than the industrial rubber samples. This finding can be attributed to the presence of EPDM in almost all of the industrial rubber samples, which were not present in the discarded tire samples. The maximum PAH concentrations obtained in the present study were equivalent to or lower than the previously reported PAH concentrations. The total concentrations of the eight PAHs included in the European Chemical Agency (ECHA) assessment of health risks were lower in the present study than those reported by the ECHA. Furthermore, elution testing was performed with four simulated biofluids (gastric and intestinal juices, saliva, and perspiration). The actual elution amounts of all compounds were less than the limits. This report provides basic data for the risk assessment of PAHs in rubber infills.

Keywords: synthetic turf, PAH, elution test

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Kawakami T, Tahara M, Ikarashi Y: A rosin-related compounds (abietic acid derivatives) concentrations in chloroprene rubber products and the amounts eluted into artificial sweat.

Dermatitis 2022;33:e47-8. doi: 10.1097/DER.0000000000000704

Rosin-related compounds are well-known contact allergens, however, few studies of these compounds in chloroprene rubber products have been reported. The objective was to elicit the concentrations of rosin-related compounds (abietic acid derivatives) in chloroprene rubber products, and the amounts eluted into artificial sweat. Seven rosin-related compounds were analyzed in 15 chloroprene rubber products, and elution tests using artificial acidic (pH 5.5) and alkaline sweat (pH 8.0) were also conducted. Five kinds of abietic acid derivatives (abietic acid, dehydroabietic acid, dihydroabietic acid, isopimaric acid, and dehydroabietic acid methyl ester) were detected (5.8 to 17,000 μg/g) in the products. These compounds, excluding dehydroabietic acid methyl ester, showed confirmed elution into artificial sweat, and the eluted amounts in alkaline sweat (0.34 to 1,400 μg/g) were higher than in acidic sweat (0.49 to 34 μg/g) because of their water solubilities based on their pKa values (4.71 to 4.74). The products investigated in this study may be used in close contact with the skin for a long time, and some products are intended for sweating. If allergic contact dermatitis due to chloroprene rubber products is confirmed, it should be considered to conduct patch test in rosin-related compounds.

Keywords: rosin, chloroprene rubber, elution test

Tahara M, Kawakami T, Sakai S, Ikarashi Y: Survey of phthalates, glycols, and several volatile organic compounds in domestic hand-pump spray products and evaluation of their effect on indoor air quality.
J Environ Chem 2022;32:84-94. doi: 10.5985/jec.32.84

The Committee on Sick House Syndrome: Indoor Air Pollution (Ministry of Health, Labour and Welfare, Japan) recommended reviewing guideline values for harmful compounds for the purpose of risk evaluation. In this study we selected domestic hand-pump spray products and focused on compounds listed as candidates for revision of guideline values and as new additions to the guideline value list. We also examined appropriate analytical methods, surveyed the product states; and after using specific products, calculated the average indoor concentrations of the detected compounds and considered their effect on indoor air quality. A total of 33 compounds [9 phthalates, 20 glycols, and 4 volatile organic compounds (VOCs)] were analyzed in 33 products. Four phthalates (0.47 to 9.8 mg/L) were detected in six products, fifteen glycols (0.46 to 3,200 mg/L) in thirty-two products, and two VOCs (0.51 to 10 mg/L) in eight products, respectively. Estimation of the average concentrations of the detected compounds in indoor air after product use showed that phthalates and VOCs were within the recommended threshold values for indoor air, indicating that those products had a low probability of being a source of indoor air pollution. However, comparatively high concentrations of glycols, diethylene glycol monomethyl ether, and diethylene glycol monoethyl ether were measured, suggesting that the use of products containing these ingredients could markedly affect indoor air quality. The findings showed that domestic hand-pump spray products may act as a significant source of glycol emissions.
Keywords: indoor air quality, domestic spray product, phthalate and glycol

Fujimoto K[*1], Higaki T[*1], Abe J[*1], Fujita M[*2], Kawakami T: Confirmation of the theoretical validity of *in chemico* skin sensitization assay "ADRA" by the analysis of products formed by nucleophilic reagents and chemicals.
Chem Res Toxicol 2022;35:2107-21. doi: 10.1021/acs.chemrestox.2c00228

Amino acid derivative reactivity assay (ADRA) is an *in chemico* assay for assessing the skin sensitization potential of chemicals by evaluating the reactivity of nucleophilic reagents that mimic skin proteins. *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (NAL), used as nucleophilic reagents, are small-molecule derivatives of two different amino acids, each with a naphthalene ring attached. The rate of decrease in the amount of NAC or NAL in the reaction solution is evaluated in this assay as an indicator of the test substance's skin sensitization ability. However, the products formed between the nucleophilic reagent and the test substance, which play an important role *in vivo*, are not directly identified. Therefore, six highly reactive chemicals, including the proficiency substances listed in the OECD test guidelines—squaric acid diethyl ester, 2-methyl-2*H*-isothiazol-3-one (MI), *p*-benzoquinone, palmitoyl chloride, diphenylcyclopropenone (DPCP), and imidazolidinyl urea (IU)—were used to determine each formed product. Samples were prepared according to the standard ADRA method, and the formed products were predicted on the basis of the reaction mechanism. Excluding DPCP, the estimated structures were validated using mass spectrometry and nuclear magnetic resonance spectrometry on the synthesized samples. In this manner, the products of each nucleophile were confirmed for all examined test substances. The estimated structure products were obtained through a series of reactions initiated by the nucleophilic attack of NAC's thiol group or NAL's amino group on the test substance's electron-deficient carbonyl carbon. However, contrary to expectations, disulfide-linked-type ring-opened products were detected in the case of MI, and products with free formaldehyde in solution were detected in the case of IU. In summary, all skin sensitizers tested herein reacted with NAC and/or NAL to give products. This supports the theoretical validity of ADRA, which provides an indirect evaluation of the formed products based on a decrease in nucleophilic reagents.
Keywords: amino acid derivative reactivity assay, nucleophilic reagent, theoretical validity

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Kawakami T, Obama T, Tahara M, Ikarashi Y: Determination of carcinogenic primary aromatic amines contained as impurity in synthetic organic coloring agents.

J AOAC Int 2023;106:49-55. doi: 10.1093/jaoacint/qsac095

Several primary aromatic amines (PAAs) have been designated carcinogenic or suspected of carcinogenicity. Several kinds of PAAs may occur either via the reduction of azo compounds or as impurities in azo colorants or other agents. Analytical method was developed and applied to determine whether certain PAAs are present as impurities in synthetic organic colorants. The developed method utilizes the ultrasound extraction of the synthetic organic colorant with a hydrochloric acid solution containing 20% methanol, followed by conversion from an acidic to alkaline solution, and then extraction using a diatomaceous earth column. Using this method, we analyzed certain PAAs in 38 synthetic organic colorants, resulting in the detection of 2,4-dimethylaniline in 4 samples at 1.2 to 19 μg/g, *o*-toluidine in 3 samples at 1.0 to 3.4 μg/g, *p*-phenylazoaniline in 2 samples at 74 to 305 μg/g, and, in one sample each, 2,4,5-trimethylaniline (13 μg/g), 5-nitro-*o*-toluidine (12 μg/g), and 2-methyl-4-(2-tolylazo)aniline (13 μg/g). Nearly all PAAs were determined to be starting materials for colorant synthesis, although *p*-phenylazoaniline in Yellow No. 407 was apparently a byproduct formed during synthesis. For Red No. 225, in which high concentrations of *p*-phenylazoaniline were detected, additional samples were purchased from five companies, and *p*-phenylazoaniline was detected at concentrations of 88 to 370 μg/g in all samples. A method to analyze certain PAAs contained as impurities in synthetic organic colorants was developed, and the actual status of them in colorants was clarified.

Keywords: primary aromatic amine, synthetic organic colorant, impurity

Kawakami T, Tahara M, Ikarashi Y: Analysis of isothiazolinone and paraben preservatives in children's toy slime in Japan.

Contact dermatitis 2023;88:80-2. doi: 10.1111/cod.14229

Many cases of contact dermatitis induced by preservatives in children's toy slime have been reported in recent years. However, there are very few reports regarding the concentration of preservatives in slime toys. The aim of this study was to determine the concentrations of seven isothiazolinone and seven paraben preservatives in 10 slimes. Liquid chromatography tandem mass spectrometry was used to measure the preservatives in slimes. 2-methyl-4-isothiazolin-3-one (MI), 5-chloro-2-methyl-4-isothiazolin-3-one, and benzisothiazolin-3-one, were detected in six products (0.69-45 μg/g), four products (1.3-3.2 μg/g), and one product (35 μg/g), respectively. Methylparaben and propylparaben, were detected in six products (100-2,200 μg/g) and one product (360 μg/g), respectively. Although the concentrations of the detected isothiazolinone and paraben preservatives in slimes were lower than the limits allowed in cosmetics, allergic contact dermatitis induced by products containing similar concentrations of MI has been reported, so care is necessary in cases of previous sensitization to the products containing these compounds. Slime manufacturers should refrain from including isothiazolinone preservatives and customers (e.g., parents) should encourage children to perform careful handwashing by children after the use of these products in order to prevent prolonged contact of slime-component residues with the skin.

Keywords: allergic contact dermatitis, slime, isothiazolinone and paraben preservative

Sugaya N*, Inoue K, Tahara M, Kawakami T: Analysis and risk assessment of vinyl chloride emitted from aerosol products.

J Environ Sci Health Part A, 2023;58,284-94. doi: 10.1080/10934529.2023.2173925

The objectives of this study were to develop a novel analytical method for quantifying vinyl chloride (VC) emitted from aerosol products, to provide analytical data on VC in aerosol products, and to evaluate consumer VC exposure by aerosol products. Our quantitative method involves absorbing VC into dimethyl sulfoxide and analyzing it using headspace gas chromatography/mass spectrometry. The correlation coefficients of the VC calibration curves were ≥ 0.9994 in the range of 0.16-80 μg/mL VC

standard gases, which were prepared under either nitrogen or emission gases containing dimethyl ether or liquid petroleum gas. VC concentrations in these emission gases were calculated using a VC calibration curve from standard gases prepared under nitrogen; they were within ± 10% of the actual concentrations. We analyzed 39 household aerosol products; VC concentrations of 0.095, 0.098, and 0.28 µg/L were detected in three polyvinyl chloride spray paints. Consumer VC inhalation exposure level was estimated through an exposure scenario, and the hazard quotient was confirmed to be very low when comparing the exposure level with a cancer risk level of 10^{-5} for inhaled VC. These results suggest that the human health risk from VC in spray paint was low.

Keywords: vinyl chloride, inhalation exposure, headspace gas chromatography/mass spectrometry

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竹脇優太郎*，岡部亮*，根本了，青柳光敏*：LC-MS/MSによる畜産物中のキンクロラックの分析法.
食品衛生学雑誌，2022;63(5):177-181. doi: https://doi.org/10.3358/shokueishi.63.177

A liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method was developed for determining quinclorac in livestock products. Quinclorac was extracted from the samples using a solution of acetone and hydrochloric acid mixed in a 99 : 1 ratio. The crude extract was purified with ethyl acetate under basic conditions, followed by quinclorac extraction with ethyl acetate under acidic conditions and analysis using LC-MS/MS. The average recoveries of quinclorac from five livestock products (n = 5) fortified at the maximum residue limits or 0.01 mg/kg ranged from 85.6 to 93.5%, with the precision of repeatability ranging from 1.7 to 6.8%. The quantification limit in this analytical method was 0.01 mg/kg. These results suggest that the developed method is useful for analyzing quinclorac in livestock products.

Keywords: quinclorac, livestock products, LC-MS/MS

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小林麻紀*，酒井奈穂子*，大町勇貴*，森田有香*，根本了，大塚健治*：LC-MS/MSによる畜産物中のクロロタロニル代謝物I分析法.
食品衛生学雑誌，2022;63(6): 195-201. doi: https://doi.org/10.3358/shokueishi.63.195

An analytical method based on LC-MS/MS was developed for the determination of chlorothalonil metabolite I in livestock products. Chlorothalonil metabolite I in livestock products was extracted with acetone. The crude extracts were defatted by acetonitrile and *n*-hexane partitioning. Cleanup was carried out using a combination of ethylene diamine-*N*-propyl silylation silica gel (PSA) and silica gel (SI) mini columns with acidic condition. The sample solution was subjected to LCMS/MS using an external solvent calibration curve. The average recovery (n = 5) of chlorothalonil metabolite I from five types of livestock products (cattle muscle, cattle fat, cattle liver, milk and egg) spike at the maximum residue limits (MRLs) or at a uniform limit of 0.01 mg/kg was 97.1-102.9%, with a relative standard deviation of 1.4-6.8%. The limit of quantitation of the developed method was calculated to be 0.01 mg/kg.

Keywords: chlorothalonil metabolite I, livestock products, LC-MS/MS

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Ishikawa K*[1], Hashimoto M*[1], Komatsu K*[1], Taguchi T, Okamoto S*[2], Ichinose K*[1]: Characterization of stereospecific enoyl reductase ActVI-ORF2 for pyran ring formation in the actinorhodin biosynthesis of Streptomyces coelicolor A3(2).
Bioorg. Med. Chem. Lett. 2022;66;128727. doi: 10.1016/j.bmcl.2022.128727

Actinorhodin (ACT) is a benzoisochromanequinone antibiotic produced by *Streptomyces coelicolor* A3(2), which has served as a favored model organism for comprehensive studies of antibiotic biosynthesis and its regulation. (*S*)-DNPA undergoes various modifications as an intermediate in the ACT biosynthetic pathway, including enoyl reduction to DDHK. It has been suggested that *act*VI-ORF2 encodes an enoyl reductase (ER). However, its function has not been characterized *in vitro*. In this study, biochemical analysis of recombinant ActVI-ORF2 revealed that (*S*)-DNPA is converted to DDHK in a stereospecific manner with NADPH acting as a cofactor. (*R*)-DNPA

was also reduced to 3-*epi*-DDHK with the comparable efficacy as (*S*)-DNPA, suggesting that the stereospecificity of ActVI-ORF2 was not affected by the stereochemistry at the C-3 of DNPA. ActVI-ORF2 is a new example of a discrete ER, which is distantly related to known ERs according to phylogenetic analysis.

Keywords: actinorhodin, *Streptomyces coelicolor*, enoyl reductase

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田口貴章，難波樹音，山下涼香，岸美紀*1，赤星千絵*1，岡部信彦*1，穐山浩*2：食品テロ対策のためのLC-MS/MSによる血液・尿等人体試料中のカーバメート系農薬の一斉分析法の検討.

日本食品化学学会誌 2022;29;77-84. doi: 10.18891/jjfcs.29.2_77

Anti-food-terrorism measures are critical for identifying toxic substances and rescuing victims of food terrorism. A rapid analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was examined to quantify 17 carbamate pesticides in human blood or urine samples. Blood or urine samples were extracted using methanol or acetone and subjected to reversed-phase LC-MS/MS. Sample preparation and LC-MS/MS analysis required approximately 25 and 20 min, respectively. The recoveries of 16 carbamates from blood and urine samples spiked with 50 ng/mL of each pesticide ranged between 13.4% and 164.1% (92.1% and 200.0%) and between 39.0% and 119.5% (36.4% and 112.1%), respectively, when methanol and acetone were used as extractants. Thiodicarb could not be recovered from the blood samples, suggesting that it was enzymatically converted into methomyl. The analytical method used in this study is simple and useful; therefore, it can be used by public health institutions as an anti-food-terrorism measure.

Keywords：食品テロ対策，血液試料，カーバメート系農薬

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Hashimoto M*1, Watari S*1, Taguchi T, Ishikawa K*1, Kumamoto T*2, Okamoto S*3, Ichinose K*1: Actinorhodin Biosynthesis Terminates with an Unprecedented Biaryl Coupling Reaction.

Angew. Chem. Int. Ed. 2023;135;e2022144. doi: 10.1002/ange.202214400

A plethora of dimeric natural products exist with diverse chemical structures and biological activities. A major strategy for dimerization is aryl coupling catalyzed by cytochrome P450 or laccase. Actinorhodin (ACT) from *Streptomyces coelicolor* A3(2) has a dimeric pyranonaphthoquinone structure connected by a C−C bond. In this study, we identified an NmrA-family dimerizing enzyme, ActVA-ORF4, and a cofactor-independent oxidase, ActVA-ORF3, both involved in the last step of ACT biosynthesis. ActVA-ORF4 is a unique NAD(P)H-dependent enzyme that catalyzes the intermolecular C−C bond formation using 8-hydroxydihydrokalafungin (DHK-OH) as the sole substrate. On the other hand, ActVA-ORF3 was found to be a quinone-forming enzyme that produces the coupling substrate, DHK-OH and the final product, ACT. Consequently, the functional assignment of all essential enzymes in the biosynthesis of ACT, one of the best-known model natural products, has been completed.

Keywords: actinorhodin, biaryl Coupling, dimerizing enzyme

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Kikuchi H, Takahashi M, Komatsu H*, Axelsen PH*: Post-Translational Chemical Modification of Amyloid-β Peptides by 4-Hydroxy-2-Nonenal.

J. Alzheimer's Dis. 2022;92:499-511. doi: 10.3233/JAD-220940

Background: The extraction and quantification of amyloid-β (Aβ) peptides in brain tissue commonly uses formic acid (FA) to disaggregate Aβ fibrils. However, it is not clear whether FA can disaggregate post-translationally modified Aβ peptides, or whether it induces artifact by covalent modification during disaggregation. Of particular interest are Aβ peptides that have been covalently modified by 4-hydroxy-2-nonenal (HNE), an oxidative lipid degradation product produced in the vicinity of amyloid plaques that

dramatically accelerates the aggregation of Aβ peptides.

Objective: Test the ability of FA to disaggregate Aβ peptides modified by HNE and to induce covalent artifacts.

Methods: Quantitative liquid-chromatography-tandem-mass spectrometry of monomeric Aβ peptides and identify covalently modified forms.

Results: FA disaggregated ordinary Aβ fibrils but also induced the time-dependent formylation of at least 2 residue side chains in Aβ peptides, as well as oxidation of its methionine side chain. FA was unable to disaggregate Aβ peptides that had been covalently modified by HNE.

Conclusion: The inability of FA to disaggregate Aβ peptides modified by HNE prevents FA-based approaches from quantifying a pool of HNE-modified Aβ peptides in brain tissue that may have pathological significance.

Keywords: amyloid-β (Aβ), 4-hydroxy-2-nonenal (HNE), LC-MS/MS

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菊地博之，坂井隆敏，大倉知子，根本了，穐山浩*，田口貴章，堤智昭：LC-MS/MSによる畜産物中のモエノマイシンAの分析法.

食品衛生学雑誌，2023;64:61-8. doi: https://doi.org/10.3358/shokueishi.64.61

LC-MS/MSを用いて畜産物中のモエノマイシンAの分析法を検討した．フラボフォスフォリポールの規制対象であるモエノマイシンAを，畜産物から効率的に抽出することが可能な溶媒の種類および温度を検討したところ，50℃に加温したアンモニア水およびメタノール（1：9，v/v）混液を用いることで検討した全ての試料から十分な回収率を得ることが可能であった．さらに，得られた抽出液を濃縮後に塩基性として，酢酸エチルで洗浄した後，トリメチルアミノプロピルシリル化シリカゲルミニカラムで精製してLC-MS/MSで定量および確認する方法を開発した．開発した分析法を用いて，豚の筋肉，豚の脂肪，豚の肝臓および鶏卵の4食品に対して，基準値濃度（0.05 mg/kg）および定量限界濃度（0.01 mg/kg）で添加回収試験を行ったところ，真度および併行精度（RSD%）は，それぞれ79～93%および0.5～2.8%と良好であった．また，各食品におけるマトリックス添加標準溶液の溶媒標準溶液に対するピーク面積比は0.81～0.98であったことから，本法は試料由来のマトリックスの影響を大きく受けることなく測定することが可能と考えられた．以上のことから，開発した分析法は，畜産物中のフラボフォスフォリポールを基準値濃度および定量限界濃度で精度良く定量することが可能と考えられた.

Keywords：モエノマイシンA，畜産物，LC-MS/MS

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Nabeshi H, Hachisuka A, Matsuda R, Teshima R, Akiyama H, Tsutsumi T: Estimation of dietary intake of 90Sr in Japan after the Fukushima Daiichi Nuclear Power Plant accident: market basket study, 2013-2018.

Food Addit Contam Part A 2022;39(12):1974-1986. doi: https://doi.org/10.1080/19440049.2022.2129099

Radionuclide contamination in foods has been a public concern in Japan after the Fukushima Daiichi Nuclear Power Plant (FDNPP) accident. To estimate time and regional trends of daily intake and annual committed effective dose of strontium-90 (^{90}Sr) after the accident, we analysed Market basket samples using a low background 2π gas-flow counter. Samples were collected from six regions, once a year from 2013 to 2018. There appeared to be little variation in estimated daily intake and annual committed effective dose of ^{90}Sr across the time periods and regions. The estimated maximum annual committed effective dose of ^{90}Sr was 0.00076 mSv/year, a value sufficiently lower than the intervention exemption level, 1 mSv/year, in foods in Japan. There was no noticeable difference between the range of estimated daily intake of ^{90}Sr in this study compared with daily intake measured before the FDNPP accident. These results suggested that no obvious increase in dietary intake of ^{90}Sr was observed after the FDNPP accident, and that the effects on commercial foods from ^{90}Sr due to the FDNPP accident were negligible.

Keywords: Fukushima Daiichi Nuclear Power Plant Accident, strontium-90, dietary intake

Saito-Shida S, Saito M, Nemoto S, Tsutsumi T: GC-MS/MS method for determining quizalofop ethyl, quizalofop tefuryl, and their metabolites in foods.

J. Food Compost. Anal. 2023;115: 105011. doi: https://doi.org/10.1016/j.jfca.2022.105011

A gas chromatography-tandem mass spectrometry (GC-MS/MS) method was developed for the quantitative analyses of quizalofop ethyl and quizalofop tefuryl herbicides as well as their metabolites (i.e., quizalofop and its conjugates). This method fulfills the residue definition established by Japan. Following sample reflux in a methanolic potassium hydroxide solution, the residues were converted to 6-chloro-2-methoxyquinoxaline (CMQ) and extracted with hexane. Cleanup was carried out using primary secondary amine and silica gel cartridges prior to GC-MS/MS analysis. The developed method was validated for quizalofop ethyl, quizalofop tefuryl, and quizalofop in six foods at 0.01 mg/kg and at their maximum residue limits set by Japan. A satisfactory analytical performance was achieved (trueness = 80-93%, relative standard deviation = 1-7%, limit of detection = 0.00025 mg/kg for all analytes), and no significant matrix effects were observed for the examined matrices. There were no interfering peaks near the retention time of CMQ, indicating the high selectivity of this method. A satisfactory recovery was also achieved for propaquizafop in soybeans, indicating that the proposed method also complies with the residue definition established by the European Union. This method is suitable for the regulatory analysis of quizalofop ethyl and quizalofop tefuryl residues.

Keywords: quizalofop ethyl, quizalofop tefuryl, GC-MS/MS

Suzuki Y, Kondo M, Akiyama H[*1], Ogra Y[*2]: Presence of nano-sized mercury-containing particles in seafoods, and an estimate of dietary exposure.
Environ. Pollut. 2022;307:119555. doi: 10.1016/j.envpol.2022.119555

The toxicity of nano-sized particles of mercury (NP-Hg), which are thought to be generated during the detoxification of methyl mercury (MeHg), may differ from that of MeHg, elemental Hg (Hg0), and inorganic Hg (I-Hg). From a human health perspective, it is important to evaluate the presence of NP-Hg in seafoods. We investigated the *in vivo* formation of NP-Hg in fish and shellfish, which are the main sources of Hg exposure in humans. NP-Hg was measured in 90 fish samples with single-particle inductively coupled plasma mass spectrometry (spICP-MS) after enzyme degradation with pancreatin and lipase. In addition to NP-Hg, total Hg (T-Hg), MeHg, and selenium (Se) concentrations were evaluated. Transient Hg signals were detected as nanoparticles from almost all samples by using spICP-MS. Higher particle number concentrations (CPN) were observed in the tuna-swordfish group than in the shellfish group (17.7×10^7 vs. 1.2×10^6 particles/g, respectively). Although the CPN and maximum particle mass increased significantly with increasing T-Hg concentration, the increase in CPN was greater than those in maximum particle mass. Assuming that the NP-Hg detected was HgSe (tiemannite) and spherical based on previous reports, the maximum particle diameter was estimated to be 89 nm. The mean dietary exposures to NP-Hg, T-Hg, and MeHg were estimated to be 0.067, 5.75, and 5.32 μg/person per day, respectively. Generation of NP-Hg was inferred to be widespread in marine animals, with a preferential increase in the number of particles rather than an increase in particle size. The mean dietary exposure to NP-Hg in Japanese people was estimated to be 1.2 ng/kg body weight (BW) per day. Compared to PTWI of 4 μg/kg BW per week (0.57 μg/kg BW per day) derived by JECFA (2011), the health risk from redissolved I-Hg from NP-Hg is small.

Keywords: nanoparticle, tiemannite, methyl mercury

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Tamura M[*1], Suzuki Y, Akiyama H[*2], Hamada-Sato N[*1,3]: Evaluation of the effect of Lactiplantibacillus pentosus SN001 fermentation on arsenic accumulation and antihypertensive effect of Sargassum horneri *in vivo*.
Naunyn Schmiedebergs Arch. Pharmacol. 2022;395:1549-1556. doi: 10.1007/s00210-022-02288-2

Sargassum horneri contains water-soluble polysaccharides, which have antihypertensive effects, and arsenic, which is harmful to the human body. Boiling and other treatments are effective in removing arsenic; however, water-soluble polysaccharides are lost during processing. Therefore, a method to remove arsenic and further increase its antihypertensive effect is required. To this end, we investigated fermentation with *Lactiplantibacillus pentosus* SN001 in this study.

Boiled and fermented *S. horneri* were administered to spontaneously hypertensive rats (SHR), and blood pressure and arsenic accumulation in organs were observed to simultaneously examine the effects of fermentation on hypertension and arsenic accumulation. The ACE (angiotensin-converting enzyme) inhibition rate, an indicator of antihypertensive effects, showed a maximum at 4 days of fermentation. Consecutive dosing studies using *S. horneri*, boiled *S. horneri*, and fermented boiled *S. horneri* in SHR were conducted. Although the boiled group showed high blood pressure values, the fermented boiled group showed lower blood pressure values than the boiled cohort. The amount of arsenic accumulated in the liver, kidney, and spleen of rats was significantly lower in the boiled and fermented boiled groups than that in the *S. horneri* group. This confirmed the arsenic removal effect of boiling pretreatment and the *in vivo* safety of fermented boiled *S. horneri*. These results suggest that fermentation of arsenic-free *S. horneri* with *L. pentosus* SN001 can enhance its antihypertensive effect *in vivo*. This is the first study to simultaneously examine the antihypertensive effect of fermentation of *S. horneri* and its effect on the arsenic accumulation *in vivo*.

Keywords: *Sargassum horneri*, arsenic, fermentation

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鈴木美成，近藤翠，北山育子，穐山浩*，堤智昭：二次元モンテカルロシミュレーションを用いた食事性鉛曝露量分布の推定：トータルダイエット試料への適用の試み．

食品衛生学雑誌，2023;64:1-12. doi: 10.3358/shokueishi.64.1

トータルダイエット（TD）試料［280試料（14食品群×10地域×２年）］を用いて，日本人の平均的な食事由来のPb曝露量を推定した．さらには，ベイズ推定を用いた二次元モンテカルロシミュレーション（2D-MCS）を行い，推定の不確かさを考慮に入れた確率論的なPb曝露量評価を試みた．推定に際しては，不検出例には下限値-上限値間の累積分布確率を用いた尤度関数を用いたベイズ推定を行った．2D-MCSによるPb曝露量の中央値は5.85 μg/person/dayであり，90％区間は2.52-17.0 μg/person/dayであった．これまでに報告されたPb曝露量分布との比較から，TD試料を用いたPb曝露量分布の推定は妥当であることが示された．Pb曝露量への寄与率は，８群（淡色野菜・海藻・きのこ類：20.0±16.1％）＞１群（米およびその加工品：12.3±19.0％）>10群（魚介類：10.5±13.9％）の順で高かったが，いずれも不確かさが大きく寄与率の大きい食品を特定することはできなかった．一方で，Pb曝露量推定の不確かさには，喫食量の不確かさよりもPb濃度の不確かさからの影響が大きく，特に１群中Pb濃度の不確かさの影響は68.2％と大きかった．曝露マージンを算出したところ，曝露マージンが１未満となる確率は，幼児への発達神経毒性：14.5％，血圧：0.13％，腎臓病：0.93％と推定され，食事性Pb曝露による健康リスクは小さいが，無視できる確率ではないと考えられた．

Keywords：鉛，不検出値，確率論的曝露評価

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Yamasaki Y, Moriwaki T*1, Ogata S*2, Ito S*3, Ohtsuki S*3, Minegishi G*4, 5, Abe S*6, Ohta Y*6, Kazuki K*6, Kobayashi K*4, 5, Kazuki Y*1, 6: Influence of MDR1 gene polymorphism (2677G>T) on expression and function of P-glycoprotein at the blood-brain barrier: utilizing novel P-glycoprotein humanized mice with mutation.

Pharmacogenet. Genomics, 2022;32(8):288-292. doi: 10.1097/FPC.0000000000000481

P-glycoprotein, the encoded product of the MDR1 / ABCB1 gene in humans, is expressed in numerous tissues including brain capillary endothelial cells and restricts the distribution of xenobiotics into the brain as an efflux pump. Although a large number of single nucleotide polymorphisms in the MDR1 gene have been identified, the influence of the nonsynonymous 2677G>T/A single nucleotide polymorphism on P-glycoprotein at the blood-brain barrier has remained unclear. In the present study, we developed a novel P-glycoprotein humanized mouse line carrying the 2677G>T mutation by utilizing a mouse artificial chromosome vector constructed by genetic engineering technology and we evaluated the influence of 2677G>T on the expression and function of P-glycoprotein at the blood-brain barrier *in vivo*. The

results of this study showed that the introduction of the 2677G>T mutation does not alter the expression levels of P-glycoprotein protein in the brain capillary fraction. On the other hand, the brain penetration of verapamil, a representative substrate of P-glycoprotein, was increased by the introduction of the 2677G>T mutation. These results suggested that the 2677G>T single nucleotide polymorphism may attenuate the function of P-glycoprotein, resulting in increased brain penetration of P-glycoprotein substrates, without altering the expression levels of P-glycoprotein protein in the blood-brain barrier. This mutant mouse line is a useful model for elucidating the influence of an MDR1 gene single nucleotide polymorphism on the expression and function of P-glycoprotein at the blood-brain barrier.

Keywords: P-glycoprotein, polymorphism, humanized mice

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Nakamura K, Chiba S, Kiuchi T, Nabeshi H, Tsutsumi T, Akiyama H, Hachisuka A: Comprehensive analysis of a decade of cumulative radiocesium testing data for foodstuffs throughout Japan after the 2011 Fukushima Daiichi Nuclear Power Plant accident.
PLOS ONE, 2022;17:e0274070. doi: 10.1371/journal.pone.0274070

The unexpected accident at the Fukushima Daiichi Nuclear Power Station in Japan, which occurred on March 11th, 2011, after the Great East Japan Earthquake and tsunami struck the north-eastern coast of Japan, released radionuclides into the environment. Today, because of the amounts of radionuclides released and their relatively long half-life, the levels of radiocesium contaminating foodstuffs remain a significant food safety concern. Foodstuffs in Japan have been sampled and monitored for $^{134, 137}$Cs since the accident. More than 2.5 million samples of foodstuffs have been examined with the results reported monthly during each Japanese fiscal year (FY, from April 1st to March 31st) from 2012 to 2021. A total of 5,695 samples of foodstuffs within the "general foodstuffs" category collected during this whole period and 13 foodstuffs within the "drinking water including soft drinks containing tea as a raw material" category sampled in FY 2012 were found to exceed the Japanese maximum permitted level (JML) set at 100 and 10 Bq/kg, respectively. No samples from the "milk and infant foodstuffs" category exceeded the JML (50 Bq/kg). The annual proportions of foodstuffs exceeding the JML in the "general foodstuffs" category varied between 0.37% and 2.57%, and were highest in FY 2012. The $^{134, 137}$Cs concentration for more than 99% of the foodstuffs monitored and reported has been low and not exceeding the JML in recent years, except for those foodstuffs that are difficult to cultivate, feed or manage, such as wild mushrooms, plants, animals and fish. The monitoring data for foodstuffs show the current status of food safety risks from $^{134, 137}$Cs contamination, particularly for cultured and aquaculture foodstuffs on the market in Japan.

Keywords: radionuclide, radiocesium, food

建部千絵，藤原由美子，鐘熙寧，久保田浩樹，多田敦子，佐藤恭子：UV-Vis法を用いた食品添加物公定書塩化物試験法に関する検討.
日本食品化学学会誌 2022;29:61-68. doi:/10.18891/jjfcs.29.2_61

第９版食品添加物公定書（公定書）の一般試験法の塩化物試験法は，食品添加物中に混在する塩化物の限度試験である．検液の濁度が比較液の濁度より濃くないかを目視により判定する試験法（目視法）であることから，目視により判別可能な塩化物濃度について検討した．その結果，目視法では，微量な塩化物濃度の差を判別することはできなかった．一方，紫外可視吸光光度計（600 nm）では，目視では濃度差の判別が困難な低濃度から高濃度（0.05～５μg/mL）の範囲で定量が可能であった．また，３種類の試料を用いたUV-Vis法による妥当性確認の結果，良好な真度（≧ 92.6%），併行精度（≦ 3.7%），室内精度（≦ 7.3%）が得られ，UV－Vis法は検液中の塩化物濃度を精度よく定量できる簡便で有用な方法であることが示された．

Keywords：食品添加物公定書，塩化物試験法，紫外可

視吸光光度計

Terami S, Kubota H, Koganesawa N[*1], Murakoshi S[*1], Satou M[*2], Sekine Y[*3], Watanabe S[*3], Tsuruoka N[*3], Sugiki M[*4], Tahara S[*4], Yasunaga M[*5], Kamimoto K[*5], Nakashima A[*6], Ihara S[*6], Takeshita T[*7], Kawahara R[*7], Takamine T[*8], Koja A[*8], Ebisu N[*8], Yanagimoto T, Tatebe C, Tada A, Sato K: Estimation of daily intake of food additives by Japanese young children using the market basket method in 2018.

Food Additives & Contaminants: Part A 2022;40:328-345. doi: 10.1080/19440049.2023.2167002

To estimate the daily intake of food additives by young children aged 1-6 years in Japan, an intake survey was conducted in 2018 using the market basket method for food additives, including twelve types of colourants, three kinds of preservatives, three kinds of sweeteners and two kinds of food manufacturing agents. A list of the daily consumption of processed foods was prepared based on a special survey (MHLW Citation2011) and used for the estimation. The results of the survey showed that the food additives with the highest daily intake were phosphorus compounds (phosphoric acid and its salts; 11.2 mg/kg bw/day, expressed as phosphorus), followed by propylene glycol (0.80 mg/kg bw/day). The daily intake of other food additives ranged from 0 to 0.20 mg/kg bw/day. The estimated daily intake of each food additives by young children was compared with the acceptable daily intake (ADI) or maximum tolerable daily intake (MTDI). The highest ratio of the estimated daily intake to ADI was 3.2% for propylene glycol, whereas the ratios of the estimated daily intake to ADI for colourants, preservatives and sweeteners ranged from 0 to 1.1% (benzoic acid). The ratio of the estimated daily intake to MTDI for phosphorus compounds was 16%.

Keywords: estimated daily intake, food additives, market basket method

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堀江正一[*]，渡邉萌[*]，多田敦子，佐藤恭子：HPLCおよびLC-MS/MSによる食品中の遊離型パントテン酸の分析.

食品衛生学雑誌 2022;64:47-52. doi: 10.3358/shokueishi.64.47

食品に含まれるパントテン酸の迅速かつ精度の高い分析法を構築した．高タンパク食品の試験溶液は，試料２ gに水20 mLを加えてホモジナイズ抽出後，15%硫酸亜鉛水溶液１ mLを加えてよく混合し，遠心分離後，上清をろ過して試験溶液とした．低タンパク食品は，試料２ gに１%ギ酸水溶液20 mLを加え，ホモジナイズ抽出後，遠心分離し，上清をろ過して試験液とした．HPLCの測定条件は，分離カラムはL-column2 ODS，移動相は0.02 mol/Lリン酸緩衝液（pH3.0）／アセトニトリル（95:5）を用い，検出波長は200 nmとした．LC-MS/MS条件は，分離カラムにL-column2 ODS，移動相に5 mMギ酸アンモニウム（0.01%ギ酸含有）／メタノール（85:15）を用い，検出には多重反応モニタリング（MRM）を用いた．本法による調製粉乳や栄養機能食品等に対する添加回収率は，85%以上と良好な結果が得られた．都内で市販されているパントテン酸含有表示のある食品を分析した結果，表示値とほぼ同じ分析値が得られ，HPLC法とLC-MS/MSで得られた値には，高い相関が認められた.

Keywords：パントテン酸，液体クロマトグラフィータンデム質量分析法，食品添加物

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食品衛生学雑誌 2022;63:97-103. doi: 10.3358/shokueishi.63.97

台所用洗浄剤中のメタノール（MeOH）分析法について，汎用性の高いキャピラリーカラムを用いた改良分析法を考案し，10試験所が参加する室間共同実験を行った．濃度非明示で２濃度の試料を配付し，プロトコール

に従い試料中のMeOHを定量した．得られた試験所の分析結果を基に，国際的なハーモナイズドガイドラインに沿って統計的に解析した．共同実験の結果として推定された室間再現相対標準偏差（RSD_R）とHorwitz/Thompson式を用いて計算した予測室間相対標準偏差（$PRSD_R$）からHorRat値を算出した．その結果，２試料のHorRat値は0.8および1.8となり，Codex委員会が分析法承認のために設定している性能規準の指標である２未満を満たした．したがって，本分析法は規格の判定を行う分析法として期待できる性能を有していると判断した．

Keywords：台所用洗浄剤，メタノール，室間共同実験

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日本食品化学学会誌 2022;29:134-145. doi: 10.18891/jjfcs.29.3_134

ポリカーボネート製器具・容器包装の溶出試験におけるビスフェノールＡの告示分析法について 23試験所が参加する室間共同実験を行った．得られた試験所の分析結果を国際的なハーモナイズドガイドラインに沿って統計的に解析した．室間共同実験の結果として推定された室間再現相対標準偏差（RSD_R）とHorwitz/Thompson式により計算される予測室間相対標準偏差（$PRSD_R$）からHorRat値を算出した．その結果，浸出用液が水，20%エタノール，４%酢酸の場合の分析法は，Codex委員会が分析法承認のために設定している性能規準の指標である２未満を満たしたが，浸出用液がヘプタンの場合の分析法は，満たさなかった．したがって，浸出用液が水，20%エタノール，４%酢酸の場合は規格の判定を行う分析法として期待できる性能を有しているが，浸出用液がヘプタンの場合の分析法については，分析法の改良が必要であると判断した．

Keywords：ポリカーボネート，ビスフェノールＡ，室間共同実験

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食品衛生学雑誌 2022;63:51-61. doi: 10.3358/shokueishi.63.51

溶出試験は器具・容器包装の規格適合性や安全性を確認するうえで重要な試験法であるが，溶出操作から定量までを含めた溶出試験全体の試験室間共同試験はほとんど実施されていない．そこで，22機関が参加し，広範なオクタノール/水分配係数を有する10物質を添加した８種類の合成樹脂製モデル試料を用いて試験室間共同試験を行い溶出試験全体の精度を検証した．その結果，

HorRat（r）は大部分が基準を満たしたが，HorRat（R）は基準を超過したものが多かった．そのため，単一試験室で行うには精度は概ね確保されるが，試験室間の精度には問題があった．この主な原因としては，試験機関間における溶出操作時の温度や時間管理等の試験溶液の調製操作の差異によるものと考えられた．

Keywords：合成樹脂製器具・容器包装，溶出試験，試験室間共同試験

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Ozaki A*, Kishi E*, Ooshima T*, Kakutani N*, Abe Y, Mutsuga M, Yamaguchi Y*, Yamano T*: Determination of potential volatile compounds in polyethylene terephthalate (PET) bottles and their short- and long-term migration into food simulants and soft drink.

Food Chemistry, 2022:397;133758. doi: 10.1016/j.foodchem.2022.133758

Head space (HS)-GC-MS was used to analyze possible migration of volatile compounds from polyethylene terephthalate (PET) bottles for soft drinks, and a total of six compounds were identified. Next, a rapid, simple, and accurate simultaneous method was established using purge-and-trap (PT)-GC-MS, to quantify their amounts in the liquid contents after short- and long-term storage in PET bottles. Starting with brand-new PET bottles, the maximum migration of 2-methyl-1,3-dioxolane into distilled water and 50% aqueous ethanol after 2 years at 25℃ were 2.3 and 19 ng/mL, respectively. In commercially available bottled mineral water sold inside and outside Japan, we were able to detect 2-methyl-1,3-dioxolane in the same way. While nonanal was also detected in some products, 2-methyl-1,3-dioxolane was confirmed as the main volatile compound. Finally, the human exposure to 2-methyl-1,3-dioxolane was estimated based on the per capita intake of soft drinks in Japan and the migration amount in this study.

Keywords: volatile compounds, polyethylene terephthalate, migration

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Forest T*1, Aeffner F*2, Bangari DS*3, Bawa B*4, Carter J*5, Fikes J*6, High WB*7, Hayashi S, Jacobsen M*8, McKinney L*9, Rudmann D*10, Steinbach T*11, Schumacher V*12, Turner OC*13, Ward JM*14, Willson CJ*15: Scientific and regulatory policy committee brief communication: 2019 survey on use of digital histopathology systems in nonclinical toxicology studies.

Toxicol Pathol, 2022;50(3): 397-401. doi: 10.1177/01926233221084621.

Histopathologic evaluation and peer review using digital whole-slide images (WSIs) is a relatively new medium for assessing nonclinical toxicology studies in Good Laboratory Practice (GLP) environments. To better understand the present and future use of digital pathology in nonclinical toxicology studies, the Society of Toxicologic Pathology (STP) formed a working group to survey STP members with the goal of creating recommendations for implementation. The survey was administered in December 2019, immediately before the COVID-19 pandemic, and the results suggested that the use of digital histopathology for routine GLP histopathology assessment was not widespread. Subsequently, in follow-up correspondence during the pandemic, many responding institutions either began investigating or adopting digital WSI systems to reduce employee exposure to COVID-19. Therefore, the working group presents the survey results as a pre-pandemic baseline data set. Recommendations for use of WSI systems in GLP

environments will be the subject of a separate publication.

Keywords: GLP, scientific and regulatory policy committee, digital histopathology

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*11 Experimental Pathology Laboratories, Inc.
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*13 Novartis
*14 Global VetPathology
*15 Inotiv-RTP

Forest T*1, Aeffner F*2, Bangari DS*3, Bawa B*4, Carter J*5, Fikes J*6, High WB*7, Hayashi S, Jacobsen M*8, McKinney L*9, Rudmann D*10, Steinbach T*11, Schumacher V*12, Turner O*13, Ward JM*14, Willson CJ*15: Scientific and Regulatory Policy Committee Points to Consider: Primary Digital Histopathology Evaluation and Peer Review for Good Laboratory Practice (GLP) Nonclinical Toxicology Studies.
Toxicol Pathol, 2022;50(4): 531-543. doi: 10.1177/092623321099273.

The Society of Toxicologic Pathology's Scientific and Regulatory Policy Committee formed a working group to consider the present and future use of digital pathology in toxicologic pathology in general and specifically its use in primary evaluation and peer review in Good Laboratory Practice (GLP) environments. Digital histopathology systems can save costs by reducing travel, enhancing organizational flexibility, decreasing slide handling, improving collaboration, increasing access to historical images, and improving quality and efficiency through integration with laboratory information management systems. However, the resources to implement and operate a digital pathology system can be significant. Given the magnitude and risks involved in the decision to adopt digital histopathology, this working group used pertinent previously published survey results and its members' expertise to create a Points-to-Consider article to assist organizations with building and implementing digital pathology workflows. With the aim of providing a comprehensive perspective, the current publication summarizes aspects of digital whole-slide imaging relevant to nonclinical histopathology evaluations, and then presents points to consider applicable to both primary digital histopathology evaluation and digital peer review in GLP toxicology studies. The Supplemental Appendices provide additional tabulated resources.

Keywords: digital pathology, GLP, histopathology

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*8 AstraZeneca
*9 US Food and Drug Administration
*10 Charles River Laboratories International, Inc.
*11 Experimental Pathology Laboratories, Inc.
*12 F. Hoffmann-La Roche Ltd
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Vij P*1, Donahue DA*2, Burke KP*2, Hayashi S, Maronpot RR*3: Lack of skin sensitization hazard potential for alpha-glycosyl isoquercitrin (AGIQ) utilizing the Local Lymph Node Assay.
Toxicol Rep, 2022;9: 1291-1296. doi: 10.1016/j.toxrep.2022.05.021.

Skin sensitization is an important aspect of safety assessment and is a key component in the toxicological evaluation of chemicals. alpha-Glycosyl isoquercitrin (AGIQ), is marketed in Japan as a food additive and is generally recognized as safe (GRAS) by the expert panel of the Flavor and Extract Manufacturers Association (FEMA) in 2005 and the U.S. Food and Drug Administration (FDA) in 2007. The Local Lymph Node Assay (LLNA) was used to assess AGIQ's potential to cause skin sensitization. Results

indicate that no excessive irritation was observed after the irritation screen (ear swelling < 25% and erythema score < 3) when AGIQ was tested at 5%, 10%, and 25% in N, N-dimethyl formamide [DMF]. Based on lack of irritation, AGIQ was further evaluated at 10%, 25%, and 50% in DMF in the main test resulting in stimulation indices of less than the positive threshold of 1.6 i.e., 1.2, 1.4, and 1.2 respectively. Therefore, AGIQ was not a dermal sensitizer in the LLNA.

Keywords: alpha-glycosyl isoquercitrin, sensitization, local lymph node assay

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佐々木貴正，米満研三[*]，朝倉宏：食鳥処理場における鶏肉のカンピロバクター汚染の定量調査.

鶏病研究会報 2022;58:17-21.

中部地方および九州地方の食鳥処理場において，ブロイラーおよび地鶏の盲腸内容物およびその鶏群に由来する胸肉のカンピロバクター汚染状況を定量的に調査した．分離株についてMultilocus sequence typingと薬剤感受性試験を実施したところ，分離株の性状は食鳥処理場間でまったく異なった．汚染鶏肉のカンピロバクター菌数および分離株の性状は，その由来となった鶏群が飼育された地域および鶏群が出荷された食鳥処理場によって特徴付けできる可能性があると考えられた.

Keywords：カンピロバクター，鶏肉，フルオロキノロン耐性

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食品衛生学雑誌 2022;63:190-194. doi: 10.3358/shokueishi.63.190

シガテラ魚類食中毒は，シガトキシン類（CTXs）を含有する魚類による動物性自然毒食中毒で，主に熱帯・亜熱帯で発生している．日本では，沖縄県を中心に発生報告があるが，日本本土の太平洋岸産魚類による食中毒事例も散発的に発生し，原因魚種のほとんどがイシガキダイ*Oplegnathus punctatus*の老成個体である．イシガキダイにおけるCTXsの含有状況を調査するために，本州，四国，九州，奄美，沖縄，および小笠原の沿岸産176個体（標準体長：13.1～60.0 cm,体重：100～6,350 g,年齢：0～11歳）を収集し，LC-MS/MSによる分析を実施した．そのうち沖縄産2個体（全試料の1.1%）からCTXsが検出され，沖縄産14個体に限定した検出率は14%であった．CTXsが検出された2個体の魚肉中の総CTX含量は，0.014 μg/kgおよび0.040 μg/kgであり，いずれも米国FDAの推奨値0.01 μg CTX1B equivalent/kg以上であったが，ヒトの最小発症量（10 MU, CTX1B換算で70 ng）に達するには，1.5 kg以上の摂食が必要であるため，シガテラ魚類食中毒発症のリスクは高くないと考えられる．沖縄産イシガキダイのCTXs組成はCTX1B系列のみで，バラフエダイやバラハタなどの肉食魚が含有するCTX1Bおよび52-*epi*-54-deoxyCTX1Bに加えて，渦鞭毛藻が産生するCTX4AおよびCTX4BがCTX1Bと同程度のレベルで検出された．なお，CTX3C系列は検出されなかった.

Keywords：イシガキダイ，シガテラ，シガトキシン

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日本食品微生物学会雑誌 2022;39:77-82. doi: 10.5803/jsfm.39.77

This study examined the thermal kinetics in wild deer and wild boar meats by low temperature cooking process as well as its bactericidal effect. The thermal processing so as to heat the inner-core of the samples at 65℃ for 15 min, 68℃ for 5 min, 75℃ for 1 min in steam convection oven exhibited faster elevation rate of the internal temperature of wild deer meat than wild boar meat, while their sterilization values after the thermal processes were estimated to be almost equal. Naturally contaminated fecal indicator bacteria were not recovered from all samples after the above-mentioned processing. Spike experiment resulted that approximately 6.6-7.8 log CFU/g of STEC O157 and/or *Salmonella* spp. were not recovered from the wild deer meats after the three types of thermal cooking. Thus, these data indicated aptitude of these low temperature

cooking conditions to minimize the microbiological risks in the game meat.

Keywords: foodborne bacteria, game meat, low temperature cooking

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Nakayama T*1, Yamaguchi T*2, Yamamoto S, Jinnai M*3, Kumeda Y*4, Hase A*5: Genome sequence of carbapenemase-producing *Enterobacter cloacae* 0102-4P-1 harboring the IncC-type plasmid with a multidrug resistance site encoding bla_{NDM-1}, isolated from commercially imported shrimp.

Microbiology Resource Announcements. 2022;11:e01058-21. doi: 10.1128/mra.01058-21

A carbapenem-resistant *Enterobacter cloacae* 0102-4P-1 strain was isolated from commercially imported shrimp in Japan. Here, we present a draft genome sequence. The complete plasmid sequence was also determined by hybrid assembly sequencing using Oxford Nanopore and Illumina methods. The assembled whole genome and plasmid were 5,164,033 bp and 162,852 bp long, respectively.

Keywords: carbapenemase-producing *Enterobacter cloacae*, imported shrimp, multidrug resistance

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Ogawa A*, Nagaoka H*, Asakura H: Draft genome sequence of *Campylobacter jejuni* ST-508 strain Shizu21005, isolated from an asymptomatic food handler in Japan, 2021.

Microbiology Resource Announcements. 2022;11:e0031622. doi: 10.1128/mra.00316-22

Here, we report a draft genome sequence of *Campylobacter jejuni* strain Shizu21005, isolated from a food handler with no symptoms in Japan on March 2021. Its genome size was 1,656,785 bp, with 2 rRNAs, 35 tRNAs, and a coverage of 330×.

Keywords: *Campylobacter jejuni*, draft genome

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Yamamoto S, Kitagawa W*1, Nakano M*1, Asakura H, Nakayama T*2, Iwabuchi E*3, Sone T*1, Asano K*1: Prevalence and characterization of gentamicin resistance genes in *Escherichia coli* isolates from beef cattle feces in Japan.

Current Microbiology. 2022;79:217. doi: 10.1007/s00284-022-02913-6

Gentamicin is an important antibiotic for the treatment of opportunistic infections in the clinical field. Gentamicin-resistant bacteria have been detected in livestock animals and can be transmitted to humans through the food supply or direct contact. We have previously revealed that gentamicin-resistant *Escherichia coli* are distributed at a comparatively high rate from beef cattle in Japan, but few studies have focused on the molecular epidemiology of gentamicin-resistant bacteria. To understand these bacteria, this study examined the prevalence of various gentamicin resistance genes in gentamicin-resistant *E. coli* isolates from beef cattle feces. Of the 239 gentamicin-resistant *E. coli* isolates, the presence of the *aacC2*, *aadB*, or *aac(3)-VIa* genes was confirmed in 147, 84, and 8 isolates, respectively. All *aac(3)-VIa*-harboring isolates had an MIC value of 64 μg/mL for gentamicin and exhibited resistance to 11 antibiotic agents. An analysis of the representative *aac(3)-VIa*-harboring *E. coli* strain GC1-3-GR-4 revealed that the *aac(3)-VIa* gene was present on the IncA/C plasmid together with the *aadA* and *blaCMY* genes. Furthermore, the upstream region of the *aac(3)-VIa* gene contained the *aadA* gene and the class 1 integron-integrase gene (*intI1*). The *aac(3)-VIa* gene was detected for the first time in Japan and is expected to be able to transfer between bacteria via the IncA/C plasmid and integron. These results reveal the expansion of the distribution or diversity of gentamicin resistance genes in Japan.

Keywords: beef cattle, *Escherichia coli*, gentamicin resistance

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Sasaki Y, Iwata T[*1], Uema M, Yonemitsu K[*2], Igimi S[*3], Asakura H: *Campylobacter* spp. prevalence and fluoroquinolone resistance in chicken layer farms.
The Journal of Veterinary Medical Science. 2022;84:743-746. doi: 10.1292/jvms.22-0047

Chicken is a major source of human campylobacteriosis. Chicken meat originates not only from broilers but also from spent layers; however, few reports have documented the prevalence and antimicrobial resistance of *Campylobacter* spp. in layers in Japan. Therefore, we investigated the prevalence and antimicrobial susceptibility of *Campylobacter* spp. in 47 layer farms in Japan. Fecal samples were collected from the youngest and oldest flocks on the farm, and *Campylobacter* spp. was isolated from 46/47 (97.9%) farms. Among the *C. jejuni* isolates, the resistance rates to ampicillin, tetracycline, and ciprofloxacin were 29.6%, 22.2%, and 19.8%, respectively. The ciprofloxacin resistance rate (7.3%) in *C. jejuni* isolated from old flocks was significantly ($P<0.01$) lower than that in young flocks (32.5%).
Keywords: *Campylobacter*, chicken layer, fluoroquinolone resistance

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Sasaki Y, Asakura H, Asai T[*]: Prevalence and fluoroquionlone resistance of *Campylobacter* spp. isolated from beef cattle in Japan.
Animal Diseases 2022;2:15. doi: 10.1186/s44149-022-00048-6

Beef is a source of human *Campylobacter* infections. Antimicrobial treatment is needed when patients are immunocompromised or have other comorbidities. Therefore, we investigated the prevalence and antimicrobial resistance of *Campylobacter* spp. in beef cattle in Japan. Rectal swab samples were collected from 164 beef cattle at an abattoir between March 2021 and August 2021, and *Campylobacter* spp. were isolated from 94 (57.3%) cattle. *C. jejuni* and *C. coli* were isolated from 68 and 26 cattle, respectively. For *C. jejuni*, the resistant rates against ampicillin, tetracycline and ciprofloxacin were 20.6, 75.0 and 64.7%, respectively. For *C. coli*, the resistant rates against ampicillin, tetracycline and ciprofloxacin were 53.8, 76.9 and 88.5%, respectively. No *Campylobacter* isolates were resistant to erythromycin. By multilocus sequence typing, *C. jejuni* and *C. coli* isolates were classified into 22 and 2 sequence types (STs). The top three STs of *C. jejuni* were ST806 (12 isolates), ST21 (nine isolates), and ST459 (eight isolates). The most frequent ST of *C. coli* was ST1068 (23 isolates). The results suggest that *Campylobacter* spp. are prevalent in the gastrointestinal tract of beef cattle slaughtered at abattoirs. Furthermore, the administration of erythromycin is effective against human campylobacteriosis caused by beef consumption. Monitoring the prevalence and antimicrobial resistance of *Campylobacter* spp. in beef cattle could be useful for managing the risk of human campylobacteriosis.
Keywords: beef cattle, *Campylobacter*, fluoroquinolone resistance

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Sasaki Y, Nozawa-Takeda T[*1,2], Yonemitsu K[*3], Asai T[*4], Asakura H, Nagai H[*5]: Characterization of *Campylobacter jejuni* in large-billed crows (*Corvus macrorhynchos*) in Tochigi prefecture, Japan.
The Journal of Veterinary Medical Science. 2022;84:1029-1033. doi: 10.1292/jvms.22-0055

As free-living crows are a potential source of *Campylobacter* infections in broilers and cattle, we characterized *Campylobacter* spp. isolated from crows using multilocus sequence typing and antimicrobial susceptibility testing. We obtained 82 samples from 27 birds captured at seven different times using a trap set in Tochigi prefecture, Japan. *Campylobacter jejuni* was isolated from 55 (67.1%) of the 82 samples and classified into 29 sequence types, of which 21 were novel. Tetracycline and streptomycin resistance rates were 18.2% and 3.6%, respectively. These results show that most types of *C. jejuni* infecting crows differ from those isolated from humans, broilers, and cattle. Thus, the importance of free-living crows as reservoirs of *Campylobacter* infections in broilers and cattle may be limited.
Keywords: antimicrobial resistance, *Campylobacter*, crow

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Yamamoto S, Hasegawa M*, Iwabuchi E*, Asakura H: Draft genome sequences of two *Listeria monocytogenes* strains isolated from raccoon feces in Japan.
Microbiology Resource Announcements. 2022;11:e0049522. doi: 10.1128/mra.00495-22

Listeria monocytogenes serotype 4b strains RF01 and RF06 were isolated from raccoon feces in Japan. Here, we report the draft genome sequences of the two isolated strains; the genome sizes were 2,918,024 and 2,872,491 bp, with 535× and 510× coverage, for the RF01 and RF06 strains, respectively.

Keywords: draft genome, *Listeria monocytogenes*

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Sasaki Y, Aoki K*1, Ishii Y*1, Tamura Y*2, Asai T*3: First isolation of ST398 methicillin-resistant *Staphylococcus aureus* carrying staphylococcal cassette chromosome mec type IVd from pig ears in Japan.
The Journal of Veterinary Medical Science. 2022;84:1211-1215. doi: 10.1292/jvms.22-0084

The emergence and increasing prevalence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) are a global concern. To investigate the prevalence and characteristics of sequence type 398 (ST398) MRSA in pig ears, 102 pig's ears were collected from 102 animals shipped from 51 farms at an abattoir. Eight ST398 MRSA isolates were isolated from the ears of eight pigs shipped from seven farms. Of the eight ST398 isolates, seven had the staphylococcal cassette chromosome mec (SCCmec) type IVd and these were obtained from seven pigs shipped from six farms. Single nucleotide polymorphisms ranging from 13 to 26 were observed in the core-genome regions in the seven SCCmec type IVd isolates. We believe that this is the first report on the isolation of ST398 MRSA SCCmec type IVd in Japan.

Keywords: methicillin-resistant *Staphylococcus aureus*, pig, sequence type 398

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Asakura H, Yamamoto S, Yamada K*1, Kawase J*2, Nakamura H*3, Abe K*4, Sasaki Y, Ikeda T*5, Nomoto R*6: Quantitative detection and genetic characterization of thermotolerant *Campylobacter* spp. in fresh chicken meats at retail in Japan.
Frontiers in Microbiology. 2022;13:1014212. doi: 10.3389/fmicb.2022.1014212

Campylobacter jejuni and *C. coli* are one of the leading causes of gastrointestinal illnesses, and which are considered to be transmitted to humans mainly from chicken meats. Considering the less availability of quantitative contamination data in the retail chicken meats in Japan, 510 fresh chicken meats retailed at five distinct regions in Japan between June 2019 and March 2021 were examined. The quantitative testing resulted that 45.7% of the samples (254/510) were positive at mean ± standard deviation of 1.15 ± 1.03 log CFU/g, whereas 43 samples (8.4%) exceeded 3.0 log CFU/g. Seasonal comparison revealed increased bacterial counts in fall compared with spring and summer. As for the chicken slaughter age, those slaughtered at >75 days old were less contaminated than those at <75 days old. Genome sequencing analyses of 111 representative *C. jejuni* isolates resulted in the detection of three antimicrobial resistance genes (*gyrA* substitution T86I, *tetO* and *blaOXA-61*) at 25.2, 27.9 and 42.3%, respectively. *In silico* MLST analysis revealed the predominance of sequence types (ST)-21 clonal complex (CC), followed by ST-45CC and ST-464CC. The single nucleotide polymorphism (SNP)-based phylogenetic tree largely classified the sequenced *C. jejuni* isolates into two clusters (I and II), where all *C. jejuni* from highly contaminated samples (STs-21CC, -22CC and -45CC) belonged to cluster I, independent of both season and slaughter age. To our knowledge, this is the first example to study the current status of *Campylobacter* contamination levels in fresh chicken meats retailed in Japan. Our data would be contributable to future quantitative microbial risk assessment, to establish effective control measures for campylobacteriosis.

Keywords: *Campylobacter jejuni/coli*, retailed poultry

meat, whole-genome sequencing

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Sasaki Y, Yonemitsu K*1, Uema M, Asakura H, Asai T*2: Prevalence and antimicrobial resistance of *Campylobacter* and *Salmonella* in layer flocks in Honshu, Japan.
The Journal of Veterinary Medical Science. 2022;84:1502-1507. doi: 10.1292/jvms.22-0257

Campylobacter and non-typhoidal *Salmonella* are the major causes of bacterial gastrointestinal infections in humans. Although antimicrobial therapy is typically not recommended in many cases of these infections, it may be life-saving in patients with severe symptoms. Since chicken eggs and meat derived from layers are destined for human consumption, we investigated the prevalence and antimicrobial resistance of these two bacterial genera in 82 layer flocks at chicken processing plants in Honshu, Japan. *Campylobacter* was isolated from 77 flocks (93.9%). Resistance to ampicillin, tetracycline, and ciprofloxacin was documented in 42.3 (30/71), 16.9 (12/71), and 14.1% (10/71) of *Campylobacter jejuni*, respectively. Multilocus-sequence typing identified ST4389 and ST5262 as the most frequent *C. jejuni* sequence types. In *C. coli*, resistance to ampicillin, tetracycline, and ciprofloxacin was found in 20.0 (7/35), 20.0 (7/35), and 25.7% (9/35), respectively. The most frequent sequence type in *C. coli* was ST8292. Erythromycin resistance was not observed among *Campylobacter* species. *Salmonella* was isolated from 14 flocks (17.1%). The two most frequent serovars were *Salmonella* Corvallis and *S.* Braenderup. Neither *S.* Enteritidis nor *S.* Infantis were isolated. Streptomycin resistance was observed in six isolates (26.1%), and all isolates were susceptible to cefotaxime and ciprofloxacin. Thus, chicken eggs and meat derived from layers are possible sources of these bacterial infections in humans. The antimicrobial susceptibility of these isolates was maintained, reflecting restrictions on the use of antimicrobial agents on layers.
Keywords: *Campylobacter*, layer, *Salmonella*

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Uema M, Yonemitsu K*, Sasaki Y, Asakura H: Detection of hepatitis E virus RNA from pig bile collected at a slaughterhouse in Japan.
AIMS Microbiology. 2022;8:566-574. doi: 10.3934/microbiol.2022036

Hepatitis E virus genes was detected from pig bile processed for food at slaughterhouse, and it was found that HEV genes were detected in 10% of pigs, indicating that HEV contaminated pork may be distributed in markets.
Keywords: bile, hepatitis E virus, pig

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Oshiro N, Nagasawa H*1, Nishimura M*2, Kuniyoshi K, Kobayashi N*1, Sugita-Konishi Y*1, Ikehara T*3, Tachihara K*4, Yasumoto T*5: Analytical Studies on Ciguateric Fish in Okinawa, Japan (II): The Grouper *Variola albimarginata*.
Journal of Marine Science and Engineering. 2023;11:242. doi: 10.3390/jmse11020242

Ciguatera fish poisoning (CFP) refers to an illness caused by ingesting fish that have accumulated ciguatoxins (CTXs). CFP frequently occurs in the tropical and subtropical Indo-Pacific Ocean and the Caribbean Sea. In Japan, CFP occurs sporadically but constantly in Okinawa and the Amami Islands. The grouper *Variola albimarginata* is regarded to be safe for consumption. To assess the real risk of *V. albimarginata*, we analyzed 133 specimens of the fish in Okinawa using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Ciguatoxin-1B, 54-deoxyciguatoxin-1B, and 52-*epi*-54-deoxyciguatoxin-1B were detected in 28 specimens (21%). In 11 of these specimens (8%), the CTX levels exceeded the US FDA guidance level (0.01 μg/kg CTX1B equivalent). However, only one fish (<1%) was found to have levels above the recommended level in Japan (0.175 μg/kg CTX1B equivalent). The amount of CTXs in the flesh (280 g) of the most toxic specimen (0.225 μg/kg) did not reach the level needed to cause

illness. The CFP risk due to the consumption of this species was thus considered to be low in Okinawa, supporting local belief. The CTX levels in the flesh were positively correlated with standard length, body weight, and age. The total CTX levels significantly fluctuated between the male and the female of the species. The estimated annual catch of *V. albimarginata* in Okinawa and Yaeyama Islands was 4909 kg or 13,636 fish. As many as 1227 fish had levels over the US FDA guidance level, but only 136 fish had levels above the Japanese recommendation. Risk management based on the Japanese recommendation level seems to be effective in protecting public health and enabling appropriate exploitation of fishery resources.

Keywords: ciguatera, ciguatoxin, *Variola albimarginata*

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Uema M, Hyuga M, Yonemitsu K*1, Hyuga S*2, Amakura Y*3, Uchiyama N, Mizoguchi K*4, Odaguchi H*2, Goda Y: Antiviral Effect of Ephedrine Alkaloids-Free Ephedra Herb Extract against SARS-CoV-2 *In Vitro*.

Microorganisms. 2023;11:534. doi:10.3390/microorganisms11020534

In vitro experiments, it was revealed that EFE suppresses the growth of the SARS-CoV-2 by more than 90%. It was also clarified that EFE shows suppressive effect against mutant viruses including the Omicron type.

Keywords: antiviral therapeutic, ephedrine alkaloids-free Ephedra Herb extract, SARS-CoV-2

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Suzuki H*, Hahoed AWR*, Midoh R*, Okada Y: Microbiological quality of pasteurized milk in Japan and testing method comparison.

Journal of Food Science and Nutrition. 2023;11:185-192. doi: 10.12691/jfnr-11-3-3

A total of 73 samples of pasteurized milk retailed in Japan, which comprised 47 low-temperature long-time pasteurized samples (LTLT; 63-66℃, 30 min), 13 high-temperature short-time pasteurized samples (HTST; 72-79℃, 15 sec), and 13 high-temperature long-time pasteurized samples (HTLT; 75-85℃, 15-30 min), were analyzed for hygiene indicator microorganisms to assess microbiological quality using the Japanese Official Method, International Standard Organization Methods, and commercial dehydrated medium sheets. Of the 73 milk samples, one LTLT milk sample was positive for both coliforms and *Enterobacteriaceae*, and another LTLT milk sample exceeded the Japanese microbiological criterion. All the samples tested were negative for *E. coli* and *S. aureus*.

Keywords: dehydrated medium sheet, method comparison, pasteurized milk

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Arai S, Ooka T*1, Shibata M*2, Nagai Y*3, Tokoi Y*4, Nagaoka H*5, Maeda R*6, Tsuchiya A*7, Kojima Y*8, Ohya K, Ohnishi T, Konishi N*9, Ohtsuka K*10, Hara-Kudo Y: Development of a novel real-time polymerase chain reaction assay to detect *Escherichia albertii* in chicken meat.

Foodborne Pathog Dis. 2022;19(12):823-829. doi: 10.1089/fpd.2022.0042.

Escherichia albertii is an emerging enteropathogen. Several foodborne outbreaks of *E. albertii* have been reported in Japan; however, foods associated with most outbreaks remain unidentified. Therefore, polymerase chain reaction (PCR) assays detecting *E. albertii* specifically and sensitively are required. Primers and probe for real-time PCR assays targeting *E. albertii*-specific gene (EA-rtPCR) was designed. With 74 strains, including 43 *E. albertii* strains and several of its close relatives, EA-rtPCR specifically amplified *E. albertii*; therefore, the sensitivity of EA-rtPCR was then evaluated. The detection limits were 2.8 and 2.0-3.2 log colony-forming unit (CFU)/mL for *E. albertii* culture and enriched chicken culture inoculated with

the pathogen, respectively. In addition, *E. albertii* was detected from 25 g of chicken meat inoculated with 0.1 log CFU of the pathogen by EA-rtPCR. The detection of *E. albertii* from chicken meat by EA-rtPCR was also evaluated by comparing with the nested-PCR assay, and 28 retail chicken meat and 193 dissected body parts from 21 chicken carcass were tested. One and three chicken meat were positive in the nested-PCR assay and EA-rtPCR, respectively. Fourteen carcasses had at least one body part that was positive for EA-rtPCR, and 36 and 48 samples were positive for the nested-PCR assay and EA-rtPCR, respectively. A total of 37 strains of *E. albertii* were isolated from seven PCR-positive samples obtained from six chicken carcass. All *E. albertii* isolates harbored *eae* gene, and were classified as *E. albertii* O-genotype (EAOg)3 or EAOg4 by EAO-genotyping. The EA-rtPCR developed in this study has potential to improve *E. albertii* detection in food and advance research on *E. albertii* infection.

Keywords: *Escherichia albertii*, real-time PCR, chicken meat, chicken carcass

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Bryła M*1, Stępniewska S*1, Modrzewska M*1, Waśkiewicz A*2, Podolska G*3, Ksieniewicz-Woźniak E*1, Yoshinari T, Stępień Ł*4, Urbaniak M*4, Roszko M*1, Gwiazdowski R*5, Kanabus J*1, Pierzgalski A*1: Dynamics of Deoxynivalenol and Nivalenol Glucosylation in Wheat Cultivars Infected with *Fusarium culmorum* in Field Conditions—A 3 Year Study (2018-2020).

J Agric Food Chem. 2022;70(14):4291-4302. doi: 10.1021/acs.jafc.2c00314.

Fusarium head blight (FHB) caused by pathogenic species of *Fusarium* fungi is one of the most important diseases of cereal plants and a factor contributing to losses in plant production. The growth of FHB-associated species is often accompanied by biosynthesis of secondary metabolites—mycotoxins, which serve as a virulence factor. The aim of the study was to evaluate the ratios between deoxynivalenol (DON) and nivalenol (NIV) and their derivatives in the ears of six cultivars of winter wheat with varying resistance to FHB, taking into account a range of factors (weather conditions, location, cultivar, and year) after inoculation with *Fusarium culmorum*, during a 3 year field experiment, 2018-2020. The presence of toxins in the ears was measured within 21 days of inoculation. The toxins were found in the ears as soon as on the third day from the start of the experiment, whereas relative humidity higher than 80% was a decisive factor for FHB incidence. All wheat cultivars showed the ability to biotransform DON and NIV present in the ears to glucosides, that is, deoxynivalenol-3-glucoside (DON-3G) and nivalenol-3-glucoside (NIV-3G).

Keywords: *Fusarium*, field experiment, modified mycotoxins, wheat plants

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Elamin A*, Enomoto H*, Watanabe M, Sakuda S*: The mechanism of ochratoxin contamination of artificially inoculated licorice root.

Toxins (Basel). 2023;15(3):219. doi: 10.3390/toxins15030219.

This study was performed to investigate the mechanism of ochratoxin (OT) contamination of licorice (*Glycyrrhiza* sp.) root. Licorice root samples were inoculated with the spores of ochratoxigenic *Aspergillus westerdijkiae*. After incubation for 10 and

20 days, the OT contents of the samples were determined by high-performance liquid chromatography, and microtome sections prepared from the samples were analyzed by desorption electrospray ionization tandem mass spectrometry, to visualize OT localization. The same sections were further examined by light microscopy and scanning electron microscopy, to investigate the path of fungal mycelial penetration of the inner roots. OT concentrations tended to increase from the upper- to the middle-root parts. OTs were located in cut areas and areas of cork layer damage; they were not present in the undamaged cork layer, indicating that the structure of this layer prevents OT contamination of the licorice root.

Keywords: ochratoxins, licorice root, *Aspergillus westerdijkiae*

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Hirose S, Nakamura Y*, Arai S, Hara-Kudo Y: The development and evaluation of a selective enrichment for the detection of *Escherichia albertii* in food.

Foodborne Pathog Dis. 2022;19(10):704-712. doi: 10.1089/fpd.2022.0048.

Escherichia albertii is an emerging pathogen causing foodborne infections with diarrhea, abdominal pain, and fever. *E. albertii* has been isolated from various food sources, such as chicken and pork. Although many foodborne outbreaks of *E. albertii* have been reported, the causative food has not been identified. It is necessary to develop effective detection methods for *E. albertii*. Because enrichment procedure as the first step of food test is important for growing pathogens, this study aimed to develop a novel effective enrichment for *E. albertii* detection in food. In this study, we investigated the optimal concentration and combination of cefixime and tellurite for supplementing modified EC broth (mEC) to effectively isolate *E. albertii* from chicken meat. The results showed that mEC supplemented with 50 lg/L cefixime and 2.5 mg/L tellurite (CT-mEC) inhibited the growth of competitive bacteria in chicken meat but not that of *E. albertii*. Therefore, it was indicated that CT- mEC had strong potential to selectively grow *E. albertii*. In an *E. albertii* foodborne outbreak, CT-mEC was evaluated. *E. albertii* was successfully isolated from a food sample, a kind of salad, by enrichment with CT- mEC but not buffered peptone water and mEC. In this study, CT-mEC as a selective enrichment broth has been developed to detect *E. albertii* in chicken meat. It was demonstrated that the selective enrichment broth was effective for the efficient detection of *E. albertii* in food.

Keywords: *Escherichia albertii*, selective enrichment, cefixime, tellurite, foodborne pathogen

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Modrzewska M*1, Błaszczyk L*2, Stępień Ł*2, Urbaniak M*2, Waśkiewicz A*3, Yoshinari T, Bryła M*1: *Trichoderma* versus *Fusarium*-Inhibition of Pathogen Growth and Mycotoxin Biosynthesis.

Molecules. 2022;27(23):8146. doi: 10.3390/molecules27238146.

This study evaluated the ability of selected strains of *Trichoderma viride*, *T. viridescens*, and *T. atroviride* to inhibit mycelium growth and the biosynthesis of mycotoxins deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEN), α-(α-ZOL) and β-zearalenol (β-ZOL) by selected strains of *Fusarium culmorum* and *F. cerealis*. For this purpose, an *in vitro* experiment was carried out on solid substrates (PDA and rice). After 5 days of co-culture, it was found that all *Trichoderma* strains used in the experiment significantly inhibited the growth of *Fusarium* mycelium. When *Fusarium* and *Trichoderma* were co-cultured on rice, *Trichoderma* strains were found to inhibit DON biosynthesis by about 73% to 98%, NIV by about 87% to 100%, and ZEN by about 12% to 100%, depending on the pathogen and antagonist strain.

Keywords: *Trichoderma* spp., *Fusarium* spp., mycotoxins, plant biocontrol

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Noda K*1,2, Hirakawa Y*3, Nishino T*1, Sekizuka R*1, Kishimoto M*2,4, Furukawa T*4, Sawane S*1, Matsunaga A*1, Kobayashi N*1, Sugita K*1, Oonaka K*1, Kawakami H*5, Otsuka Y*5, Yamamoto T*5,

Yamamoto T[*5], Yoshiya T[*5], Watanabe M, Saka M[*1,6], Momma K[*3], Kushiro M[*4], Miyake S[*1]: Preparation of Monoclonal Antibodies Specifically Reactingwith the Trichothecene Mycotoxins Nivalenol and 15-Acetylnivalenol via the Introduction of a Linker Moleculeinto Its C-15 Position.

Toxins (Basel). 2022;14(11):747. doi: 10.3390/toxins14110747.

To establish an immunoassay, we prepared nivalenol (NIV), introduced a linker, and generated antibodies against it. NIV was prepared from a culture of *Fusarium kyushuense* obtained from pressed barley through chromatographic procedures with synthetic adsorbents and silica gel. NIV was reacted with glutaric anhydride, and the reaction was stopped before mono-hemiglutaryl-NIV was changed to di-hemiglutaryl-NIV. 15-*O*-Hemiglutaryl-NIV was isolated via preparative HPLC and bound to keyhole limpet hemocyanin (KLH) using the active ester method. Two different monoclonal antibodies were prepared by immunizing mice with the NIV-KLH conjugate. The 50% inhibitory concentration values were 36 and 37 ng/mL. These antibodies also showed high reactivity in a direct competitive enzyme-linked immunosorbent assay and specifically reacted with NIV and 15-acetyl-NIV but not with deoxynivalenol and 4-acetyl-NIV.

Keywords: *Fusarium* head blight, hapten, nivalenol

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Ohnishi T, Banzai A[*1], Hara-Kudo Y, Sugiyama Y[*2]: Prevalence and abundance of *Anisakis larvae* in ready-to-eat mackerel products in Japan.

J Int Food Microbiol. 2023;395:110181. doi: 10.1016/j.ijfoodmicro.2023.110181.

The risk of contracting anisakiasis from consuming ready-to-eat (RTE) mackerel products in Japan was investigated by examining the prevalence and abundance of *Anisakis simplex* and its sibling species in these products. From 2019 to 2021, a total of 448 RTE mackerel products were purchased in Japan. *Anisakis* larvae were isolated from 244 of the 448 samples (54%), and live larvae were isolated from 161 of the 448 samples (36%). In total, 3170 *Anisakis* larvae, which included 919 live larvae, were isolated. The isolated *Anisakis* larvae consisted of 3118 *A. simplex* (s. s.), 27 *A. pegreffii*, and 25 hybrid genotype (*A. simplex* [s. s.] × *A. pegreffii*) larvae. No *A. berlandi* larvae were isolated. The prevalence of larvae in samples of mackerel caught in the Southern Japan region and Sea of Japan was much lower than that in mackerel caught in other areas. Both the prevalence of *Anisakis* larvae in all samples and their abundance in larvae-positive samples exhibited specific seasonal variations, being high in spring.

Keywords: *Anisakis*, food-borne disease, contamination

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Oshikata C[*1,2], Watanabe M, Hashimoto K[*3], Yamazaki A[*4], Kobayashi N[*5], Konuma R[*6], Ishida M[*7], Kobayashi S[*7], Shimada T[*8], Kaneko T[*2], Kamata Y[*9], Kuriyama S[*10], Kure S[*10], Yanai M[*7], Tsurikisawa N[*1,2]: Effects of mite allergen avoidance in children in two distant towns in Japan.

Rev Fr Allergol. 2022;62:661-669. doi: 10.1016/j.reval.2022.03.012.

We investigated the prevalence of asthma, rhinitis, and atopic dermatitis and evaluated changes in mite allergen levels on mattresses and allergic symptoms in children after allergen avoidance in two distant regions of Japan after the Great East Japan Earthquake. We performed an International Study of Asthma and Allergies in Childhood survey of 49 school children aged 6-10 years in Iwanuma in 2017 and 57 school children aged 6-10 years in Oiso in 2018, and measured levels of Dermatophagoides group 1 (Der 1) in their bedding. Children in the intervention group attended an allergen avoidance seminar. In the same season the following year, we again measured Der 1 level in bedding and examined changes in allergic symptoms. The Der 1 level in the intervention group in both towns significantly decreased after implementing allergen avoidance ($P < 0.01$) but did not decrease in either non-intervention group. In each town, symptoms of allergic rhinitis were significantly lower ($P < 0.05$)

among children whose Der 1 level were reduced to less than 10% of initial levels. Allergen avoidance ameliorated allergic symptoms in school children.
Keywords: allergic symptoms, children, Der 1

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Watanabe M, Ohnishi T, Arai S, Kawakami T, Hayashi K, Ohya K, Hirose S, Yoshinari T, Taharaguchi S[*1], Mekata H[*2], Taniguchi T[*3], Ikarashi T, Honma M, Goda Y, Hara-Kudo Y: Survival of SARS-CoV-2 and bovine coronavirus on common surfaces of living environments.
Sci Rep. 2022;12(1):10624. doi: 10.1038/s41598-022-14552-9.

To understand the survival potential of the virus, the viral titers of bovine coronavirus (BCoV), as a model virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were measured on porous and non-porous surfaces. The amount of infectious BCoV recovered remained relatively high on non-porous substrates. However, it quickly decreased on several non-porous surfaces such as nitrile rubber. On porous substrates other than non-woven masks, the amount of virus recovered quickly decreased, and then remained at a low level. The decrease in the amount of infectious virus recovered was similar to that of BCoV, although that of SARS-CoV-2 was more rapid. RNA derived from SARS-CoV-2 remained on surfaces much longer than infectious virus, on all substrates. Therefore, it is important to measure the viral titer to avoid the overestimation of infectious virus contamination in the environments. Our results suggest that the surface structure was not directly related to viral survivability.
Keywords: SARS-CoV-2, BCoV, porous, surface structure

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Yoshinari T, Watanabe M, Hara-Kudo Y: Cross-genus inhibitory activity of polyoxins against aflatoxin production by *Aspergillus parasiticus* and fumonisin production by *Fusarium fujikuroi*.
FEMS Microbiol Lett. 2022;369(1):fnac048. doi: 10.1093/femsle/fnac048.

Co-exposure to aflatoxin and fumonisin is a health concern where corn is a staple food, and a method to prevent co-contamination of these mycotoxins in foods is urgently needed. Polyoxins are chitin synthase inhibitors produced by *Streptomyces cacaoi* var. *asoensis*. The aflatoxin production inhibitory activity of a commercially available polyoxin D and four polyoxins purified from polyoxin AL water-soluble powder, an agricultural chemical containing polyoxins, was tested. The five polyoxins dose-dependently inhibited aflatoxin production by *Aspergillus parasiticus* and the IC_{50} values of polyoxin A, B, D, K and L were 16, 74, 110, 9 and 280 μmol/L, respectively. Polyoxins also inhibited fumonisin production by *Fusarium fujikuroi*, and the IC_{50} values of polyoxin B, D, K and L were 270, 42, 65 and 62 μmol/L, respectively. These results suggest that a mixture of polyoxins may effectively prevent co-contamination of aflatoxin and fumonisin in foods.
Keywords: aflatoxin, fumonisin, polyoxin, inhibitor

オブライエン悠木子[*1]，渡辺麻衣子，池内隼佑[*1]，Tran Vu Linh[*1,2]，Bui Thi Hien[*1]，林谷秀樹[*1]：本邦で市販される愛玩鳥の餌における真菌汚染の状況.
日本獣医師会雑誌 2023;76:e51-e54. doi: 10.12935/jvma.76.e51.

平成21年に「愛がん動物用飼料の安全性の確保に関する法律」（ペットフード安全法）が施行され，犬猫の餌は一定の安全性が担保された一方，愛玩鳥の餌は同法の範疇外であり，微生物や化学物質汚染の実態は調べられていない．本研究では，愛玩鳥の餌を汚染する可能性のある微生物のうち，特に真菌に焦点を当て分離・同定を行った．その結果，愛玩鳥の餌72検体のうち58検体（80.6％）から真菌が分離された．また，分離された真菌182菌株中157菌株が同定され，*Aspergillus*属の真菌が最も多く89菌株（56.7％）であった．愛玩鳥の餌は海外からの輸入が多く，亜熱帯・熱帯由来の*Aspergillus*はカ

ビ毒産生株，特にアフラトキシン産生性が高い菌株が多いことから，今後分離株のカビ毒産生性を調べる必要がある．

Keywords：真菌，*Aspergillus*属，ペットフード安全法，愛玩鳥餌

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都丸亜希子*，登田美桜，工藤由起子：日本のヒスタミン食中毒事例における魚種およびヒスタミン生成菌に関する文献情報解析．

食品衛生学会誌 2022;63(3):109-116. doi: 10.3358/shokueishi.63.109.

1998年から2020年に国内で発生したヒスタミン食中毒事例の傾向を解析した結果，ヒスタミン食中毒は毎年発生し，１年あたりの平均事例数は9.7件，患者数は195.3名であった．施設別による事例数は，飲食店が最も多く，患者数では給食施設が最も多かった．食中毒の原因となった魚種は，マグロ，カジキおよびサバが主であった．文献情報調査の結果，国内に流通する魚種から単離されたヒスタミン生成菌は，23属であり，最も報告が多かった菌種は腸内細菌科である*Morganella morganii*であった．また，海洋性細菌である*Photobacterium damselae*の報告も多かったが，低温性の*Morganella psychrotolerans*や*Photobacterium phosphoreum*の報告もみられた．

Keywords：ヒスタミン食中毒，文献情報解析，魚種，ヒスタミン産生菌

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Fujita M, Tsuchiya K, Kurohara T, Fukuhara K*, Misawa T, Demizu Y: *In silico* optimization of peptides that inhibit Wnt/β-catenin signaling

Bioorg. Med. Chem. 2023;*84*:117264. doi: 10.1016/j.bmc.2023.117264

The Wnt/β-catenin signaling pathway causes transcriptional activation through the interaction between β-catenin and T cell-specific transcription factor (TCF) and regulates a wide variety of cellular responses, including proliferation, differentiation and cell motility. Excessive transcriptional activation of the Wnt/β-catenin pathway is implicated in developing or exacerbating various cancers. We have recently reported that liver receptor homolog-1 (LRH-1) -derived peptides inhibit the β-catenin/TCF interaction. In addition, we developed a cell-penetrating peptide (CPP)-conjugated LRH-1-derived peptide that inhibits the growth of colon cancer cells and specifically inhibits the Wnt/β-catenin pathway. Nonetheless, the inhibitory activity of the CPP-conjugated LRH-1-derived peptide was unsatisfactory (ca. 20 μM), and improving the bioactivity of peptide inhibitors is required for their *in vivo* applications. In this study, we optimized the LRH-1-derived peptide using *in silico* design to enhance its activity further. The newly designed peptides showed binding affinity toward β-catenin comparable to the parent peptide. In addition, the CPP-conjugated stapled peptide, Penetratin-st6, showed excellent inhibition (ca. 5 μM). Thus, the combination of *in silico* design by MOE and MD calculations has revealed that logical molecular design of PPI inhibitory peptides targeting β-catenin is possible. This method can be also applied to the rational design of peptide-based inhibitors targeting other proteins.

Keywords: *in silico* design, helical peptide, stapled peptide

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Osawa H, Kurohara T, Ito T, Shibata N, Demizu Y: CRBN ligand expansion for Hematopoietic Prostaglandin D_2 Synthase (H-PGDS) targeting PROTAC design and their *in vitro* ADME profiles

Bioorg. Med. Chem. 2023;84:117259. doi: 10.1016/j.bmc.2023.117259

An increasing number of research reports are describing modifications of the E3 ligand, in particular, cereblon (CRBN) ligands, to improve the chemical and metabolic stabilities as well as the physical properties of PROTACs. In this study, phenyl-glutarimide (PG) and 6-fluoropomalidomide (6-F-POM), recently used as CRBN ligands for PROTAC design, were applied to hematopoietic prostaglandin D2 synthase (H-PGDS) -targeted PROTACs. Both PROTAC-5 containing PG and PROTAC-6 containing 6-F-POM were found to have potent activities to induce H- PGDS degradation. Furthermore, we obtained *in vitro* ADME data on the newly designed PROTACS as well as our previously reported PROTACs(H-PGDS) series. Although all PROTACs(H-PGDS) are relatively stable toward metabolism, they had poor PAMPA values.

Nevertheless, PROTAC-5 showed Papp values similar to TAS-205, which is in Phase 3 clinical trials, and is expected to be the key to improving the pharmacokinetics of PROTACs.
Keywords: PROTAC, H-PGDS, ADME

Takada M, Ito T, Kurashima M, Matsunaga N, Demizu Y, Misawa T: Structure-Activity Relationship Studies of Substitutions of Cationic Amino Acid Residues on Antimicrobial Peptides.
Antibiotics (Basel). 2022;12(1):19. doi: 10.3390/antibiotics12010019.

Antimicrobial peptides (AMPs) have received considerable attention as next-generation drugs for infectious diseases. Amphipathicity and the formation of a stabilized secondary structure are required to exert their antimicrobial activity by insertion into the microbial membrane, resulting in lysis of the bacteria. We previously reported the development of a novel antimicrobial peptide, 17KKV, based on the Magainin 2 sequence. The peptide was obtained by increasing the amphipathicity due to the replacement of amino acid residues. Moreover, we studied the structural development of 17KKV and revealed that the secondary structural control of 17KKV by the introduction of non-proteinogenic amino acids such as α,α-disubstituted amino acids or side-chain stapling enhanced its antimicrobial activity. Among them, peptide 1, which contains 2-aminobutyric acid residues in the 17KKV sequence, showed potent antimicrobial activity against multidrug-resistant Pseudomonus aeruginosa (MDRP) without significant hemolytic activity against human red blood cells. However, the effects of cationic amino acid substitutions on secondary structures and antimicrobial activity remain unclear. In this study, we designed and synthesized a series of peptide 1 by the replacement of Lys residues with several types of cationic amino acids and evaluated their secondary structures, antimicrobial activity, hemolytic activity, and resistance against digestive enzymes.
Keywords: antimicrobial peptides (AMPs), cationic amino acid, chemical stability

Kurohara T, Ito T, Tsuji G, Misawa T, Yokoo H, Kawamura M, Shoda T, Hanajiri-Kikura R, Demizu Y: Comprehensive synthesis of 20 fentanyl derivatives for use as reference materials
Heterocycles 2023;106:82-93. doi: 10.3987/COM-22-14760

Fentanyl, a selective agonist of opioid μ receptors, is a broadly used clinical agent for anesthesia and pain relief. Despite its clinical benefits, the abuse of fentanyl and its derivatives causes a number of health concerns, which are increasing at an alarming rate; its abuse has become a serious social problem. These compounds are often difficult to obtain as reagents, which hinders forensic toxicological analysis. Therefore, it is important to address their unavailability by synthesizing the structural derivatives of fentanyl. In this study, we synthesized 20 fentanyl derivatives, such as o- or p-fluorofentanyl and furanylfentanyl, and determined their purities using HPLC (95.2%-100%). Moreover, the GC-MS analysis of the synthesized fentanyl derivatives was performed for the rapid differentiation of the synthesized fentanyl derivatives. We demonstrate that our method achieves a convenient and efficient synthesis of fentanyl derivatives.
Keywords: fentanyl, HPLC analysis, GC-MS analysis

辻厳一郎，伊藤貴仁，内山奈穂子，細江潤子，出水庸介，合田幸広：日本薬局方の国際化を目的とした各条の試験法変更に関する研究（第３報）：クロモグリク酸ナトリウムおよびトリヘキシフェニジル塩酸塩のHPLCによる定量法設定に向けた検討
Yakugaku Zasshi, 2023;143: 297-307. doi: 10.1248/yakushi.22-00188

The Japanese Pharmacopoeia (JP) is an official normative publication for establishing the authenticity and properties and maintaining the quality of pharmaceutical products in Japan. The JP is revised every five years and partially revised in order to respond to the progress of science and technology, the demand for medical care, and international harmonization. Thus, "Internationalization of the JP" is one of the most important issues to address for the revision of the JP, which is also referred to the basic principles for the preparation of the JP 19th edition. For instance, the incorporation of the test methods that have been used in other pharmacopeias, such as the European Pharmacopoeia (EP) and the United States Pharmacopeia (USP), into the JP is one of promising approaches. From this perspective, we have

recently reported changes in test methods, establishment of a quantitative test method for the JP-listed clonidine hydrochloride as well as lorazepam from using a potentiometric titration method to using HPLC method. As our ongoing study to change test methods for internationalization, we selected sodium cromoglicate and trihexyphenidyl hydrochloride. Each pharmaceutical product is analyzed using a potentiometric titration method as listed in the 18th JP; however, both the EP and the USP use HPLC method for quantitative analysis of these drugs.

In this study, we synthesized the related impurities of sodium cromoglicate and trihexyphenidyl hydrochloride listed in the EP and determined their purities using quantitative NMR. The separation conditions of these compounds were examined using HPLC and simultaneous analyses were performed.

Keywords: international harmonization, the Japanese Pharmacopoeia, HPLC

Murakami Y, Osawa H, Kurohara T, Yanase Y, Ito T, Yokoo H, Shibata N, Naito M[*1], Aritake K[*2], Demizu Y: Structure-activity relationship study of PROTACs against hematopoietic prostaglandin D_2 synthase

RSC Med. Chem. 2022;13:1495-1503. doi: 10.1039/d2md00284a

Degradation of hematopoietic prostaglandin D2 synthase (H-PGDS) by proteolysis-targeting chimeras (PROTACs) is expected to be important in the treatment of allergic diseases and Duchenne's muscular dystrophy. We recently reported that PROTAC(H-PGDS)-7 (PROTAC1), which is composed of H-PGDS inhibitor (TFC-007) and cereblon (CRBN) E3 ligase ligand (pomalidomide), showed potent H-PGDS degradation activity. Here, we investigated the structure-activity relationships of PROTAC1, focusing on the C4- or C5-conjugation of pomalidomide, in addition, the H-PGDS ligand exchanging from TFC-007 with the biaryl ether to TAS-205 with the pyrrole. Three new PROTACs were evaluated for H-PGDS affinity, H-PGDS degrading activity, and inhibition of prostaglandin D2 production. All compounds showed high H-PGDS degrading activities, but PROTAC (H-PGDS)-4-TAS-205 (PROTAC3) was slightly less active than the other compounds. Molecular dynamics simulations suggested that the decrease in activity of PROTAC3 may be due to the lower stability of the CRBN-PROTAC-H-PGDS ternary complex.

Keywords: PROTAC, H-PGDS, structure-activity relationship

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Tsuchiya K, Kiyoshi M, Hashii N, Fujita M, Kurohara T, Ishii-Watabe A, Fukuhara K[*], Misawa T, Demizu Y: Development of a penetratin-conjugated stapled peptide that inhibits Wnt/β-catenin signaling.

Bioorg Med Chem. 2022;73:117021. doi: 10.1016/j.bmc.2022.117021

Wnt/β-catenin pathway triggers the formation of a complex between β-catenin and T cell-specific transcription factor (TCF), which induces transcriptional activation. Excessive transcriptional activation of this pathway is associated with the development, cause, and deterioration of various cancers. Therefore, the Wnt/β-catenin pathway is an attractive drug target for cancer therapeutics and small molecule- and peptide-based protein-protein interaction (PPI) inhibitors have been developed. However, peptide-based PPI inhibitors generally have low cell-membrane permeability because of their large molecular size. To improve cell-membrane permeability, conjugating cell-penetrating peptides (CPPs) to PPI-inhibiting peptides is a useful method for developing intracellularly targeted PPI inhibitors. In this study, we focused on the interaction between β-catenin and liver receptor homologue-1 (LRH-1) and designed and synthesized a series of LRH-1-derived peptides to develop inhibitors against Wnt/β-catenin signaling. The results showed that a penetratin-conjugated LRH-1-derived peptide (Penetratin-st7) predominantly inhibited DLD-1 cell growth at 20 μM treatment via inhibition of the Wnt signaling pathway. This result suggests that Penetratin-st7 is one of promising PPI inhibitors between TCF and β-catenin.

Keywords: CPP-conjugated peptide, cell-penetrating peptide, protein-protein interaction

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Takyo M, Sato Y, Hirata N, Tsuchiya K, Ishida H[*], Kurohara T, Yanase Y, Ito T, Kanda Y, Yamamoto

K*, Misawa T, Demizu Y: Oligoarginine-Conjugated Peptide Foldamers Inhibiting Vitamin D Receptor-Mediated Transcription.
ACS Omega. 2022;7:46573-46582. doi: 10.1021/acsomega.2c05409.

The vitamin D receptor (VDR) is a nuclear receptor, which is involved in several physiological processes, including differentiation and bone homeostasis. The VDR is a promising target for the development of drugs against cancer and bone-related diseases. To date, several VDR antagonists, which bind to the ligand binding domain of the VDR and compete with the endogenous agonist 1α,25(OH)D_3, have been reported. However, these ligands contain a secosteroidal skeleton, which is chemically unstable and complicated to synthesize. A few VDR antagonists with a nonsecosteroidal skeleton have been reported. Alternative inhibitors against VDR transactivation that act via different mechanisms are desirable. Here, we developed peptide-based VDR inhibitors capable of disrupting the VDR-coactivator interaction. It was reported that helical SRC2-3 peptides strongly bound to the VDR and competed with the coactivator *in vitro*. Therefore, we designed and synthesized a series of SRC2-3 derivatives by the introduction of nonproteinogenic amino acids, such as β-amino acids, and by side-chain stapling to stabilize helical structures and provide resistance against digestive enzymes. In addition, conjugation with a cell-penetrating peptide increased the cell membrane permeability and was a promising strategy for intracellular VDR inhibition. The nonaarginine-conjugated peptides **24** with side-chain stapling and **25** with cyclic β-amino acids showed strong intracellular VDR inhibitory activity, resulting in suppression of the target gene expression and inhibition of the cell differentiation of HL-60 cells. Herein, the peptide design, structure-activity relationship (SAR) study, and biological evaluation of the peptides are described.
Keywords: vitamin D receptors, cell-penetrating peptides, protein-protein interaction

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Yokoo H, Misawa T, Kato T*1, Tanaka M*2, Demizu Y, Oba M*3: Development of delivery carriers for plasmid DNA by conjugation of a helical template to oligoarginine.
Bioorg. Med. Chem., 2022;72:116997. doi: 10.1016/j.bmc.2022.116997

Arginine (Arg)-rich peptides can penetrate the cell membrane and deliver nucleic acid-based therapeutics into cells. In this study, a helical template designed with a repeating sequence composed of two L-leucines (L-Leu) and a 2-aminoisobutyric acid (Aib) (L-Leu-L-Leu-Aib) was conjugated to nona-arginine on either the C- or N- terminus, designated as Block 1 and Block 2. Each terminal modification induced helical structure formation and improved the physicochemical properties of peptide/plasmid DNA (pDNA) complexes, resulting in efficient intracellular pDNA delivery. The introduction of a helical template may be effective for the endosomal escape of pDNA and pDNA release from complexes in cells. These results emphasized the potency of a helical template for the development of novel cell-penetrating peptides for pDNA delivery.
Keywords: cell-penetrating peptide, arginine, gene transfer

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Katagiri K*, Kuriyama M*, Yamamoto K*, Demizu Y, Onomura O*: Organocatalytic synthesis of phenols from diaryliodonium salts with water under metal-free conditions.
Org. Lett. 2022;24:5149-54. doi: 10.1021/acs.orglett.2c01989

The metal-free synthesis of phenols from diaryliodonium salts with water was developed by using N-benzylpyridin-2-one as an organocatalyst. In this process, sterically congested, functionalized, and heterocycle-containing iodonium salts were smoothly converted to the desired products, and the clofibrate and mecloqualone derivatives were also synthesized in high yields. In addition, the gram-scale experiment was successfully carried out with 10 mmol of a sterically congested substrate.
Keywords: organocatalyst, phenol, metal-free condition

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Xu H, Kurohara T, Takano R, Yokoo H, Shibata N, Ohoka N, Inoue T, Naito M*, Demizu Y: Development of rapid and facile solid-phase synthesis of PROTACs via a variety of binding styles
ChemistryOpen 2022;11:e303300131. doi: 10.1002/open.202200131

Optimizing linker design is important for ensuring efficient degradation activity of proteolysis-targeting chimeras (PROTACs). Therefore, developing a straightforward synthetic ap- proach that combines the protein-of-interest ligand (POI ligand) and the ligand for E3 ubiquitin ligase (E3 ligand) in various binding styles through a linker is essential for rapid PROTAC syntheses. Herein, a solid-phase approach for convenient PROTAC synthesis is presented. We designed azide intermediates with different linker lengths to which the E3 ligand, pomalidomide, is attached and performed facile PROTACs synthesis by forming triazole, amide, and urea bonds from the intermediates.

Keywords: BRD4, H-PGDS, PROTAC

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Takada H, Tsuchiya K, Demizu Y: Helix-stabilized cell-penetrating peptides for delivery of antisense morpholino oligomers: Relationships among helicity, cellular uptake, and antisense activity.
Bioconjug. Chem. 2022;38:1311-18. doi: 10.1021/acs.bioconjchem.2c00199

The secondary structures of cell-penetrating peptides (CPPs) influence their properties including their cell-membrane permeability, tolerability to proteases, and intracellular distribution. Herein, we developed helix-stabilized arginine-rich peptides containing α,α-disubstituted α-amino acids and their conjugates with antisense phosphorodiamidate morpholino oligomers (PMOs), to investigate the relationships among the helicity of the peptides, cellular uptake, and antisense activity of the peptide-conjugated PMOs. We demonstrated that helical CPPs can efficiently deliver the conjugated PMO into cells compared with non-helical CPPs and that their antisense activities are synergistically enhanced in the presence of an endosomolytic reagent or endosomal escape domain peptide.

Keywords: cell-penetrating peptide, antisense oligonucleotide, peptide-conjugated PMO

Yamamoto K*, Suganomata Y*, Inoue T*, Kuriyama M*, Demizu Y, Onomura O*: Copper-catalyzed asymmetric oxidative desymmetrization of 2-substituted 1,2,3-triols.
J. Org. Chem. 2022;87:6479-91. doi: 10.1021/acs.joc.2c00398

Asymmetric oxidative desymmetrization of 2-substituted glycerols has been achieved by using a new chiral bisoxazoline ligand/copper catalyst system with 1,3-dibromo-5,5-dimethylhydantoin and MeOH. The present transformation smoothly proceeds with readily accessible 2-(hetero)aryl- and alkyl-substituted glycerols and provides straightforward access toward various glycerate derivatives in good to high yields with high enantioselectivities. The synthetic utility of the present protocol was demonstrated by the transformation of the optically active glycerol into a glyceraldehyde derivative.

Keywords: ssymmetric oxidative desymmetrization, catalyst, glycerol

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Tsuji G, Nakajima S*[1,2], Watashi K*[1,2,3], Torii S*[4,5], Suzuki R*[6], Fukuhara T*[6], Ohoka N, Inoue T, Demizu Y: Antiviral activity of ciclesonide acetal derivatives blocking SARS-CoV-2 RNA replication.
J. Pharmacol. Sci. 2022;149:81-4. doi: 10.1016/j.jphs.2022.04.001

Ciclesonide (**Cic**) is approved as an inhalant for asthma and was clinically tested as a candidate therapy for coronavirus disease 2019 (COVID-19). Its active metabolite **Cic2** was recently reported to suppress genomic RNA replication of severe acute respiratory syndrome coronavirus 2. In this study, we designed and synthesized a set of ciclesonide-acetal (Cic-acetal) derivatives. Among designated compounds, some Cic-acetal derivatives with a linear alkyl chain exhibited strong viral copy-number

reduction activities compared with **Cic2**. These compounds might serve as lead compounds for developing novel anti-COVID-19 agents.
Keywords: SARS-CoV2, antiviral activity, ciclesonide

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Yanase Y, Tsuji G, Nakamura M, Shibata N, Demizu Y: Control of STING agonistic/antagonistic activity using amine skeleton-based c-di-GMP analogues.
Int. J. Mol. Sci. 2022;23:6847. doi: 10.3390/ijms23126847

Stimulator of Interferon Genes (STING) is a type of endoplasmic reticulum (ER)-membrane receptor. STING is activated by a ligand binding, which leads to an enhancement of the immune-system response. Therefore, a STING ligand can be used to regulate the immune system in therapeutic strategies. However, the natural (or native) STING ligand, cyclic-di-nucleotide (CDN), is unsuitable for pharmaceutical use because of its susceptibility to degradation by enzymes and its low cell-membrane permeability. In this study, we designed and synthesized CDN derivatives by replacing the sugar-phosphodiester moiety, which is responsible for various problems of natural CDNs, with an amine skeleton. As a result, we identified novel STING ligands that activate or inhibit STING. The cyclic ligand **7**, with a cyclic amine structure containing two guanines, was found to have agonistic activity, whereas the linear ligand **12** showed antagonistic activity. In addition, these synthetic ligands were more chemically stable than the natural ligands.
Keywords: STING, cyclic dinucleotide, amines

Tsuchiya K, Kurohara T, Fukuhara K*, Misawa T, Demizu Y: Helical Foldamers and stapled peptides as new modalities in drug discovery: Modulators of protein-protein interactions.
Processes, 2022:10;924. doi: 10.3390/pr10050924

A “foldamer” is an artificial oligomeric molecule with a regular secondary or tertiary structure consisting of various building blocks. A “stapled peptide” is a peptide with stabilized secondary structures, in particular, helical structures by intramolecular covalent side-chain cross-linking. Helical foldamers and stapled peptides are potential drug candidates that can target protein-protein interactions because they enable multipoint molecular recognition, which is difficult to achieve with low-molecular-weight compounds. This mini-review describes a variety of peptide-based foldamers and stapled peptides with a view to their applications in drug discovery, including our recent progress.
Keywords: foldamers, protein-protein interaction, helical structure

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辻厳一郎，内山奈穂子，合田幸広，出水庸介：薬局方各条における有害試薬の可及的排除に関する研究（第４報）
医薬品医療機器レギュラトリーサイエンス 2022;53:37-52. doi: 10.51018/pmdrs.53.1_37

日本薬局方（JP）は，医薬品の性状及び品質の適正を図るために定められた，日本の医薬品の公的な規範書であり，JPで規定される医薬品の試験法には普遍性が要求される．JPは５年ごとに改正が行われており，科学技術の進展並びに国際調和に対応するため，部分改正等を適宜行っている．筆者らは，第十八改正日本薬局方作成基本方針に掲げられた「保健医療上重要な医薬品の全面的収載」，「最新の学問・技術の積極的導入による質的向上」，「医薬品のグローバル化に対応した国際化の一層の推進」，「必要に応じた速やかな部分改正及び行政によるその円滑な運用」，「日本薬局方改正過程における透明性の確保及び日本薬局方の普及」の５本柱の中で，「最新の学問・技術の積極的導入による質的向上」として，薬局方試験法における有害試薬の可及的排除を目的とした代替試験法，定量法改定等の検討を行ってきた．令和元年度は，各条（クロニジン塩酸塩）を対象として，低毒性溶媒への検討を行うとともに，定量法として設定されている電位差滴定をより簡便HPLC法への変更（米国薬局方（USP）や欧州薬局方（EP）では既にHPLC法が設定されている）についての検討を行った．このように，USPやEPなど他局で規定されているより優れた試験方法についてJPでの採用の可能性を検討し，国内外の関係者に利用されるようJPの普及を図ることは，医

薬品各条の国際調和という観点で重要である．第十九改正日本薬局方作成基本方針（案）においても，引き続き「最新の学問・技術の積極的導入による質的向上」が５本の柱として掲げられている．また，「日本薬局方改正過程における透明性の確保及び日本薬局方の国内外への普及」も挙げられている．令和２年度は，ロラゼパムを対象として，合成した類縁物質の定量NMR（qNMR）による純度決定，定量法の設定に向けたHPLCクロマトグラム上における各化合物の完全分離の条件を検討した．更に，トリヘキシフェニジル塩酸塩及びクロモグリク酸ナトリウムについても同様に，試験法の設定を目的として類縁物質の合成，qNMRによる純度決定，HPLCにおける各化合物の完全分離条件の検討を行った．

Keywords: international harmonization, the Japanese Pharmacopoeia, HPLC

Naganuma M, Ohoka N, Tsuji G, Tsujimura H, Matsuno K[*1], Inoue T, Naito M[*2], Demizu Y:
Development of chimeric molecules that degrade the estrogen receptor using decoy oligonucleotide ligands.
ACS Med. Chem. Lett. 2022;13:134-9. doi: 10.1021/acsmedchemlett.1c00629

Targeted protein degradation using chimeric small molecules, such as proteolysis-targeting chimeras (PROTACs) and specific and nongenetic inhibitors of apoptosis protein (IAP)-dependent protein erasers (SNIPERs), has attracted attention as a method for degrading intracellular target proteins via the ubiquitin-proteasome system (UPS). These chimeric molecules target a variety of proteins using small molecules that can bind to the proteins. However, it is difficult to develop such degraders in the absence of suitable small-molecule ligands for the target proteins, such as for transcription factors (TFs). Therefore, we constructed the chimeric molecule **LCL-ER(dec)**, which consists of a decoy oligonucleotide that can bind to estrogen receptor α (ERα) and an IAP ligand, LCL161 (LCL), in a click reaction. **LCL-ER(dec)** was found to selectively degrade ERα via the UPS. These findings will be applicable to the development of other oligonucleotide-type degraders that target different TFs.

Keywords: ubiquitin-proteasome system protein knockdown decoy

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Takabatake R[*1], Egi T[*2], Soga K, Narushima J, Yoshiba S, Shibata N, Nakamura K, Kondo K, Kishine M[*1], Mano J[*1], Kitta K[*1]:
Development and interlaboratory validation of a novel reproducible qualitative method for GM soybeans using comparative Cq-based analysis for the revised non-GMO labeling system in Japan.
Analytical Chemistry. 2022;94:13447-54. doi: 10.1021/acs.analchem.2c02447

Many countries have implemented the labeling system of genetically modified organisms (GMO). In Japan, the regulatory threshold for non-GMO labeling will be revised and restricted to undetectable by April 2023. The practical criterion for the revised system is based on the limit of detection (LOD). However, determining whether the commingling of GMO levels exceeds the LOD is challenging because GM contents close to the LOD are usually below the limit of quantification. In this study, we developed a qualitative method based on comparative Cq-based analysis targeting cauliflower mosaic virus 35S promoter and GM soybean MON89788 event-specific sequences that could be applicable to the revised non-GMO labeling. ΔCq values between the target and endogenous sequences were calculated, and the ΔΔCq value obtained was used as a criterion to determine analytical samples with GM contents exceeding the threshold. To improve the reproducibility of the method, we used a standard plasmid that yields equivalent and stable ΔCq values comparable with those obtained from LOD samples. The developed method was validated with an interlaboratory study. The new qualitative detection concept would be useful for ensuring robust and reproducible results among laboratories, particularly for detecting low-copy-number DNA samples.

Keywords: genetically modified soybean, real-time PCR, limit of detection

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Soga K, Nakamura K, Egi T[*1], Narushima J, Yoshiba S, Kishine M[*2], Mano J[*2], Kitta K[*2], Takabatake R[*2], Shibata N, Kondo K: Development and validation of a new robust detection method for low-content DNA using ΔΔCq-based real-time PCR with optimized standard plasmids as a control sample.

Analytical Chemistry. 2022;94:14475-83. doi: 10.1021/acs.analchem.2c03680

Real-time polymerase chain reaction (PCR) is the gold standard for DNA detection in many fields, including food analysis. However, robust detection using a real-time PCR for low-content DNA samples remains challenging. In this study, we developed a robust real-time PCR method for low-content DNA using genetically modified (GM) maize at concentrations near the limit of detection (LOD) as a model. We evaluated the LOD of real-time PCR targeting two common GM maize sequences (P35S and TNOS) using GM maize event MON863 containing a copy of P35S and TNOS. The interlaboratory study revealed that the LOD differed among laboratories partly because DNA input amounts were variable depending on measurements of DNA concentrations. To minimize this variability for low-content DNA samples, we developed ΔΔCq-based real-time PCR. In this study, ΔCq and ΔΔCq are as follows: ΔCq = Cq (P35S or TNOS) − Cq (SSIIb; maize endogenous gene), ΔΔCq = ΔCq (analytical sample) − ΔCq (control sample at concentrations near the LOD). The presence of GM maize was determined based on ΔΔCq values. In addition, we used optimized standard plasmids containing SSIIb, P35S, and TNOS with ΔCq equal to the MON863 genomic DNA (gDNA) at concentrations near the LOD as a control sample. A validation study indicated that at least 0.2% MON863 gDNA could be robustly detected. Using several GM maize certified reference materials, we have demonstrated that this method was practical for detecting low-content GM crops and thus for validating GM food labeling. With appropriate standards, this method would be applicable in many fields, not just food.

Keywords: genetically modified maize, real-time PCR, limit of detection

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Narushima J, Kimata S, Shiwa Y[*1], Gondo T[*2], Akimoto S, Soga K, Yoshiba S, Nakamura K, Shibata N, Kondo K: Unbiased prediction of off-target sites in genome-edited rice using SITE-Seq analysis on a web-based platform.

Genes to Cells. 2022;27:706-18. doi: 10.1111/gtc.12985

Genome-editing using the CRISPR-Cas9 system has the potential to substantially accelerate crop breeding. Since off-target editing is one of problems, a reliable method for comprehensively detecting off-target sites is needed. A number of *in silico* methods based on homology to on-target sequence have been developed, however the prediction without false negative is still under discussion. In this study, we performed a SITE-Seq analysis to predict potential off-target sites. SITE-Seq analysis is a comprehensive method that can detect double-strand breaks *in vitro*. Furthermore, we developed a systematic method using SITE-Seq in combination with web-based Galaxy system (Galaxy for Cut Site Detection), which can perform reproducible analyses without command line operations. We conducted a SITE-Seq analysis of a rice genome targeted by *OsFH15* gRNA-Cas9 as a model, and found 41 candidate off-target sites in the annotated regions. Detailed amplicon-sequencing revealed mutations at one off-target site in actual genome-edited rice. Since this off-target site has an uncommon protospacer adjacent motif, it is difficult to predict using *in silico* methods alone. Therefore, we propose a novel off-target assessment scheme for genome-edited crops that combines the prediction of off-target candidates by SITE-Seq and *in silico* programs and the validation of off-target sites by amplicon-sequencing.

Keywords: genome-editing, off-target, SITE-Seq

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Shibata N, Soga K, Sugino M, Narushima J, Yoshiba S, Egi T[*1], Takabatake R[*2], Kondo K: Evaluation of conversion factor for rapid quantification of authorized genetically modified maize and soybean in Japan.

Biological and Pharmaceutical Bulletin Reports. 2022;

5:115-20. doi: 10.1248/bpbreports.5.5_11

With the increasing development of genetically modified (GM) crops authorized for use in food, a rapid and accurate method of quantifying the weight-based amount of GM crops is needed to ensure consumers' rights to choose. Conversion factor (C_f) value is the ratio of the copy number of a GM-specific sequence to an endogenous sequence in the GM crop and is used to convert a copy number ratio of the GM-specific sequence to the endogenous sequence of a sample into weight-based amount of GM crops. However, in the current Japanese official method for GM crops, determining C_f values using real-time PCR instruments capable of rapid measurements has not been established. In this study, C_f values for GM maize and soybean authorized for use as food in Japan were experimentally determined using an Applied Biosystems 7500 Fast Real-Time PCR System, which is capable of rapid measurement. The C_f values were almost the same as those of the PCR instruments described in the Japanese official method, and the weight-based amount of GM maize MON810 measured using this C_f value showed similar results. These results suggest that rapid quantification by this PCR instrument has the same performance as the recommended PCR instruments and may contribute to the labeling regulation of GM crops in Japan.

Keywords: genetically modified food, rapid quantitative method, conversion factor

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Yamada T*1, Furutaka K*1, Hachinohe M*2 and Hachisuka A: Applicability of non-destructive equipment for radioactivity measurement to screening radio-cesium in foods.
Applied Radiation and Isotope. 2023;194:110671. doi: 10.1016/j.apradiso.2023.110671

This study aimed to evaluate the applicability of non-destructive radioactivity measurement equipments for screening radio-cesium in whole foods without sample preparation procedures. Wild mushrooms and bamboo shoots were collected and studied using five different non-destructive radioactivity devices, and activity concentration was determined by conventional gamma-ray spectrometry using a Ge-detector. Linear regression analyses of activity concentrations were conducted and prediction intervals determined as uncertainties. Overall, non-destructive radioactivity measurement devices found to be suitable for screening radioactive cesium contamination in foods with an effective screening level.

Keywords: radioactivity measurement, non-destructive inspection, screening, cesium, gamma-ray spectrometry

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Okazaki F*1, Momma K*2, Hirakawa Y*2, Kawai N*3, Yamaguchi-Murakami Y*3, Adachi R, Yuji Mori Y*4, Kondo Y*4, Narita H*2,3: Determination of Severe Peach Allergens, Gibberellin-Regulated Protein, and Lipid Transfer Protein, Using Monoclonal Antibodies.
J Nutr Sci Vitaminol. 2022;68:221-227. doi: 10.3177/jnsv.68.221.

In this study, monoclonal antibodies against two major fruit allergens-gibberellin-regulated protein (GRP) and lipid transfer protein (LTP)-were established. Sandwich enzyme-linked immunosorbent assays (ELISAs) for the quantification of peach GRP and LTP were constructed using these antibodies. Both ELISAs reacted with the respective antigens when heated at 100℃ for 20 min, but not when reduced with sodium sulfite, indicating that GRP and LTP are heat-stable, while disulfide bonds play an important role in their native steric structures. GRP and LTP in peaches and peach-containing foods were quantified by these ELISAs. In both cases, there were few differences among peach cultivars normally available on the market; however, concentrations were higher when the peach was ripe. GRP was localized in the pulp of the peach, while LTP was present in the peel. They could be quantified in peach-containing beverages, as well as in dried and canned peaches. GRP in Japanese apricots could also be determined using this ELISA, as its amino acid sequence is the same as that of peach GRP. Then, high concentrations of GRP were detected in umeboshi, a traditional Japanese pickled apricot. Peach leaves were found to have a high LTP content, accordingly, LTP was also observed in lotions containing peach leaf extract. The

ability to quantitatively detect GRP and LTP in this study will, therefore, contribute to the improvement of component-resolved diagnoses and quality of life in patients allergic to peaches.

Keywords: GRP, LTP, allergen labeling

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Nishimaki-Mogami T, Ito S*, Cui H, Akiyama T, Tamehiro N, Adachi R, Wakamatsu K*, Ikarashi Y, Kondo K: A cell-based evaluation of human tyrosinase-mediated metabolic activation of leukoderma-inducing phenolic compounds.

J Dermatol Sci. 2022;108:77-86. doi: 10.1016/j.jdermsci.2022.12.002

Background: Chemical leukoderma is a skin depigmentation disorder induced through contact with certain chemicals, most of which have a p-substituted phenol structure similar to the melanin precursor tyrosine. The tyrosinase-catalyzed oxidation of phenols to highly reactive o-quinone metabolites is a critical step in inducing leukoderma through the production of melanocyte-specific damage and immunological responses.

Objective: Our aim was to find an effective method to evaluate the formation of o-quinone by human tyrosinase and subsequent cellular reactions.

Methods: Human tyrosinase-expressing 293T cells were exposed to various phenolic compounds, after which the reactive o-quinones generated were identified as adducts of cellular thiols. We further examined whether the o-quinone formation induces reductions in cellular GSH or viability.

Results: Among the chemicals tested, all 7 leukoderma-inducing phenols/catechol (rhododendrol, raspberry ketone, monobenzone, 4-tert-butylphenol, 4-tert-butylcatechol, 4-S-cysteaminylphenol and p-cresol) were oxidized to o-quinone metabolites and were detected as adducts of cellular glutathione and cysteine, leading to cellular glutathione reduction, whereas 2-S-cysteaminylphenol and 4-n-butylresorcinol were not. *In vitro* analysis using a soluble variant of human tyrosinase revealed a similar substrate-specificity. Some leukoderma-inducing phenols exhibited tyrosinase-dependent cytotoxicity in this cell model and in B16BL6 melanoma cells where tyrosinase expression was effectively modulated by siRNA knockdown.

Conclusion: We developed a cell-based metabolite analytical method to detect human tyrosinase-catalyzed formation of o-quinone from phenolic compounds by analyzing their thiol-adducts. The detailed analysis of each metabolite was superior in sensitivity and specificity compared to cytotoxicity assays for detecting known leukoderma-inducing phenols, providing an effective strategy for safety evaluation of chemicals.

Keywords: Chemical leukoderma, Tyrosinase, ortho-Quinone

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Sugano Y*, Sakata K, Nakamura K, Hosokawa A*, Kouguchi H*, Suzuki T*, Kondo K: Loop-mediated isothermal amplification (LAMP) for rapid and easy identification of *Omphalotus japonicus*.

Food Chemistry: Molecular Sciences. 2022;5:100115. doi: 10.1016/j.fochms.2022.100115

Omphalotus japonicus is a major toxic mushroom in Japan. When food poisoning caused by *O. japonicus* occurs, quick and accurate identification using a method that does not rely on morphological discrimination is required. Because the loop-mediated isothermal amplification (LAMP) method meets these requirements, we developed a LAMP method for detecting *O. japonicus*. Amplification occurred within 60 min, and the presence or absence of *O. japonicus* was confirmed within 2 h, including the DNA extraction protocol. The LAMP method did not show cross-reactivity with 13 species of edible mushrooms, had high specificity toward *O. japonicus*, and had sufficient detection sensitivity even in a mixed mushroom sample containing 1% *O. japonicus*. Additionally, *O. japonicus* could be detected in simulated food poisoning samples of heated and digested mushrooms, and in actual food poisoning residual samples.

Keywords: loop-mediated isothermal amplification, toxic mushroom, genetic identification

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Watanabe T, Kataoka Y, Hayashi K, Matsuda R, Uneyama C: Dietary exposure of the Japanese general population to elements: Total diet study 2013-2018.

Food Safety. 2022;10:83-101. doi: 10.14252/foodsafetyfscj.D-22-00003

Some countries have conducted a total diet study (TDS) focused on the estimation of specific trace elements. Although some results of a Japanese TDS examining trace elements were published, there have been no reports of a nationwide TDS across Japan over a multi-year period to estimate the level of exposure to multiple elements. In the present study, a TDS using a market basket approach was performed to estimate the dietary exposure levels of the general population of Japan to 15 elements, including aluminum (Al), total arsenic (tAs), boron (B), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), total mercury (THg), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), tin (Sn), and uranium (U). Samples prepared in eight regions across Japan over a 6-year period were analyzed using validated methods. The robust mean exposure estimates for Al, tAs, B, Ba, Cd, Co, Cr, THg, Mo, Ni, Pb, Sb, Se, Sn, and U were 48, 4.2, 29, 8.6, 0.35, 0.17, 0.49, 0.14, 4.2, 2.8, 0.15, 0.022, 1.8, 0.10, and 0.021 μg/kg body weight/day, respectively. Although the variability in exposure estimates varied greatly from element to element, the relative standard deviations calculated from the robust means and robust standard deviations were ≤ 50% for all elements except Sn. Compared against the health-based guidance values, none of the robust and precise estimates obtained for the target elements would be associated with urgent health risk concern. In addition, the estimated exposure levels were generally in agreement with previously reported estimates, indicating that health risks associated with exposure to these elements have not changed markedly nationwide in Japan in recent years.

Keywords: total diet study, exposure assessment, element

Kageyama D[†*1], Harumoto T[†*2,3], Nagamine K[*1], Fujiwara A[*4,5], Sugimoto TN[*1], Jouraku A[*1], Tamura M, Katoh TK[*6,7], Watada M[*6,7]: A male-killing gene encoded by a symbiotic virus of *Drosophila*.

Nature Communications. 2023;14:1357. doi: 10.1038/s41467-023-37145-0.

In most eukaryotes, biparentally inherited nuclear genomes and maternally inherited cytoplasmic genomes have different evolutionary interests. Strongly female-biased sex ratios that are repeatedly observed in various arthropods often result from the male-specific lethality (male-killing) induced by maternally inherited symbiotic bacteria such as *Spiroplasma* and *Wolbachia*. However, despite some plausible case reports wherein viruses are raised as male-killers, it is not well understood how viruses, having much smaller genomes than bacteria, are capable of inducing male-killing. Here we show that a maternally inherited double-stranded RNA (dsRNA) virus belonging to the family Partitiviridae (designated DbMKPV1) induces male-killing in *Drosophila*. DbMKPV1 localizes in the cytoplasm and possesses only four genes, i.e., one gene in each of the four genomic segments (dsRNA1-dsRNA4), in contrast to ca. 1000 or more genes possessed by *Spiroplasma* or *Wolbachia*. We also show that a protein (designated PVMKp1; 330 amino acids in size), encoded by a gene on the dsRNA4 segment, is necessary and sufficient for inducing male-killing. Our results imply that male-killing genes can be easily acquired by symbiotic viruses through reassortment and that symbiotic viruses are hidden players in arthropod evolution. We anticipate that host-manipulating genes possessed by symbiotic viruses can be utilized for controlling arthropods.

Keywords: pest control, male-killing, symbiotic virus

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Sun Y, Nitta S[*1], Saito K, Hosogai R[*1], Nakai K[*1], Goda R[*2], Shimizu H[*3], Fujita H[*3], Kakehi M[*3], Murata K[*4], Yamaguchi T[*4], Okuzono T[*5], Yamane S[*5], Kawabata M[*6], Matsunuma T[*7], Takahara K[*7], Kato N[*8], Yamada M[*8], Yoshida T, Inoue T, Saito Y: Development and multicenter validation of an LC/MS-based bioanalytical method for antisense therapeutics.

Bioanalysis. 2022;14:113-122. doi: 10.4155/bio-2022-0126.

Background: Many bioanalytical methods for antisense oligonucleotides (ASOs) using LC-MS have been reported. However, no data have been available on the reproducibility and robustness of a single bioanalytical method for ASOs. As such, in the current study, we evaluated the reproducibility and robustness of LC-MS-based bioanalytical methods for ASOs in multiple laboratories. Methods/Results: Seven independent laboratories were included in this study. Mipomersen was measured by ion-pairing LC-MS (IP-LC-MS) as a model ASO using different LC-MS. The validation results of calibration curve, accuracy, precision and selectivity met the criteria of conventional bioanalytical method validation guidelines using LC/GC-MS for drugs in all laboratories. Meanwhile, carryover (>20%) was detected in three laboratories. Conclusion: We first demonstrated the multicenter-validated IP-LC-MS bioanalytical method for ASOs. Our data showed that the method was sensitive, robust and reproducible. However, the occurrence of carryover should be carefully monitored in its future application.

Keywords: antisense oligonucleotide, clarity OTX, multilaboratory validation

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Arakawa N, Ushiki A[*1], Abe M[*2], Matsuyama S, Saito Yoshinobu[*3], Kashiwada T[*3], Horimasu Y[*4], Gemma A[*3], Tatsumi K[*2], Hattori N[*5], Tsushima K[*6], Miyashita K, Saito K, Nakamura R, Toyoda T, Ogawa K, Sato M[*7], Takamatsu K[*7], Mori K[*8], Nishiya T[*9], Izumi T[*10], Ohno Y[*10], Saito Yoshiro, Hanaoka M[*1]: Stratifin as a novel diagnostic biomarker in serum for diffuse alveolar damage.

Nat Commun. 2022;13(1):5854. doi: 10.1038/s41467-022-33160-9.

Among the various histopathological patterns of drug-induced interstitial lung disease (DILD), diffuse alveolar damage (DAD) is associated with poor prognosis. However, there is no reliable biomarker for its accurate diagnosis. Here, we show stratifin/14-3-3σ (SFN) as a biomarker candidate found in a proteomic analysis. The study includes two independent cohorts (including totally 26 patients with DAD) and controls (total 432 samples). SFN is specifically elevated in DILD patients with DAD, and is superior to the known biomarkers, KL-6 and SP-D, in discrimination of DILD patients with DAD from patients with other DILD patterns or other lung diseases. SFN is also increased in serum from patients with idiopathic DAD, and in lung tissues and bronchoalveolar lavage fluid of patients with DAD. *In vitro* analysis using cultured lung epithelial cells suggests that extracellular release of SFN occurs via p53-dependent apoptosis. We conclude that serum SFN is a promising biomarker for DAD diagnosis.

Keywords: drug-induced interstitial lung disease, diffuse alveolar damage, stratifin, diagnostic marker, respiratory distress syndrome, proteome

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Arakawa N, Matsuyama S, Matsuoka M[*1], Kitamura I[*2], Miyashita K, Kitagawa Y[*1], Imai K[*3], Ogawa K, Maeda T[*1], Saito Y, Hasegawa C[*2]: Serum stratifin and presepsin as candidate biomarkers for early detection of COVID-19 disease progression.

J Pharmacol Sci. 2022;150(1):21-30. doi: 10.1016/j.jphs.2022.06.002.

The prognosis of patients with severe cases of COVID-19 is poor; thus, biomarkers for earlier prediction of COVID-19 progression are vital. We measured levels of five lung injury-related biomarkers, SP-D, KL-6, presepsin, kallistatin and stratifin, in serum samples collected serially during hospitalization from 31 patients with mild/moderate or severe/critical COVID-19 pneumonia, and their predictive performances were compared. Like the previously reported presepsin, a new biomarker candidate, stratifin, was significantly elevated with the onset of severe or critical symptoms in COVID-19 patients and decreased with symptom improvement. Notably, changes in stratifin and presepsin levels were distinctly earlier than those in SP-D, KL-6 and even SpO_2/FiO_2 values. Furthermore, serum levels of these biomarkers were significantly higher at the pre-severe stage (before the start of oxygen support) of patients who eventually advanced to severe/critical stages than in the patients who remained at the mild/moderate stage. These results were confirmed in an independent cohort, including 71 mild/moderate and 14 severe/critical patients, for whom the performance of stratifin and presepsin in discriminating between mild/moderate and pre-severe conditions of COVID-19 patients was superior to that of the SpO_2/FiO_2 ratio. Therefore, we concluded that stratifin and presepsin could be used as prognostic biomarkers for severe COVID-19 progression.

Keywords: COVID-19, predictive biomarker, presepsin, stratifin

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Maeda K[*1], Takikawa H[*2], Aiso M[*2], Tsuji K[*3], KagawaT[*4], Watanabe M[*5], Sato K[*6], Sakisaka S[*7], Hiasa Y[*8], Takei Y[*9], Ohira H[*10], Hashimoto E[*11], Ayada M[*12], Ikegami T[*13], Arakawa N, Kusuhara H[*14], Saito Y, Sugiyama Y[*15]: Frequency of null genotypes of glutathione S-transferase M1 and T1 in Japanese patients with drug-induced liver injury.

Hepatol Res. 2022;52(10):882-887. doi: 10.1111/hepr.13812.

Aim: Previous reports suggested that null genotype (*0/*0) of glutathione S-transferase (GST) M1 and/or GSTT1 could be one of the risk factors of drug-induced liver injury (DILI), however multi-institutional pharmacogenetic research with various suspected drugs on this issue had rarely been performed in Japan. Therefore, the aim of this study is to investigate the role of GSTM1 and GSTT1 null genotype in the occurrence of DILI in Japanese patients. Methods: Blood samples of 270 DILI cases from 23 hospitals all over Japan collected between 2010 and 2018 were subjected to the genotyping of null genotypes of GSTM1 and GSTT1 using SmartAmp-2 method. We also collected an information on DILI types, time to onset of DILI, pharmacological classification of suspected drugs and DDW-Japan score as well as genotypes of GSTM1 and GSTT1 in each DILI patient. Results: The distribution of a combination of null genotypes of GSTM1 and GSTT1 in Japanese DILI patients was significantly different from the reported one in general Japanese population. Especially, the incidence of GSTM1 null genotype in DILI patients was significantly higher than that of control population. The significant relationship between the frequency of GSTM1 and GSTT1 null genotypes and pharmacological classification of suspected drugs, time to onset of DILI and DDW-Japan scores was not observed. Conclusions: GSTM1 null genotype was considered to increase the incidence of DILI in Japanese patients.

Keywords: drug-induced liver injury, glutathione S-transferase M1, glutathione S-transferase T1

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Tanaka Y, Takahashi K*1, Hattori N*2, Yokoyama H*3, Yamaguchi K*3, Shibui Y*2, Kawaguchi S*2, Shimazaki T*3, Nakai K*1, Kusuhara H*4, Saito Y: The influence of serial 50 μL microsampling on rats administered azathioprine, the immunosuppressive drug.

Toxicol Rep. 2023;10: 334-340. doi: 10.1016/j.toxrep.2023.02.016

According to the ICH S3A Q&A, microsampling is applicable to pharmaceutical drugs and toxicological analysis. Few studies have reported the effect of microsampling on the toxicity of immunotoxicological drugs. The aim of this multicenter study was to evaluate the toxicological effects of serial microsampling on rats treated with azathioprine as a model drug with immunotoxic effects. Fifty microliters of blood were collected from the jugular vein of Sprague-Dawley rats at six time points from day 1 to 2 and 7 time points from day 27 to 28. The study was performed at three organizations independently. The microsampling effect on clinical signs, body weights, food consumption, hematological parameters, biochemical parameters, urinary parameters, organ weights, and tissue pathology was evaluated. Azathioprine-induced changes were observed in certain hematological and biochemical parameters and thymus weight and pathology. Microsampling produced minimal or no effects on almost all parameters; however, at 2 organizations, azathioprine-induced changes were apparently masked for two leukocytic, one coagulation, and two biochemical parameters. In conclusion, azathioprine toxicity could be assessed appropriately as overall profiles even with blood microsampling. However, microsampling may influence azathioprine-induced changes in certain parameters, especially leukocytic parameters, and its usage should be carefully considered.

Keywords: Microsampling, Azathioprine, Rat, Toxicokinetics, Jugular vein, Hematological parameter

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Yamamoto N*1, Tanno Y*1, Tanaka Y, Hira D*2, Terada T*2, Saito Y, Yokozawa Y*1: Development of Novel Mass Spectrum-based Assay for Simultaneous Detection of 36 Variants in the 14 Pharmacogenetic Genes for the Japanese Population.

Biol Pharm Bull. 2023;46:511-516. doi: 10.1248/bpb.b22-00810.

Pharmacogenetics (PGx) enhances personalized care, often reducing medical costs, and improving patients' Quality of Life (QOL). Unlike single variant analysis, multiplex PGx panel tests can result in applying comprehensive PGx-guided medication to maximize drug efficacy and minimize adverse reactions. Among PGx genes, drug-metabolizing enzymes and drug transporters have significant roles in the efficacy and safety of various pharmacotherapies. In this study, a genotyping panel has been developed for the Japanese population called PGx_JPN panel comprising 36 variants in 14 genes for drugmetabolizing enzymes and drug transporters using a mass spectrometry-based genotyping method, in which all the variants could be analyzed in two wells for multiplex analysis. The verification test exhibited good concordance with the results analyzed using the other standard genotyping methods (microarray, TaqMan assay, or another mass spectrometrybased commercial kit). However, copy number variations such as *CYP2D6*5* could not apply to this system. In this study, we demonstrated that the mass spectrometry-based multiplex method could be useful for in the simultaneous genotyping of more than 30 variants, which are essential among the Japanese population in two wells, except for copy number variations. Further study is needed to assess our panel to demonstrate the clinical use of pharmacogenomics for precision medicine in the Japanese population.

Keywords: mass spectrometry-based method, multiplex panel, pharmacogenetics, variant detection

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Zhao J*1, Ueki M*1, Sawai S*1, Sugiyama M*1, Tetashita Y*1, Hirabayashi S*1, Cho Y*1, Kobayashi R*2, Tanaka Y, Manabe A*1,3: The heterozygous *NUDT15* variant is not associated with the severity of 6-mercaptopurine-related side effects in early

intensification therapy for childhood acute lymphoblastic leukemia.

EJC Paediatric Oncology. 2023;1:100006. doi: 10.1016/j.ejcped.2023.100006.

Background: In the treatment of childhood acute lymphoblastic leukemia (ALL), 6-mercaptopurine (6-MP) is essential for early intensification and maintenance of therapy. Recently, an association between genetic variants of nudix hydrolase 15 (NUDT15) rs116855232 and 6-MP-induced severe myelotoxicity was reported. Given that the *NUDT15* variant is relatively common in East Asian and Hispanic populations, it is important to evaluate its impact on 6-MP treatment and determine the dose for an effective therapy and avoid side effects in those populations. Since all the previous reports have focused on maintenance therapy, we studied the clinical impact of the NUDT15 variants in the early intensification therapy for the first time. Methods: Thirty patients with ALL who received early intensification therapy were retrospectively registered in this study. Clinical and laboratory data were collected from their clinical records, and genetic analysis of the *NUDT15* variant was performed. Results: Twenty-four patients had CC (wild-type), six patients had CT (heterozygous variant), and none had TT (homozygous variant) of *NUDT15* rs116855232. All patients showed myelotoxicity and hepatotoxicity but a correlation between these side effects and the*NUDT15* haplotype was not observed. Conclusion: The heterozygous *NUDT15* variant was not associated with 6-MP toxicity during early intensification therapy and a dose modification of 6-MP may not be recommended in children with the heterozygous variant of *NUDT15*.

Keywords: leukemia, children, NUDT15, mercaptopurine, side effects

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Br J Haematol. 2022;199:260-269. doi: 10.1111/bjh.18405.

Inherited genetic variation is associated with 6-mercaptopurine (6-MP) dose reduction and frequent 6-MP induced toxicities. However, tolerable dose for 6-MP is not completely predicted by the known variation in *NUDT15* and *TPMT* among Asian children with acute lymphoblastic leukemia (ALL). We performed a genome-wide association study (GWAS) related to 6-MP dose among Japanese children with ALL. This GWAS comprised 224 patients previously enrolled in Tokyo Children's Cancer Study Group clinical studies with replication attempted in 55 patients. Genome-wide single nucleotide polymorphism (SNP) genotypes were evaluated for association with 6-MP average dose during initial 168 days of maintenance therapy. Possible associations were observed across 5 gene coding regions, among which only variants at 13q14.2 were genome-wide significant and replicated (rs116855232, *NUDT15*, β=−10.99, P=3.7 $\times 10^{-13}$). Notable findings were observed for variants in *AFF3* (rs75364948, P=2.05 $\times 10^{-6}$) and CHST11 (rs1148407, P=2.09 $\times 10^{-6}$), but were not replicated possibly due to small numbers. A previously reported candidate SNP in *MTHFR* was associated with higher 6-MP average dose (rs1801133, P=0.045), and FOLH1 (rs12574928) was associated in a candidate region evaluation (Padjust=0.013). This study provides strong evidence that rs116855232 in *NUDT15* is the prominent genetic factor associated with 6-MP tolerable dose in Japanese.

Keywords: 6-mercaptopurine, tolerance, genetic variant, genome-wide association study, Japanese

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Ohtsuka H*1, Takahashi K*2, Kitaura H*1, Kandori H*1, Danbayashi K*1, Higuchi T*1, Jinno F*1, Nitta S*3, Mori K*3, Iwai A*2, Nakai K*2, Saito K, Saito Y: No obvious toxicological influences of 50 μL microsampling from rats administered phenacetin as a drug with hematological toxicity.

J Toxicol. Sci. 2022;47:193-199. DOI: 10.2131/jts.47.193

According to ICH S3A Q&A focusing on microsampling, its application should be avoided in main study animals for test drugs that could exacerbate hematological parameters with frequent blood sampling. However, no study has reported the effects of microsampling on toxicity parameters of drugs known to induce hematological toxicity. Therefore, we assessed the toxicological effects of serial microsampling on rats treated with phenacetin as a model drug. In a common 28-day study, 50 μL of microsampling was performed at 6-time points on days 1 to 2 and 7-time points on days 27 to 28 from the jugular vein of Sprague Dawley rats. The study was performed independently by two organizations. The toxicological influence of microsampling was evaluated on body weight, food consumption, hematology, blood clinical chemistry, urine parameters, organ weights, and tissue pathology. Phenacetin treatments induced significant changes of various hematological parameters (including hemoglobin and reticulocytes), some organ weights (including liver and spleen), and some hematology-related pathological parameters in the liver, spleen and bone marrow. Meanwhile, serial microsampling exhibited minimal influence on the assessed parameters, although 20 parameters showed statistical differences mostly at one organization. The current results support the notion that serial 50 μL microsampling from the jugular vein had minimal impacts on overall toxicological profiles even in rats treated with a drug inducing hematological toxicity, but the potential adverse effect on certain parameters could not be fully excluded. Accordingly, this microsampling technique has possibility to be employed even for non-clinical rat toxicity studies using drugs with potentially hematological toxicity.

Keywords: Hematological toxicity, Jugular vein, Microsampling, Rats

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Muta K*, Saito K, Kemmochi Y*, Masuyama T*, Kobayashi A*, Saito Y, Sugai S*: Phosphatidylcholine (18:0/20:4), a potential biomarker to predict ethionamide-induced hepatic steatosis in rats.

J Appl Toxicol. 2022;42:1533-1547. DOI: 10.1002/jat.4324

Ethionamide (ETH), a second-line drug for multidrug-resistant tuberculosis, is known to cause hepatic steatosis in rats and humans. To investigate predictive biomarkers for ETH-induced steatosis, we performed lipidomics analysis using plasma and liver samples collected from rats treated orally with ETH at 30 and 100 mg/kg for 14 days. The ETH-treated rats developed hepatic steatosis with Oil Red O staining-positive vacuolation in the centrilobular hepatocytes accompanied by increased hepatic contents of triglycerides (TG) and decreased plasma TG and total cholesterol levels. A multivariate analysis for lipid profiles revealed differences in each of the 35 lipid species in the plasma and liver between the control and the ETH-treated rats. Of those lipids, phosphatidylcholine (PC) (18:0/20:4) decreased dose-dependently in both the plasma and liver. Moreover, serum TG-rich very low-density lipoprotein (VLDL) levels, especially the large particle fraction of VLDL composed of PC containing arachidonic acid (20:4) involved in hepatic secretion of TG, were decreased dose-dependently. In conclusion, the decreased PC (18:0/20:4) in the liver, possibly leading to suppression of hepatic TG secretion, was considered to be involved in the pathogenesis of the ETH-induced hepatic steatosis. Therefore, plasma PC (18:0/20:4) levels are proposed as mechanism-related biomarkers for ETH-induced hepatic steatosis.

Keywords: DILI, lipid profile, lipidomics, triglyceride, very low-density lipoprotein

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Saito K, Ishikawa R, Kitamura I[*1], Ogawa K, Arakawa N, Sun Y, Imai K[*2], Maeda T[*3], Saito Y, Hasegawa C[*1]: Characterization of serotonin as a candidate biomarker of severity and prognosis of COVID-19 using LC/MS analysis.

J Pharmacol Sci. 2022;50:49-55. DOI: 10.1016/j.jphs.2022.06.005

The coronavirus disease 2019 (COVID-19) pandemic has been associated with high mortality worldwide. Owing to its complicated pathophysiology, diagnostic and prognostic biomarkers for effective patient management remain scarce. We analyzed kynurenine, tryptophan, and serotonin levels in the serum of patients with COVID-19 via liquid chromatography/mass spectrometry analysis. Serum serotonin levels were decreased in patients with more severe COVID-19, along with increased kynurenine and decreased tryptophan concentrations. Patients with moderate disease who subsequently worsened showed significantly lower serotonin concentrations compared with those who did not experience severe disease. Serum serotonin levels may represent a valuable biomarker for COVID-19 severity and prognosis.

Keywords: Biomarker, COVID-19, Disease severity, Prognosis, Serotonin

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Sci. Rep. 2022,12:19819. DOI: 10.1038/s41598-022-24406-z

Drug-induced interstitial lung disease (DILD) occurs when drug exposure causes inflammation of the lung interstitium. DILD can be caused by different types of drugs, and some DILD patterns results in a high mortality rate; hence, DILD poses a serious problem in clinical practice as well as drug development, and strategies to diagnose and distinguish DILD from other lung diseases are necessary. We aimed to identify novel biomarkers for DILD by performing lipidomics analysis on plasma samples from patients with acute and recovery phase DILD. Having identified lysophosphatidylcholines (LPCs) as candidate biomarkers for DILD, we determined their concentrations using validated liquid chromatography/mass spectrometry biomarker assays. In addition, we evaluated the ability of LPCs to discriminate patients with acute phase DILD from those with recovery phase DILD, DILD-tolerant, or other lung diseases, and characterized their association with clinical characteristics. Lipidomics analysis revealed a clear decrease in LPC concentrations in the plasma of patients with acute phase DILD. In particular, LPC (14:0) had the highest discriminative index against recovery phase and DILD-tolerant patients. LPC(14:0) displayed no clear association with causal drugs, or subjects' backgrounds, but was associated with disease severity. Furthermore, LPC(14:0) was able to discriminate between patients with DILD and other lung diseases, including idiopathic interstitial pneumonia and lung disease associated with connective tissue disease. LPC(14:0) is a promising biomarker for DILD that could improve the diagnosis of DILD and help to differentiate DILD from other lung diseases, such as idiopathic interstitial pneumonia and connective tissue disease.

Keywords: lysophosphatidylcholine, drug-induced interstitial lung disease, biomarker, drug exposure, inflammation, lung interstitium

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青木良子，佐井君江，勝田由紀子，鈴木美佳，鈴木康夫*，石井明子，斎藤嘉朗：本邦における抗体及び抗体関連医薬品のバイオシミラー採用及び処方に関する医師の意識調査.

薬学雑誌 2022;142:547-560. doi: 10.1248/yakushi.21-00216.

Biosimilars are less expensive than their originators, and Japanese government policies call for their development and promotion. However, the adoption and prescription of some biosimilars, especially antibody/its-related ones, have been delayed for use in Japan, possibly due to concerns on the differences in quality attributes such as glycan structures between the originators and their biosimilars, and that clinical efficacy/safety studies are conducted for usually one disease and its results extrapolated to other indications. We conducted a questionnaire survey among physicians in four disease areas (hematology, medical oncology, rheumatoid arthritis, and inflammatory bowel disease), where biosimilars of antibody/its-related drugs have been approved, regarding their thoughts on the adoption and prescription of biosimilars in Japan from January to April 2020. We received totally 1024 responses. When adopting biosimilars and explaining them to patients, physicians requested specific information including the comparative results of phase III clinical trials and quality characteristics between biosimilars and their originators; the results of clinical studies on switching from originators to their biosimilars; and a comparison of the estimated cost on patients in consideration of the high medical cost payment system. Priority differed depending on the studied disease areas. In terms of post-marketing information, physicians requested a variety of information. When explaining biosimilars to the patients, physicians would like to use general material from government describing the comparability between originators and their biosimilars. These results suggest that physicians sought more comparative information on the quality, efficacy, and patients' cost between originators and their biosimilars when adopting or prescribing biosimilars.

Keywords: biosimilar, questionnaire survey, antibody therapeutics

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Yuda M*, Aizawa S*, Tsuboi I*, Hirabayashi Y, Harada T*, Hino H*, Hirai S*: Imbalanced M1 and M2 Macrophage Polarization in Bone Marrow Provokes Impairment of the Hematopoietic Microenvironment in a Mouse Model of Hemophagocytic Lymphohistiocytosis.

Biol Pharm Bull. 2022;45:1602-1608. doi: 10.1248/bpb.b22-00108

Lipopolysaccharide (LPS) treatment induced hemophagocytic lymphohistiocytosis in senescence-accelerated mice (SAMP1/TA-1), but not in senescence-resistant control mice (SAMR1). SAMP1/TA-1 treated with LPS exhibited functional impairment of the hematopoietic microenvironment, which disrupted the dynamics of hematopoiesis. Macrophages are a major component of the bone marrow (BM) hematopoietic microenvironment, which regulates hematopoiesis. Qualitative and quantitative changes in activated macrophages in LPS-treated SAMP1/TA-1 are thought to contribute to the functional deterioration of the hematopoietic microenvironment. Thus, we examined the polarization of pro-inflammatory (M1) and anti-inflammatory (M2) macrophages, and the dynamics of macrophage production in the BM of SAMP1/TA-1 and SAMR1 after LPS treatment. After LPS treatment, the proportions of M1 and M2 macrophages and the numbers of macrophage progenitor (CFU-M) cells increased in both SAMP1/TA-1 and SAMR1. However, compared to the SAMR1, the increase in the M1 macrophage proportion was prolonged, and the increase in the M2 macrophage proportion was delayed. The increase in the number of CFU-M cells was prolonged in SAMP1/TA-1 after LPS treatment. In addition, the levels of transcripts encoding an M1 macrophage-inducing cytokine (interferon-γ) and macrophage colony-stimulating factor were markedly increased, and the increases in the levels of transcripts encoding M2 macrophage-inducing cytokines (interleukin (IL)-4, IL-10, and IL-13) were delayed in SAMP1/TA-1 when compared to SAMR1. Our results suggest that LPS treatment led to the severely imbalanced polarization of activated M1/M2 macrophages accompanied by a prolonged increase in macrophage production in the BM of SAMP1/TA-1, which led to the impairment of the hematopoietic microenvironment, and disrupted the dynamics of hematopoiesis.

Keywords: hematopoietic microenvironment, hemophagocytic lymphohistiocytosis, senescence-accelerated mouse

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Kuwagata M, Tsuboi M*, Igarashi T, Tsurumoto M, Nishimura T, Taquahashi Y, Kitajima S: A 90-day dose Toxicity Study of 2-(2H-benzotriazol-2-yl) -6-dodecyl-4-methylphenol in Rats.

Fundam Toxicol Sci. 2023;10(2):59-68. doi:10.2131/fts.10.59

2-(2H-benzotriazol-2-yl)-6-dodecyl-4-methylphenol (BTMLP, CAS No. 125304-04-3) is widely used as a liquid ultraviolet absorber that prevents deterioration of synthetic resins and so on. To investigate its toxicological properties and determine the no-observed-adverse-effect level (NOAEL), a 90-day repeated oral toxicological study of BTMLP was conducted in Crl:CD (SD) rats at doses of 0 (vehicle control, corn oil), 100, 300, and 1000 mg/kg/day. There was no observed mortality or abnormal clinical signs related to the treatment of any group. Body weight and food consumption were not affected by BTMLP treatment. In males, significant prolongations of prothrombin time and activated partial thrombin time were observed in the BTMLP-treated groups. Histopathological examination revealed a slight increase of the eosinophilic bodies and hyaline droplets in the renal cortical tubules in the 1000 mg/kg group in males. As mentioned above, the toxic effect of the BTMLP was noted in the blood coagulation system and kidneys only in males. Based on these findings, the NOAEL was judged to be less than 100 mg/kg/day in males and 1000 mg/kg/day in females under this study's condition.

Keywords: 2-(2H-benzotriazol-2-yl)-6-dodecyl-4-methylphenol, a 90-day repeated oral toxicity study, rats

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Kuwagata M, Tsuboi M*, Igarashi T, Tsurumoto M, Nishimura T, Taquahashi Y, Kitajima S: A 90-day repeated oral dose toxicity study of 2-Butylbenzo[d] isothiazol-3(2H)-one in Rats.

Fundam Toxicol Sci. 2023;10 (2):69-82. doi:10.2131/fts.10.69

2-Butylbenzo[d]isothiazol-3(2H)-one (BBIT, CAS No. 4299-07-4) is widely used as an industrial antiseptic and antifungal agent. To investigate its toxicological properties and determine the no-observed-adverse-effect level (NOAEL), a 90-day repeated oral toxicological study of BBIT was conducted in Crl:CD (SD) rats at doses of 0 (vehicle control, corn oil), 30, 90, or 270 mg/kg/day. There was no mortality or abnormal clinical signs related to treatment in any group. Slightly decreased body weight and food consumption were observed in the 270 mg/kg group in females. Increased urine volume and kidney weight, increased liver weight, and thickening of the forestomach mucosa in autopsy were observed in both sexes in the 270 mg/kg group. Histopathological examination revealed that hyperplasia of the squamous epithelium of the forestomach with parakeratosis and/or hyperkeratosis was observed in both sexes in all the BBIT-treated groups. Moreover, centrilobular hypertrophy of hepatocytes was observed in both sexes of the 270 mg/kg group. Similarly, increased depositions of eosinophilic bodies and/or hyaline droplets in the proximal tubules of the kidney were observed among the male in the 270 mg/kg group. Based on the forestomach changes, NOAEL was judged to be less than 30 mg/kg/day in both sexes under this study's conditions.

Keywords: 2-Butylbenzo[d]isothiazol-3(2H)-one, a 90-day repeated oral toxicity study, rats

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Kuwagata M, Sato A*1, Izumi Y*2, Chihara K*3, Yamasaki H*4, Katsumata Y*5, Ooshima Y*5, Buschmann J*6, Fujiwara M*7: Current activities between the DevTox Berlin workshops and the Japanese Teratology Society Terminology Committee in harmonizing the terminology for classifying anomalies in laboratory animals in developmental toxicity studies: Report from the Satellite Workshop of the 60th Annual Meeting of the Japanese Teratology Society.

Congenit Anom (Kyoto). 2022;62(5):198-202. doi:10.1111/cga.12480.

In recent years, the Japanese Teratology Society has worked with the DevTox Berlin Workshops project to

provide internationally consistent terminology for teratogenic effects. This paper summarizes a satellite workshop of the 60th Annual Meeting of the Japanese Teratology Society, which was entitled "Current activities between DevTox Berlin Workshops to develop a harmonized terminology for classifying anomalies in laboratory animals in developmental toxicity studies." The Japanese Teratology Society - Laboratory Animal Terminology Project (JTS-LATP) reviewed "gray zone" anomalies and focused on developing criteria for reclassifying a large number of gray zone anomalies to clarify them and to make it easier to judge fetal categories. This effort will lead to international agreement, based on shared conceptions. The present article aimed to provide the reader with a summary of the issues discussed at the 2020 satellite meeting, which included discussions on open issues from the DevTox Berlin Workshops, ongoing work by the JTS-LATP on gray zone (GZ) anomalies, current industrial concerns, and future challenges.

Keywords: DevTox Berlin workshops, harmonization of terminology in developmental toxicology, the terminology committee in a laboratory animal terminology project

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Hojo M*1, Maeno A*1, Sakamoto Y*1, Ohnuki A*1, Tada Y*1, Yamamoto Y*1, Ikushima K*1, Inaba R*1, Suzuki J*1, Taquahashi Y, Yokota S, Kobayashi N, Ohnishi M*2, Goto Y*2, Numano T*3, Tsuda H*4, Alexander DB*4, Kanno J, Hirose A, Inomata A*1, Nakae D*5, 6: Two-year intermittent exposure of a multiwalled carbon nanotube by intratracheal instillation induces lung tumors and pleural mesotheliomas in F344 rats.

Part Fibre Toxicol. 19(1):38. (2022) doi: 10.1186/s12989-022-00478-7

Background: A mounting number of studies have been documenting the carcinogenic potential of multiwalled carbon nanotubes (MWCNTs); however, only a few studies have evaluated the pulmonary carcinogenicity of MWCNTs *in vivo*. A 2-year inhalation study demonstrated that MWNT-7, a widely used MWCNT, was a pulmonary carcinogen in rats. In another 2-year study, rats administered MWNT-7 by intratracheal instillation at the beginning of the experimental period developed pleural mesotheliomas but not lung tumors. To obtain data more comparable with rats exposed to MWNT-7 by inhalation, we administered MWNT-7 to F344 rats by intratracheal instillation once every 4-weeks over the course of 2 years at 0, 0.125, and 0.5 mg/kg body weight, allowing lung burdens of MWNT-7 to increase over the entire experimental period, similar to the inhalation study. Results: Absolute and relative lung weights were significantly elevated in both MWNT-7-treated groups. Dose- and time-dependent toxic effects in the lung and pleura, such as inflammatory, fibrotic, and hyperplastic lesions, were found in both treated groups. The incidences of lung carcinomas, lung adenomas, and pleural mesotheliomas were significantly increased in the high-dose group compared with the control group. The pleural mesotheliomas developed mainly at the mediastinum. No MWNT-7-related neoplastic lesions were noted in the other organs. Cytological and biochemical parameters of the bronchoalveolar lavage fluid (BALF) were elevated in both treated groups. The lung burden of MWNT-7 was dose- and time-dependent, and at the terminal necropsy, the average value was 0.9 and 3.6 mg/lung in the low-dose and high-dose groups, respectively. The number of fibers in the pleural cavity was also dose- and time-dependent.

Conclusions: Repeated administration of MWNT-7 by intratracheal instillation over the 2 years indicates that MWNT-7 is carcinogenic to both the lung and pleura of rats, which differs from the results of the 2 carcinogenicity tests by inhalation or intratracheal instillation.

Keywords: MWCNT, carcinogenicity, intratracheal instillation

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Kanno S*, Mizota K*, Okubo Y, Kageyama T*, Yan L*, Fukuda J*: Luciferase assay system to monitor fibroblast growth factor signal disruption in human iPSCs.
STAR Protoc. 2022;7(2):101439. doi: 10.1016/j.xpro.2022.101439

We describe a protocol for a live-cell luciferase assay system for continuously monitoring fibroblast growth factor (FGF) signal disruption in human-induced pluripotent stem cells (iPSCs). Signal disrupting effects of chemicals are used as an indicator to evaluate toxicity. The assay is reliably predictive of the effects of limb malformation chemicals (AUC = 0.93). The current approach is limited to FGF signal disruption, and combinations with other types of signaling will be required to detect the effects of different toxicants. For complete details on the use and execution of this protocol, please refer to Kanno et al. (2022a).
Keywords: luciferase assay, FGF signal, human iPS cells

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Isano Y*1, Fujita H*2, Murakami K*1, Ni S*1, Kurotaki Y *1, Takano T*1, Isoda Y*1, Matsuda R*1, Nakamura F*1, Nishitai Y*1, Ochirkhuyag N.*1, Inoue K*1, Kawakami H*1, Okubo Y, Ueno K*3, Fujie T*2, Ota H*1: Transparent and Breathable Ion Gel - Based Sensors toward Multimodal Sensing Ability.
Adv Mater Technol. 2022;7 (11):2200209. doi: 10.1002/admt.202200209

Polymer thin-film sensors have attracted considerable attention in various applications owing to their highly transparent, flexible, and gas-permeable features. However, conventional thin-film sensors based on nanomaterials suffer from poor selectivity in sensing targets and scalability of functions. Therefore, a new approach is required for achieving higher selectivity with simple processibility. This study proposes highly transparent, ultra-flexible, and gas-permeable polymer thin-film sensors using ion gels as the sensing material; the sensors demonstrated the capacity for selective detections. Particularly, this study demonstrates simultaneous and independent sensing of temperature and humidity as a proof of concept. The sensors are fabricated using a simple spray coating method on a thin silicone rubber film (≈ 25 μm thickness). Owing to their thin-film shape, the sensors exhibit more than 80% visible light transmittance and a higher gas permeability than the human transepidermal water loss. The temperature and humidity are simultaneously detected with a high sensitivity of 15.4% ℃-1 and 2.0% per percentage of the relative humidity, respectively, using gels containing two different ionic liquids (ILs). The results suggest that the easily modifiable nature of ILs enables the fabrication of ultra-flexible and transparent sensors that can detect various objects using a simple method.
Keywords: multimodal sensing ability, flexible, gas-permeable

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Kageyama T*, Shimizu A*, Anakama R*, Nakajima R*, Suzuki S*, Okubo Y, Fukuda J*: Reprogramming of three-dimensional microenvironments for *in vitro* hair follicle induction.
Sci Adv. *2022*;8(42):eadd4603. doi: 10.1126/sciadv.add4603

During embryonic development, reciprocal interactions between epidermal and mesenchymal layers trigger hair follicle morphogenesis. This study revealed that microenvironmental reprogramming via

control over these interactions enabled hair follicle induction *in vitro*. A key approach is to modulate spatial distributions of epithelial and mesenchymal cells in their spontaneous organization. The de novo hair follicles with typical morphological features emerged in aggregates of the two cell types, termed hair follicloids, and hair shafts sprouted with near 100% efficiency *in vitro*. The hair shaft length reached ~3 mm in culture. Typical trichogenic signaling pathways were up-regulated in hair follicloids. Owing to replication of hair follicle morphogenesis *in vitro*, melanosome production and transportation were also monitored in the hair bulb region. This *in vitro* hair follicle model might be valuable for better understanding hair follicle induction, evaluating hair growth and inhibition of hair growth by drugs, and modeling gray hairs in a well-defined environment.

Keywords: hair follicloids, melanosome, microenvironmental reprogramming

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Miyaso H[*1], Takano K[*2], Nagahori K[*2], Li Z[*2], Kawata S[*2], Kuramasu M[*2], Ogawa Y[*2], Yoshioka H[*1], Matsuno Y[*3], Yokota S, Itoh M[*2]: Neonatal corticosterone administration increases p27-positive Sertoli cell number and decreases Sertoli cell number in the testes of mice in prepuberty.

Sci Rep. 2022;12(1):19402. doi:10.1038/s41598-022-23695-8

Cortisol and corticosterone (CORT) are steroid, antistress hormones and one of the glucocorticoids in humans and animals, respectively. This study evaluated the effects of CORT administration on the male reproductive system in early life stages. CORT was subcutaneously injected at 0.36 (low-), 3.6 (middle-), and 36 (high-dosed) mg/kg body weight from postnatal day (PND) 1 to 10 in ICR mice. We observed a dose-dependent increase in serum CORT levels on PND 10, and serum testosterone levels were significantly increased only in high-dosed-CORT mice. Triiodothyronine levels were significantly higher in the low-dosed mice but lower in the middle- and high-dosed mice. However, testicular weights did not change significantly among the mice. Sertoli cell numbers were significantly reduced in low- and middle-dosed mice, whereas p27-positive Sertoli cell numbers increased in low- and middle-dosed mice. On PND 16, significant increases in testicular and relative testicular weights were observed in all-dosed-CORT mice. On PND 70, a significant decrease in testicular weight, Sertoli cell number, and spermatozoa count was observed. These results revealed that increased serum CORT levels in early life stages could induce p27 expression in Sertoli cells and terminate Sertoli cell proliferation, leading to decreased Sertoli cell number in mouse testes.

Keywords: developmental toxicity, Sertoli cell, corticosterone

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Yokota S, Miyaso H[*1], Hirai T[*2], Suga K, Wakayama T[*3], Taquahashi Y, Kitajima S: Development of a non-invasive method to evaluate testicular toxicity using a novel compact magnetic resonance imaging system.

J Toxicol Sci. 2023;48(2):57-64. doi:10.2131/jts.48.57

In non-clinical animal studies for drug discovery, histopathological evaluation is the most powerful tool to assess testicular toxicity. However, histological analysis is extremely invasive; many experimental animals are needed to evaluate changes in the pathology and anatomy of the testes over time. As an alternative, small animal magnetic resonance imaging (MRI) offers a non-invasive methodology to examine testicular toxicity without radiation. The present study demonstrated the suitability of a new, ready-to-use compact MRI platform using a high-field permanent magnet to assist with the evaluation of testicular toxicity. To validate the utility of the MRI platform, male mice were treated with busulfan (40 mg/kg, intraperitoneal injection). Twenty-eight days after treatment, both testes in busulfan-treated and control mice (n = 6/group) were non-invasively scanned in situ by MRI at 1 tesla. On a T1-weighted 3D gradient-echo MRI sequences (voxel size: 0.23 × 0.23 × 0.50 mm), the total testicular volume in busulfan-

treated mice was significantly smaller than in controls. On T1-weighted images, the signal intensity of the testes was significantly higher in busulfan-treated mice than in controls. The mice were sacrificed, and the testes were isolated for histopathological analysis. The weight of the testes in busulfan-treated mice significantly decreased, similar to the results of the non-invasive analysis. Additionally, periodic acid-Schiff stain-positive effusions were observed in the interstitium of the busulfan-treated mouse testes, potentially explaining T1 shortening due to a high concentration of glycoproteinaceous content. The present data demonstrated a rapid evaluation of testicular toxicity *in vivo* by compact MRI.

Keywords: magnetic resonance imaging, busulfan, testicular toxicity

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Sasaki T*1, Saito H, Furukawa Y, Tominaga T*2, Kitajima S, Kanno J, Tanemura K*1: Exposure to bisphenol A or its phenolic analogs during early life induces different types of anxiety-like behaviors after maturity in male mice.

J Toxicol Sci. 2023;48(4), 211-219. doi: 10.2131/jts.48.211

Products used in daily life contain multiple chemicals capable of inducing endocrine disruption in animals, including humans. One such typical substance is bisphenol A (BPA). BPA has been widely used in epoxy resins and polycarbonate plastics and can exert several adverse effects. Furthermore, given their structural similarity to BPA, phenolic analogs of BPA, i.e., synthetic phenolic antioxidants (SPAs), are considered to exhibit similar toxicity; however, the effects of early SPA exposure on the adult central nervous system remain poorly clarified. In the present study, we aimed to evaluate and compare the neurobehavioral effects of early life exposure to BPA and two selected SPAs, 4,4'-butylidenebis (6-tert-butyl-m-cresol) (BB) and 2,2'-methylenebis (6-tert-butyl-p-cresol) (MB). We exposed mice to low levels of these chemicals through drinking water during prenatal and postnatal periods. Subsequently, we examined the adverse effects of these chemicals on the central nervous system using a mouse behavioral test battery, comprising the open field test, light/dark transition test, elevated plus-maze test, contextual/cued fear conditioning test, and prepulse inhibition test, at 12-13 weeks old. Based on the behavioral analysis, SPAs, like BPA, may cause affective disorders even at low doses, although qualitative differences were noted in anxiety-related behaviors. In conclusion, our findings could be valuable for clarifying the potential adverse developmental risks of SPA exposure in early life.

Keywords: central nervous system, developmental neurotoxicity, endocrine disrupting chemicals

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Saito H, Tanemura K*, Furukawa Y, Sasaki T, Kanno J, Kitajima S: Behavioral effects induced by the oral administration of acetamiprid in male mice during the postnatal lactation period or adulthood.

J Toxicol Sci. 2023;48(4), 203-210. doi: 10.2131/jts.48.203

Acetamiprid (ACE), a neonicotinoid chemical, is widely used as a pesticide due to its rapid insecticidal activity. Although neonicotinoids exert very low toxicity in mammals, the effects of early exposure to neonicotinoids on the adult central nervous system are poorly understood. This study investigated the effects of ACE exposure in early life on brain function in adult mice. We exposed male C57BL/6N mice to ACE (10 mg/kg) orally when they were two (postnatal lactation) or 11 weeks old (adult). We examined the effects of ACE on the central nervous system using the mouse behavioral test battery, consisting of the open field test, light/dark transition test, elevated plus-maze test, contextual/cued fear conditioning test, and pre-pulse inhibition test at 12-13 weeks old. In the mouse behavioral test battery, learning memory abnormalities were detected in the mature treatment group. In addition, learning memory and emotional

abnormalities were detected in the postnatal lactation treatment group. These results suggest that the behavioral effects of postnatal lactation treatment with ACE were qualitatively different from the behavioral abnormalities in the mature treatment group.

Keywords: acetamiprid, central nervous system, developmental neurotoxicity

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Tsukiboshi Y[*1], Ogata A[*1], Noguchi A[*1], Mikami Y[*2], Yokota S, Ogata K[*2], Yoshioka H[*1]: Sasa veitchii extracts protect phenytoin-induced cell proliferation inhibition in human lip mesenchymal cells through modulation of miR-27b-5p.

Biomed Res (Tokyo). 2023;44 (2):73-80. doi: 10.2220/biomedres.44.73

A cleft lip, with or without a cleft palate, is a common birth defect caused by environmental factors or genetic mutations. Environmental factors, such as pharmaceutical exposure in pregnant women, are known to induce cleft lip, with or without cleft palate in the child. This study aimed to investigate the protective effect of Sasa veitchii extract (SE) on phenytoin-induced inhibition of cell proliferation in human lip mesenchymal cells (KD cells) and human embryonic palatal mesenchymal cells (HEPM cells). We demonstrated that cell proliferation was inhibited by phenytoin in a dose-dependent manner in both KD and HEPM cells. Co-treatment with SE restored phenytoin-induced toxicity in KD cells but did not protect HEPM cells against phenytoin-induced toxicity. Several microRNAs (miR-27b, miR-133b, miR-205, miR-497-5p, and miR-655-3p) is reported to associate with cell proliferation in KD cells. We measured the seven kinds of microRNAs (miR27b-3p, miR-27b-5p, miR-133b, miR-205-3p, miR-205-5p, miR-497-5p, and miR-655-3p) and found that SE suppressed miR-27b-5p induced by phenytoin in KD cells. Furthermore, co-treatment with SE enhanced the expression of miR-27b-5p downstream genes (PAX9, RARA, and SUMO1). These results suggest that SE protects phenytoin-induced cell proliferation inhibition by modulating miR-27b-5p.

Keywords: human lip, development, microRNA

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Kaneko S[*1], Okada Y[*2], Yokota S, Takamatsu K[*1]: Reactive Blue Dye: Highlights of Vacuoles in Human Sperm.

J Med Diagn Meth. 2023;12(2):400. doi: 10.35248/2168-9784.23.12.400

Objectives: The WHO reference manual for andrology considers the outline of sperm head as a key parameter for assessing male infertility and classifies internal vacuoles as head defects. The features of vacuoles were heterogeneous among interindividual as well as inter-sperm. The present study reported some unique properties of Reactive Blue 2 (RB2). The dye exhibited pH-dependent cellular specificity for human sperm and spermatid.

Materials and Methods: Human sperm and spermatid were stained with RB2 at pH 10.0.

Results: RB2 stained human sperm and spermatids, which appeared as translucent bluish bodies, but did not stain lymphocytes, while inner vacuoles appeared as toneless spots. RB2 staining also revealed degraded spermatids that had undergone protamination but were arrested prior to tail elongation. The majority of azoospermic semen specimens (16/23 cases) included sperm or degraded spermatids. Although the features of the head outline and vacuoles were heterogenous among interindividual, RB2 staining in the presence of 2.0 mmol/L Dithiothreitol universally re-formed the outline into an oval shape and led to the disappearance of the vacuoles, regardless of their original features.

Conclusion: Three sulfate residues in RB2 interacted selectively with guanidyl residues in Arg of protamines at pH 10. The cellular specificity was due to the Arg content of the nucleoproteins. RB2 revealed the head outline and internal vacuoles of the sperm and arrested spermatids those had undergone protamination. Local failure of disulfide cross-linkage might play a critical role in vacuole formation. RB2 staining opened new methods for exploring spermiogenesis.

Keywords: human sperm, sperm vacuole

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Yoshioka H*1, Yokota S, Tominaga S*2, Tsukiboshi Y*1, Suzui M*2, Shinohara Y*3, Yoshikawa M*3, Sasaki H*4, Sasaki N*4, Maeda T*3, Miura N*5: Involvement of Bmal1 and Clock in bromobenzene metabolite-induced diurnal renal toxicity.
Biol Pharm Bull. 2023 *In press* doi:10.2220/biomedres.44.73

Circadian rhythms are endogenous oscillators that regulate 24 h behavioral and physiological processes. Our previous investigation demonstrated that bromobenzene metabolite (4- bromocatechol:4-BrCA) exhibited chronotoxicity (i.e., the nephrotoxicity induced by 4-BrCA was observed during the dark phase, while not observed at light phase in mice). However, the molecular mechanism is still unknown. The aim of the present study is to investigate the cellular molecule(s) involved in the 4-BrCA-induced nephrotoxicity using mouse renal cortex tubular cell lines (MuRTE61 cells).

We found that 4-BrCA showed dose dependent (0.01-1 mM) cell proliferation defect in MuRTE61 cells. By treating with 0.03 mM 4-BrCA, we demonstrated that major clock genes (Bmal1, Clock, Cry1, Cry2, Per1, and Per2) were significantly downregulated. Interestingly, the expression levels of two genes, Bmal1 and Clock, continued to decrease after 3 h of treatment with 4-BrCA, while Cry1, Per1, and Per2 were unchanged until 24 h of treatment. Moreover, BMAL1 and CLOCK levels are higher at light phase. We speculated that BMAL1 and CLOCK might function defensively against 4-BrCA-induced nephrotoxicity since the expression levels of Bmal1 and Clock were rapidly decreased. Finally, overexpression of Bmal1 and Clock restored 4-BrCA-induced cell proliferation defect in MuRTE61 cells. Taken together, our results suggest that Bmal1 and Clock have protective roles against 4-BrCA-induced nephrotoxicity.

Keywords: 4-bromocathecol, circadian rhythm, chronotoxicity

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Tang X*1,2,3, Nishimura A*1,2,3, Ariyoshi K*4, Nishiyama K*4, Kato Y*4, Vasileva EA*5, Mishchenko NP*5, Fedoreyev SA*5, Stonik VA*5, Kim HK*6, Han J*6, Kanda Y, Umezawa K*7, Urano Y*8,9, Akaike T*10, Nishida M*1,2,3,4: Echinochrome Prevents Sulfide Catabolism-Associated Chronic Heart Failure after Myocardial Infarction in Mice.
Marine Drugs. 2023;21:52.

Abnormal sulfide catabolism, especially the accumulation of hydrogen sulfide (H2S) during hypoxic or inflammatory stresses, is a major cause of redox imbalance-associated cardiac dysfunction. Polyhydroxynaphtoquinone echinochrome A (Ech-A), a natural pigment of marine origin found in the shells and needles of many species of sea urchins, is a potent antioxidant and inhibits acute myocardial ferroptosis after ischemia/reperfusion, but the chronic effect of Ech-A on heart failure is unknown. Reactive sulfur species (RSS), which include catenated sulfur atoms, have been revealed as true biomolecules with high redox reactivity required for intracellular energy metabolism and signal transduction. Here, we report that continuous intraperitoneal administration of Ech-A (2.0 mg/kg/day) prevents RSS catabolism-associated chronic heart failure after myocardial infarction (MI) in mice. Ech-A prevented left ventricular (LV) systolic dysfunction and structural remodeling after MI. Fluorescence imaging revealed that intracellular RSS level was reduced after MI, while H2S/HS- level was increased in LV myocardium, which was attenuated by Ech-A. This result indicates that Ech-A suppresses RSS catabolism to H2S/HS- in LV myocardium after MI. In addition, Ech-A reduced oxidative stress formation by MI. Ech-A suppressed RSS catabolism caused by hypoxia in neonatal rat cardiomyocytes and human iPS cell-derived cardiomyocytes. Ech-A also

suppressed RSS catabolism caused by lipopolysaccharide stimulation in macrophages. Thus, Ech-A has the potential to improve chronic heart failure after MI, in part by preventing sulfide catabolism.

Keywords: cardiac remodeling, echinochrome, hydrogen sulfide

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Nakayama-Kitamura K, Shigemoto-Mogami Y, Toyoda H*1, Mihara I*1, Moriguchi H*1, Naraoka H*1, Furihata T*2, Ishida S*3, Sato K: Usefulness of a humanized tricellular static transwell blood-brain barrier model as a microphysiological system for drug development applications. - A case study based on the benchmark evaluations of blood-brain barrier microphysiological system.

Regen Ther. 2023 Feb 24;22:192-202. doi: 10.1016/j.reth.2023.02.001. eCollection 2023 Mar. PMID: 36891355

Microphysiological system (MPS), a new technology for *in vitro* testing platforms, have been acknowledged as a strong tool for drug development. In the central nervous system (CNS), the blood–brain barrier (BBB) limits the permeation of circulating substances from the blood vessels to the brain, thereby protecting the CNS from circulating xenobiotic compounds. At the same time, the BBB hinders drug development by introducing challenges at various stages, such as pharmacokinetics/pharmacodynamics (PK/PD), safety assessment, and efficacy assessment. To solve these problems, efforts are being made to develop a BBB MPS, particularly of a humanized type. In this study, we suggested minimal essential benchmark items to establish the BBB-likeness of a BBB MPS; these criteria support end users in determining the appropriate range of applications for a candidate BBB MPS. Furthermore, we examined these benchmark items in a two-dimensional (2D) humanized tricellular static transwell BBB MPS, the most conventional design of BBB MPS with human cell lines. Among the benchmark items, the efflux ratios of P-gp and BCRP showed high reproducibility in two independent facilities, while the directional transports meditated through Glut1 or TfR were not confirmed. We have organized the protocols of the experiments described above as standard operating procedures (SOPs). We here provide the SOPs with the flow chart including entire procedure and how to apply each SOP. Our study is important developmental step of BBB MPS towards the social acceptance, which enable end users to check and compare the performance the BBB MPSs.

Keywords: microphysiological system, blood–brain barrier, humanized model

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Takahashi K, Chen L*1, Sayama M*1, Wu M*1, Kato-Hayashi M*2, Irie T, Ohwada T*1, Sato K: Leucine 434 is essential for docosahexaenoic acid-induced augmentation of L-glutamate transporter current.

J Biol Chem. 2023 Jan;299(1):102793. doi: 10.1016/j.jbc.2022.102793. Epub 2022 Dec 9. PMID: 36509140

Astrocytic excitatory amino acid transporter 2 (EAAT2) plays a major role in removing the excitatory neurotransmitter L-glutamate (L-Glu) from

synaptic clefts in the forebrain to prevent excitotoxicity. Polyunsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6 n-3) enhance synaptic transmission, and their target molecules include EAATs. Here, we aimed to investigate the effect of DHA on EAAT2 and identify the key amino acid for DHA/EAAT2 interaction by electrophysiological recording of L-Glu-induced current in Xenopus oocytes transfected with EAATs, their chimeras, and single mutants. DHA transiently increased the amplitude of EAAT2 but tended to decrease that of excitatory amino acid transporter subtype 1 (EAAT1), another astrocytic EAAT. Single mutation of leucine (Leu) 434 to alanine (Ala) completely suppressed the augmentation by DHA, while mutation of EAAT1 Ala 435 (corresponding to EAAT2 Leu434) to Leu changed the effect from suppression to augmentation. Other polyunsaturated fatty acids (docosapentaenoic acid, eicosapentaenoic acid, arachidonic acid, and α-linolenic acid) similarly augmented the EAAT2 current and suppressed the EAAT1 current. Finally, our docking analysis suggested the most stable docking site is the lipid crevice of EAAT2, in close proximity to the L-Glu and sodium binding sites, suggesting that the DHA/Leu434 interaction might affect the elevator-like slide and/or the shapes of the other binding sites. Collectively, our results highlight a key molecular detail in the DHA-induced regulation of synaptic transmission involving EAATs.

Keywords: glutamate transporter, polyunsaturated fatty acid (PUFA), electrophysiolosy

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Kato Y*1, Nishiyama K*1, Man Lee J*2, Ibuki Y*3, Imai Y*4, Noda T*5, 6, 7, 8, Kamiya N*9, 10, Kusakabe T*11, Kanda Y, Nishida M*1, 12: TRPC3-Nox2 Protein Complex Formation Increases the Risk of SARS-CoV-2 Spike Protein-Induced Cardiomyocyte Dysfunction through ACE2 Upregulation.

International Journal of Molecular Sciences. 2022;24:102.

Myocardial damage caused by the newly emerged coronavirus (SARS-CoV-2) infection is one of the key determinants of COVID-19 severity and mortality. SARS-CoV-2 entry to host cells is initiated by binding with its receptor, angiotensin-converting enzyme (ACE) 2, and the ACE2 abundance is thought to reflect the susceptibility to infection. Here, we report that ibudilast, which we previously identified as a potent inhibitor of protein complex between transient receptor potential canonical (TRPC) 3 and NADPH oxidase (Nox) 2, attenuates the SARS-CoV-2 spike glycoprotein pseudovirus-evoked contractile and metabolic dysfunctions of neonatal rat cardiomyocytes (NRCMs). Epidemiologically reported risk factors of severe COVID-19, including cigarette sidestream smoke (CSS) and anti-cancer drug treatment, commonly upregulate ACE2 expression level, and these were suppressed by inhibiting TRPC3-Nox2 complex formation. Exposure of NRCMs to SARS-CoV-2 pseudovirus, as well as CSS and doxorubicin (Dox), induces ATP release through pannexin-1 hemi-channels, and this ATP release potentiates pseudovirus entry to NRCMs and human iPS cell-derived cardiomyocytes (hiPS-CMs). As the pseudovirus entry followed by production of reactive oxygen species was attenuated by inhibiting TRPC3-Nox2 complex in hiPS-CMs, we suggest that TRPC3-Nox2 complex formation triggered by panexin1-mediated ATP release participates in exacerbation of myocardial damage by amplifying ACE2-dependent SARS-CoV-2 entry.

Keywords: NADPH oxidase, SARS-CoV-2, chemical stress

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Uchiyama Y*1, Yamazaki D, Kobayashi N*2, Kanda Y, Sugita-Konishi Y*3: Electrophysiological Effect of Citreoviridin on Human InducedPluripotent Stem Cell-derived Cardiomyocytes.

Shokuhin Eiseigaku Zasshi. 2022;63:210-217.

Citreoviridin (CTV) is a mycotoxin produced by various fungi, including Penicillium citreonigrum. One of the toxicities reportedly associated with CTV is neurotoxicity. CTV is also suspected to be associated with acute cardiac beriberi (also known as "Shoshin-kakke") and Keshan disease, which can have adverse effects on the heart, so the *in vivo* and *in vitro* toxicity of CTV on the heart or cardiomyocytes in experimental animal models have been reported. However, the toxicity of CTV for the human heart, especially its electrophysiological effect, remains poorly understood. Therefore, to investigate the electrophysiological effect of CTV on the human cardiomyocytes, we conducted a multi-electrode array (MEA) using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The MEA revealed that 30 μmol/L of CTV stopped the beating of hiPSC-CMs, and the field potential duration and first peak amplitude were shortened at 10 μmol/L. Before the hiPSC-CMs stopped beating, the length of the inter-spike interval varied two- to four-fold. These results demonstrated that CTV induced an electrophysiological disturbance on human cardiomyocytes. This is first paper to elucidate the electrophysiological effect of CTV on human heart directly and may aid in analyzing the risk associated with CTV to ensure food safety.

Keywords: cardiotoxicity, citreoviridin, hiPSC-CM

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Yasuhiko Y, Ishigami M*, Machino S*, Fujii T*, Aoki M*, Irie F*, Kanda Y, Yoshida M*: Comparison of the lower limit of benchmark dose confidence interval with no-observed-adverse-effect level by applying four different software for tumorigenicity testing of pesticides in Japan.

Regul Toxicol Pharmacol 2022 Aug;133:105201. DOI: 10.1016/j.yrtph.2022.105201.

The benchmark dose (BMD) approach is updated to create an international harmonizing process following rapid theoretical sophistication. We calculated the lower limit of BMD confidence interval (BMDL) for carcinogenicity based on 193 tumorigenicity bioassay data published in 50 pesticide risk assessment reports by the Food Safety Commission of Japan (FSCJ) to validate the appropriateness and necessity for the refinement of the FSCJ-established BMD guidance. Three well-known BMD software, PROAST, BMDS, and BBMD were used to compare their BMDLs with no-observed-adverse-effect levels (NOAELs) for carcinogenicity. Recently implemented methodologies such as model averaging or Bayesian inference were also used. Our results indicate that the BMD approach provides a point of departure similar to the NOAEL approach if the data used exhibit a clear dose-response relationship. In some cases, particularly in software with a frequentist approach, the calculation failed to provide BMDL or provided considerably lower BMDLs than NOAELs. However, most of the datasets that resulted in failed calculations or extremely low BMDLs exhibited unclear dose-response relationships, i.e., non-monotonous and sporadic responses. The expert review on the shape of the dose-response plot would help better apply the BMD approach. Furthermore, we observed that Bayesian approaches provided fewer failed or extreme BMD calculations than the frequentist approaches.

Keywords: benchmark dose (BMD), pesticide, risk assessment

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Hirata N, Horinouchi T*, Kanda Y: Effects of cigarette smoke extract derived from heated tobacco products on the proliferation of lung cancer stem cells.
Toxicology Reports. 2022;9:1273-1280.

Epidemiological studies have suggested that cigarette smoking can increase a person's risk of developing several types of cancer, including lung cancer. Lung cancer originates from cancer stem cells (CSCs), which constitute a minor cell population in tumors, and contribute to drug resistance and recurrence. Heated tobacco products (HTPs) produce aerosols that contain nicotine and toxic chemicals. Current evidence, however, is insufficient to accurately determine if HTPs are less harmful than burned cigarettes. This study has investigated the effects of cigarette smoke extract (CSE) from HTPs on lung CSCs in lung cancer cell lines. We found that CSEs induced the proliferation of lung CSCs and increased the expression levels of stem cell markers. In addition, CSE induced epithelial-mesenchymal transition (EMT) expression and cytokine production. These results suggest that HTPs can induce lung CSCs *in vitro*.
Keywords: aldehyde dehydrogenase, cancer stem cell, cigarette smoke extract

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Yamada S, Masuno H*[1], Kagechika H*[1], Tanatani A*[2], Kanda Y: A Novel Lithocholic Acid Derivative Upregulates Detoxification-Related Genes in Human Induced Pluripotent Stem Cell-Derived Intestinal Organoids.
Biological and Pharmaceutical Bulletin. 2022;45:1720-1724.

Vitamin D is a fat-soluble micronutrient that plays essential roles in a range of biological processes, including cell proliferation, inflammation, and metabolism. In this study, we investigated the effects of a novel synthetic lithocholic acid derivative with vitamin D activity (Dcha-20) on pharmacokinetic gene expression in human induced pluripotent stem cell-derived intestinal organoids. Compared with vitamin D3 treatment, Dcha-20 was found to upregulate the expression and enzyme activity of the drug-metabolizing enzyme CYP3A4, an indicator of intestinal functional maturation. In addition, Dcha-20 specifically increased expression levels of the xenobiotic detoxification enzyme UGT1A and excretion transporter MRP2. These results suggest that Dcha-20 promotes activity of the intrinsic defense system of the intestinal epithelium.
Keywords: Dcha-20, detoxification, intestine

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Ishida K*[1], Tatsumi K*[1], Minamigawa Y*[1], Mori K*[1], Matsumaru D*[1], Nagase H*[2], Kanda Y, Takuma K*[3], Nakanishi T*[1]: Neuronal differentiation reporter mice as a new methodology for detecting *in vivo* developmental neurotoxicity.
Biochemical Pharmacology. 2022;206:115332.

Current *in vivo* developmental neurotoxicity (DNT) tests are not performed routinely for chemical risk assessment because they are time and resource intensive and require many animals. Therefore, new methodologies are required that can detect and evaluate the DNT potential of chemicals in a more simple, quantitative, and objective manner. Toward this end, we generated transgenic mice expressing reporter genes (luciferase and lacZ) under the control of the rat synapsin 1 promoter (Syn-Rep mice) and evaluated their usefulness as a DNT detection tool. Brain luciferase expression levels in Syn-Rep mice increased dramatically from just before to after birth, peaked early in the postnatal period, subsequently decreased sharply, and then remained low after weaning. This pattern is analogous to the generally recognized temporal changes in synapse numbers in the developing mammal brain. To evaluate further the responsiveness of Syn-Rep mice during DNT induction, we administered valproic acid (VPA), a reference DNT-inducing chemical, to pregnant mice and evaluated its effect on reporter gene expression in the developing brains of Syn-Rep pups. *In vivo* luminescence in the brains of VPA-exposed pups was significantly lower than in controls from postnatal days 4 to 13. Moreover, luciferase activity in the prefrontal cortexes of 8-week-old VPA-exposed offspring was significantly lower than in controls, reflecting the

reduced number of neurons in the prefrontal cortex. These results suggest that the Syn-Rep mice are potentially useful tools for streamlined detection of chemical-induced DNT in the developing mammalian brain.

Keywords: DNT testing, *in vivo* imaging, luciferase

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Satsuka A, Hayashi S, Yanagida S, Ono A*, Kanda Y: Contractility assessment of human iPSC-derived cardiomyocytes by using a motion vector system and measuring cell impedance.
Journal of Pharmacological and Toxicological Methods. 2022;118:107227.

Predicting drug-induced cardiotoxicity during the non-clinical stage is important to avoid severe consequences in the clinical trials of new drugs. Human iPSC-derived cardiomyocytes (hiPSC-CMs) hold great promise for cardiac safety assessments in drug development. To date, multi-electrode array system (MEA) has been a widely used as a tool for the assessment of proarrhythmic risk with hiPSC-CMs. Recently, new methodologies have been proposed to assess *in vitro* contractility, such as the force and velocity of cell contraction, using hiPSC-CMs. Herein, we focused on an imaging-based motion vector system (MV) and an electric cell-substrate impedance sensing system (IMP). We compared the output signals of hiPSC-CMs from MV and IMP in detail and observed a clear correlation between the parameters. In addition, we assessed the effects of isoproterenol and verapamil on hiPSC-CM contraction and identified a correlation in the contractile change of parameters obtained with MV and IMP. These results suggest that both assay systems could be used to monitor hiPSC-CM contraction dynamics.

Keywords: iPSC, motion vector, impedance

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Chiba K*1,2, Kambayashi R*1, Onozato M*3, Goto A*1, Izumi-Nakaseko H*1, Takei Y*1, Matsumoto A*4, Tanaka K*2, Kanda Y, Fukushima T*3, Sugiyama A*1,4: Imatinib induces diastolic dysfunction and ventricular early-repolarization delay in the halothane-anesthetized dogs: Class effects of tyrosine kinase inhibitors.
Journal of Pharmacological Sciences. 2022;150:154-162.

Imatinib has been reported to induce heart failure and/or QTc prolongation. To better understand their underlying mechanisms, we assessed its effects on cardiohemodynamic, electrocardiographic and echocardiographic variables along with biomarkers of myocardial damage. Imatinib mesylate in doses of 1 and 10 mg/kg was intravenously administered to the halothane-anesthetized beagle dogs (n = 4). Effects of imatinib on each phase of isovolumetric contraction, ejection, isovolumetric relaxation and filling were studied, whereas its electrophysiological effects on early and late repolarization were analyzed by measuring J-Tpeak and Tpeak-Tend, respectively. The low and high doses of imatinib provided peak plasma concentrations of 3.23 and 17.39 μg/mL, reflecting clinically-relevant and supratherapeutic concentrations, respectively. Neither lethal ventricular tachyarrhythmia nor cardiohemodynamic collapse was observed. Imatinib decreased amplitude of peak -dP/dt, indicating suppression of isovolumetric relaxation, whereas no significant change was detected in the other phases. Imatinib prolonged QTc and J-Tpeakc without altering Tpeak-Tend, indicating increase of net inward current, which leads to intracellular Ca2+ overload. Thus, imatinib suppressed ventricular active relaxation and early repolarization, which may suggest the association of mitochondrial dysfunction-associated inhibition of ATP production. Since those findings were also reported for dasatinib, sunitinib and lapatinib, they could be common cardiac phenotype of tyrosine kinase inhibitors *in vivo*.

Keywords: diastolic dysfunction, imatinib, QTc

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Li M*1,2, Nawa Y*1,2, Ishida S*1,3, Kanda Y, Fujita S*1,2, Fujita K*1,2,4: Label-free chemical imaging of cytochrome P450 activity by Raman microscopy.
Communications Biology. 2022;5:778.

Although investigating drug modulation of cytochrome P450 (CYP) activity under physiological conditions is crucial in drug development to avoid severe adverse drug reactions, the current evaluation approaches that rely on the destructive and end-point analysis can be misleading due to invasive treatments and cellular heterogeneity. Here, we propose a non-destructive and high-content method for visualizing and quantifying intracellular CYP activity under drug administration by Raman microscopy. The redox-state and spin-state sensitive Raman measurement indicated that the induced CYPs in living hepatocytes were in oxidized and low-spin state, which is related to monooxygenase function of CYP. Moreover, glycogen depletion associated with CYP induction was simultaneously observed, indicating a relevant effect on glucose metabolism. By deciphering the overall changes in the biochemical fingerprints of hepatocytes, Raman microscopy offers a non-destructive and quantitative chemical imaging method to evaluate CYP activity at the single-cell level with the potential to facilitate future drug development schemes.

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Goto A*1, Sakamoto K*2, Kambayashi R*1, Izumi-Nakaseko H*1, Kawai S*3, Takei Y*1, Matsumoto A*4, Kanda Y, Sugiyama A*1,3,4: Validation of Risk-Stratification Method for the Chronic Atrioventricular Block Cynomolgus Monkey Model and Its Mechanistic Interpretation Using 6 Drugs With Pharmacologically Distinct Profile.
Toxicological Sciences. 2022;190:99-109.

Validation of risk-stratification method for the chronic atrioventricular block cynomolgus monkey model and its mechanistic interpretation was performed using 6 pharmacologically distinct drugs. The following drugs were orally administered in conscious state, astemizole: 1, 5, and 10 mg/kg (n = 6); haloperidol: 1, 10, and 30 mg/kg (n = 5); amiodarone: 30 mg/kg (n = 4); famotidine: 10 mg/kg (n = 4); levofloxacin: 100 mg/kg (n = 4); and tolterodine: 0.2, 1, and 4.5 mg/kg (n = 4). Astemizole of 5 and 10 mg/kg significantly prolonged ΔΔQTcF, whereas no significant change was observed by the others. Torsade de pointes (TdP) was induced by astemizole of 5 and 10 mg/kg in 3/6 and 6/6, and by haloperidol of 10 and 30 mg/kg in 1/5 and 1/5, respectively, which was not observed in the others. Torsadogenic risk of the drugs was quantified using the criteria for the monkey model specified in our previous study. Namely, high-risk drugs induced TdP at ≤ 3 times of their maximum clinical daily dose. Intermediate-risk drugs did not induce TdP at this dose range, but induced it at higher doses. Low/no-risk drugs never induced TdP at any dose tested. The magnitude of risk was intermediate for astemizole and haloperidol, and low/no risk for the others. The prespecified, risk-stratification method for the monkey model may solve the issue existing between nonclinical models and patients with labile repolarization, which can reinforce the regulatory decision-making and labeling at time of marketing application of nondouble-negative drug candidate (hERG assay positive and/or *in vivo* QT study positive).

Keywords: *in silico* model, CiPA, atrioventricular block

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Yanagida S, Satsuka A, Hayashi S, Ono A*, Kanda Y: Proarrhythmia risk assessment using electromechanical window in human iPS cell-derived cardiomyocytes.
Biological and Pharmaceutical Bulletin. 2022;45:940-

947

Evaluation of drug-induced cardiotoxicity is still challenging to avoid adverse effects, such as torsade de pointes (TdP), in non-clinical and clinical studies. Numerous studies have suggested that human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a useful platform for detecting drug-induced TdP risks. Comprehensive *in vitro* Proarrhythmia Assay (CiPA) validation study suggested that hiPSC-CMs can assess clinical TdP risk more accurately than the human ether-a-go-go-related assay and QT interval prolongation. However, there were still some outliers, such as bepridil, mexiletine, and ranolazine, among the CiPA 28 compounds in the CiPA international multi-site study using hiPSC-CMs. In this study, we assessed the effects of the positive compound dofetilide, the negative compound aspirin, and several CiPA compounds (bepridil, mexiletine, and ranolazine) on the electromechanical window (E-M window), which were evaluated using multi-electrode array assay and motion analysis, in hiPSC-CMs. Similar to previous *in vivo* studies, dofetilide, which has a high TdP risk, decreased the E-M window in hiPSC-CMs, whereas aspirin, which has a low TdP risk, had little effect. Bepridil, classified in the high TdP-risk group in CiPA, decreased the E-M window in hiPSC-CMs, whereas ranolazine and mexiletine, which are classified in the low TdP-risk group in CiPA, slightly decreased or had little effect on the E-M window of hiPSC-CMs. Thus, the E-M window in hiPSC-CMs can be used to classify drugs into high and low TdP risk.

Keywords: electro-mechanical window, induced pluripotent stem cell, proarrhythmia

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Nishiuchi S*1, 2, Yagi K*3, Saito H*1, Zamami Y*1, 4, Niimura T*3, Miyata K*1, Sakamoto Y*1, Fukunaga K*1, Ishida S*2, Hamano H*1, 4, Aizawa F*2, Goda M*1, 4, Chuma M*5, Izawa-Ishizawa Y*6, Nawa H*7, Yanagawa H*3, Kanda Y, Ishizawa K*1, 2, 3: Investigation of drugs for the prevention of doxorubicin-induced cardiac events using big data analysis.

European Journal of Pharmacology. 2022;928:175083.

Aim: Doxorubicin, an anthracycline anti-tumour agent, is an essential chemotherapeutic drug; however, the adverse events associated with doxorubicin usage, including cardiotoxicity, prevent patients from continuing treatment. Here, we used databases to explore existing approved drugs with potential preventative effects against doxorubicin-induced cardiac events and examined their efficacy and mechanisms.

Methods: The Gene Expression Omnibus (GEO), Library of Integrated Network-based Cellular Signatures (LINCS), and Food and Drug Administration Adverse Events Reporting System (FAERS) databases were used to extract candidate prophylactic drugs. Mouse models of doxorubicin-induced cardiac events were generated by intraperitoneal administration of 20 mg/kg of doxorubicin on Day 1 and oral administration of prophylactic candidate drugs for 6 consecutive days beginning the day before doxorubicin administration. On Day 6, mouse hearts were extracted and examined for mRNA expression of apoptosis-related genes.

Results: GEO analysis showed that doxorubicin administration upregulated 490 genes and downregulated 862 genes, and LINCS data identified sirolimus, verapamil, minoxidil, prednisolone, guanabenz, and mosapride as drugs capable of counteracting these genetic alterations. Examination of the effects of these drugs on cardiac toxicity using FAERS identified sirolimus and mosapride as new prophylactic drug candidates. In model mice, mosapride and sirolimus suppressed the Bax/Bcl-2 mRNA ratio, which is elevated in doxorubicin-induced cardiotoxicity. These drugs also suppressed the expression of inflammatory cytokines Il1b and Il6 and markers associated with myocardial fibrosis, including Lgal3 and Timp1.

Conclusion: These findings suggest that doxorubicin-induced cardiac events are suppressed by the administration of mosapride and sirolimus.

Keywords: cardiology, chemotherapy, data analysis

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Yamada S, Noda T*1, 2, Okabe K*1, Yanagida S, Nishida M*3, 4, Kanda Y: SARS-CoV-2 induces barrier damage and inflammatory responses in the human iPSC-derived intestinal epithelium.
Journal of Pharmacological Sciences. 2022;149:139-146.

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly spread and led to global health crises. COVID-19 causes well-known respiratory failure and gastrointestinal symptoms, such as diarrhea, nausea, and vomiting. Thus, human gastrointestinal cell models are urgently needed for COVID-19 research; however, it is difficult to obtain primary human intestinal cells. In this study, we examined whether human induced pluripotent stem cell (iPSC)-derived small intestinal epithelial cells (iPSC-SIECs) could be used as a SARS-CoV-2 infection model. We observed that iPSC-SIECs, such as absorptive and Paneth cells, were infected with SARS-CoV-2, and remdesivir treatment decreased intracellular SARS-CoV-2 replication in iPSC-SIECs. SARS-CoV-2 infection decreased expression levels of tight junction markers, ZO-3 and CLDN1, and transepithelial electrical resistance (TEER), which evaluates the integrity of tight junction dynamics. In addition, SARS-CoV-2 infection increased expression levels of proinflammatory genes, which are elevated in patients with COVID-19. These findings suggest iPSC-SIECs as a useful *in vitro* model for elucidating COVID-19 pathology and drug development.
Keywords: barrier function, SARS-CoV-2, small intestinal epithelial cell

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Kato Y*1, Nishiyama K*1, Nishimura A*2, 3, 4, Noda T*5, 6, 7, 8, Okabe K*5, Kusakabe T*9, Kanda Y, Nishida M*1, 2, 3: Drug repurposing for the treatment of COVID-19.
Journal of Pharmacological Sciences. 2022;149:108-114.

Coronavirus disease 2019 (COVID-19) remains prevalent worldwide since its onset was confirmed in Wuhan, China in 2019. Vaccines against the causative virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have shown a preventive effect against the onset and severity of COVID-19, and social and economic activities are gradually recovering. However, the presence of vaccine-resistant variants has been reported, and the development of therapeutic agents for patients with severe COVID-19 and related sequelae remains urgent. Drug repurposing, also called drug repositioning or eco-pharma, is the strategy of using previously approved and safe drugs for a therapeutic indication that is different from their original indication. The risk of severe COVID-19 and mortality increases with advancing age, cardiovascular disease, hypertension, diabetes, and cancer. We have reported three protein-protein interactions that are related to heart failure, and recently identified that one mechanism increases the risk of SARS-CoV-2 infection in mammalian cells. This review outlines the global efforts and outcomes of drug repurposing research for the treatment of severe COVID-19. It also discusses our recent finding of a new protein-protein interaction that is common to COVID-19 aggravation and heart failure.
Keywords: cardiomyocyte, eco-pharma, NADPH oxidase

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Hirata N, Yamada S, Yanagida S, Ono A*, Yasuhiko Y, Kanda Y: Transforming Growth Factor Beta Promotes the Expansion of Cancer Stem Cells via S1PR3 by Ligand-Independent Notch Activation.
Biological and Pharmaceutical Bulletin. 2022;45: 649-658.

Growing evidence suggests that cancer originates from cancer stem cells (CSCs), which can be identified by aldehyde dehydrogenase (ALDH) activity-based flow cytometry. However, the regulation of CSC growth is not fully understood. In the present study, we investigated the effects of Transforming Growth Factor-β (TGFβ) in breast CSC expansion. Stimulation with TGFβ increased the ALDH-positive breast CSC population via the phosphorylation of sphingosine kinase 1 (SphK1), a sphingosine-1-phosphate (S1P) -producing enzyme, and subsequent S1P-mediated S1P receptor 3 (S1PR3) activation. These data suggest that TGFβ promotes breast CSC expansion via the ALK5/SphK1/S1P/S1PR3 signaling pathway. Our findings provide new insights into the role of TGFβ in the regulation of CSCs.
Keywords: ALK5, Notch, Transforming Growth Factor-β (TGFβ)

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Piantino M*1, Kang DH*1, Furihata T*2, Nakatani N*3, Kitamura K, Shigemoto-Mogami Y, Sato K, Matsusaki M*1: Development of a three-dimensional blood-brain barrier network with opening capillary structures for drug transport screening assays.
Mater Today Bio. 2022 Jun 15;15:100324. doi: 10.1016/j.mtbio.2022.100324. eCollection 2022 Jun. PMID: 35757028

The blood-brain barrier (BBB), a selective barrier regulating the active and passive transport of solutes in the extracellular fluid of the central nervous system, prevents the delivery of therapeutics for brain disorders. The BBB is composed of brain microvascular endothelial cells (BMEC), pericytes and astrocytes. Current *in vitro* BBB models cannot reproduce the human structural complexity of the brain microvasculature, and thus their functions are not enough for drug assessments. In this study, we developed a 3D self-assembled microvascular network formed by BMEC covered by pericytes and astrocyte end feet. It exhibited perfusable microvasculature due to the presence of capillary opening ends on the bottom of the hydrogel. It also demonstrated size-selective permeation of different molecular weights of fluorescent-labeled dextran, as similarly reported for *in vivo* rodent brain, suggesting the same permeability with actual *in vivo* brain. The activity of P-glycoprotein efflux pump was confirmed using the substrate Rhodamine 123. Finally, the functionality of the receptor-mediated transcytosis, one of the main routes for drug delivery of large molecules into the brain, could be validated using transferrin receptor (TfR) with confocal imaging, competition assays and permeability assays. Efficient permeability coefficient (Pe) value of transportable anti-TfR antibody (MEM-189) was seven-fold higher than those of isotype antibody (IgG1) and low transportable anti-TfR antibody (13E4), suggesting a higher TfR transport function than previous reports. The BBB model with capillary openings could thus be a valuable tool for the screening of therapeutics that can be transported across the BBB, including those using TfR-mediated transport.
Keywords: blood-brain barrier, *in vitro* model, transferrin receptor

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Yosuke Uchiyama[*1], Daiju Yamazaki, Naoki Kobayashi[*2], Yasunari Kanda, Yoshiko Sugita-Konishi[*3]: Electrophysiological Effect of Citreoviridin on Human Induced Pluripotent Stem Cell-derived Cardiomyocytes.

Shokuhin Eiseigaku Zasshi 2022;63:210-217. doi: 10.3358/shokueishi.63.210

Citreoviridin (CTV) is a mycotoxin produced by various fungi, including Penicillium citreonigrum. One of the toxicities reportedly associated with CTV is neurotoxicity. CTV is also suspected to be associated with acute cardiac beriberi (also known as "Shoshin-kakke") and Keshan disease, which can have adverse effects on the heart, so the *in vivo* and *in vitro* toxicity of CTV on the heart or cardiomyocytes in experimental animal models have been reported. However, the toxicity of CTV for the human heart, especially its electrophysiological effect, remains poorly understood. Therefore, to investigate the electrophysiological effect of CTV on the human cardiomyocytes, we conducted a multi-electrode array (MEA) using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The MEA revealed that 30 μmol/L of CTV stopped the beating of hiPSC-CMs, and the field potential duration and first peak amplitude were shortened at 10 μmol/L. Before the hiPSC-CMs stopped beating, the length of the inter-spike interval varied two- to four-fold. These results demonstrated that CTV induced an electrophysiological disturbance on human cardiomyocytes. This is first paper to elucidate the electrophysiological effect of CTV on human heart directly and may aid in analyzing the risk associated with CTV to ensure food safety.

Keywords: cardiotoxicity, citreoviridin, hiPSC-CMs

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Chem Res Toxicol. 2022;35:1625-30. doi: 10.1021/acs.chemrestox.2c00226.

Several aromatic amine compounds, including 4-aminobiphenyl, are urinary bladder carcinogen with metabolic activation followed by DNA adduct formation as carcinogenic mechanism. Activated metabolites and DNA adducts of polycyclic aromatic amines such as 4-aminobiphenyl have been identified, whereas that of monocyclic aromatic amines such as *o*-toluidine (*o*-Tol) and *o*-anisidine (*o*-Ans), aniline (Ani) have not been fully determined. We have recently reported that *o*-Tol or *o*-Ans were metabolically converted *in vitro* and *in vivo* to cytotoxic and mutagenic *p*-semidine type dimers, 2-methyl-N^4-(2-methylphenyl) benzene-1,4-diamine (MMBD) and 2-methoxy-N^4-(2-methoxyphenyl) benzene-1,4-diamine (MxMxBD), respectively, suggesting roles in urinary bladder carcinogenesis. In this study, we found that when *o*-Tol and *o*-Ans were incubated with S9 mix, not only MMBD and MxMxBD, but also two isomeric heterodimers named MMxBD and MxMBD were formed. Therefore, any two of *o*-Tol, *o*-Ans and Ani (10 mM each) were incubated with S9 mix up to 24 hours, then applied to LC-MS to investigate their metabolic kinetics. Metabolic conversions to all 9 kinds of *p*-semidine type homo- and heterodimers were observed, peaking at 6 hours of incubation with S9 mix, especially MxMxBD, reaching 6.1 ± 1.4 μM. Homo- and heterodimers containing *o*-Ans moiety in the diamine structure rather than *o*-Tol or Ani showed faster dimerization ratio, whereas levels of these dimers such as MxMxBD were markedly declined with further incubation. Dimers containing *o*-Tol and Ani were relatively stable even after an incubation for 24 hours. The electron donating group of *o*-Ans moiety may be involved in a rapid metabolic conversion. In cytotoxic assay, dimers with *o*-Ans moiety in the diamine structure and MMBD showed about 2-4 fold stronger cytotoxicity than other dimers in human bladder cancer T24 cells. These chemical and biological properties of homo- and heterodimers of monocyclic aromatic amine might be important to consider the combined exposure risk of bladder carcinogenesis.

Keywords: aromatic amine, cytotoxicity, urinary bladder

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Takasu S, Ishii Y, Namiki M, Nakamura K, Mitsumoto T, Takimoto N, Nohmi T, Ogawa K: Comprehensive analysis of the general toxicity, genotoxicity, and carcinogenicity of 3-acetyl-2,5-dimethylfuran in male *gpt* delta rats.

Food Chem Toxicol. 2022;172:113544. doi: 10.1016/j.fct.2022.113544.

The safety of flavoring agents has been evaluated according to classification by chemical structure and using a decision tree approach. The genotoxic potential found in some flavoring agents has highlighted the importance of efficient toxicity studies. We performed a comprehensive toxicity analysis using reporter gene transgenic rats to assess the safety of 3-acetyl-2,5-dimethylfuran (ADF), a flavoring agent exhibiting genotoxic potential in *in silico* and *in vitro* assays. Male F344 *gpt* delta rats were given 0, 30, or 300 mg/kg body weight/day ADF by gavage for 13 weeks. In serum biochemistry analyses, triglyceride, total cholesterol, phospholipid, and total protein levels and albumin/globulin ratios were significantly altered in the 30 and 300 mg/kg groups. Histopathologically, nasal cavity toxicity and hepatocellular hypertrophy were observed in the 300 mg/kg group. In the livers of 300 mg/kg group, a significant increase in *gpt* mutant frequencies were observed along with ADF-specific DNA adduct formation. The number and area of glutathione S-transferase placental form-positive foci were significantly increased in the same group. Thus, ADF affected nasal cavity, liver, and lipid metabolism and showed genotoxicity and possible carcinogenicity in the liver. Overall, our comprehensive toxicity study using *gpt* delta rats provided insights into the safety evaluation of ADF.

Keywords: 3-acetyl-2,5-dimethylfuran, flavoring agent, *gpt* delta rat

Kuroda K, Ishii Y, Takasu S, Matsushita K, Kijima K, Nohmi T, Umemura T: Toxicity, genotoxicity, and carcinogenicity of 2-methylfuran in a 90-day comprehensive toxicity study in *gpt* delta rats.

Food Chem Toxicol. 2022;168:113365. doi: 10.1016/j.fct.2022.113365.

2-Methylfuran (2-MF) exists naturally in foods and is used as a flavoring agent. Furan, the core structure of 2-MF, possesses hepatocarcinogenicity in rodents. Accumulation of toxicological information on furan derivatives is needed to elucidate their carcinogenic mode of action. In the current study, we examined the comprehensive toxicological studies of 2-MF using *gpt* delta rats. 2-MF was intragastrically administered to groups of 10 male and 10 female Sprague-Dawley *gpt* delta rats at a dose of 0, 1.2, 6, or 30 mg/kg/day for 13 weeks. Effects of 2-MF on the hepatobiliary system including an increase in serum alkaline phosphatase were observed in the 6 and 30 mg/kg groups, and cholangiofibrosis was found in the 30 mg/kg group. The no observed adverse effect level was set at 1.2 mg/kg/day for both sexes and 1.14 mg/kg/day was determined as the benchmark dose low. The acceptable daily intake was calculated to be 11.4 μg/kg/day. Increases in the number and areas of glutathione S-transferase placental form-positive foci in the 30 mg/kg group were apparent, suggesting the hepatocarcinogenicity of 2-MF in rats. By contrast, the lack of increase in *in vivo* mutagenicity in the liver implied that 2-MF hepatocarcinogenesis may not involve genotoxic mechanisms.

Keywords: 2-methylfuran, carcinogenesis, *gpt* delta rat

Ishii Y, Nakamura K, Mitsumoto T, Takimoto N, Namiki M, Takasu S, Ogawa K: Visualization of the distribution of anthraquinone components from madder roots in rat kidneys by desorption electrospray ionization-time-of-flight mass spectrometry imaging.

Food Chem Toxicol. 2022;161:112851. doi: 10.1016/j.fct.2022.112851.

Madder color (MC), a natural dye isolated from Rubia tinctorum, is a potent carcinogen that targets the outer stripe of outer medulla (OSOM) in the kidneys of rats. To clarify the role of MC components in renal carcinogenesis, we examined distributions of MC components and metabolites in the kidneys of rats treated with MC using desorption electrospray ionization-mass spectrometry imaging (DESI-MSI). Alizarin, lucidin, munjistin, nordamnacanthal, purpurin, pseudopurpurin, rubiadin, and some other metabolites detected and identified by liquid chromatography time-of-flight MS analysis of rat serum 1 h after MC administration were subjected to DESI-MSI. This analysis enabled visualization of the distribution of

anthraquinones in the kidney, and the ion images showed a characteristic distribution according to their chemical structure. Among the components, lucidin and rubiadin specifically localized in the OSOM, suggesting that their genotoxicity was a direct cause of MC carcinogenesis. Alizarin showed greater distribution in the OSOM than the cortex and may therefore participate in renal carcinogenicity owing to its tumor-promoting activity. Overall, our data suggested that the distribution of carcinogenic components to the OSOM was responsible for the site-specific renal carcinogenicity of MC and that DESI-MSI analysis may be a powerful tool for exploring the mechanisms of chemical carcinogenesis.

Keywords: desorption electrospray ionization-mass spectrometry imaging, genotoxicity, madder color

Akane H, Toyoda T, Mizuta Y, Cho YM, Ide T, Kosaka T*, Tajima H*, Aoyama H*, Ogawa K: Histopathological and immunohistochemical evaluation for detecting changes in blood hormone levels caused by endocrine disruptors in a 28-day repeated-dose study in rats.

J Appl Toxicol. 2022;42:1603-17. doi: 10.1002/jat.4327.

Although measurements of blood hormone levels in rodent toxicological studies can provide important information on the mechanisms of toxicity and extrapolation to humans, there are several difficulties such as large individual differences and limited sample volume. To develop a more simplified method that does not depend solely on blood samples, we examined the possible application of immunohistochemistry for detecting endocrine disruptors in short-term studies. Aminotriazole (AMT), propylthiouracil (PTU), phenobarbital, aminoglutethimide (AGT), estradiol, and vitamin D3 were administered orally to 6-week-old male and female SD rats (five/group) for 28 days. Measurements of serum hormone levels revealed decreases in triiodothyronine (T3) and thyroxine (T4) in the AMT and PTU groups, an increase in thyroid stimulating hormone (TSH) in the AMT, PTU, and AGT groups, and an increase in adrenocorticotrophic hormone in the AGT group. Increased thyroid, pituitary, and adrenal gland weights; histopathological lesions, including follicular hypertrophy/hyperplasia, hypertrophy/vacuolation of anterior pituitary cells, and increased adrenocortical vacuolation were observed in association with the hormone level changes. Immunohistochemical analysis revealed a decreased T4 level in the thyroid gland of the AMT and PTU groups and an increased area of TSH positive immunostaining in the pituitary gland of the AMT, PTU, and AGT groups, consistent with the changes in serum T4 and TSH levels, respectively. These results suggest that histopathological analysis and immunohistochemistry for T4 and TSH might be useful and sensitive methods of detecting thyroid dysfunction, and that combining organ weight measurements is a reliable parameter of detecting endocrine disruptors.

Keywords: endocrine disruptor, immunohistochemistry, thyroid gland

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Toyoda T, Kobayashi T*, Miyoshi N*, Matsushita K, Akane H, Morikawa T, Ogawa K: Toxicological effects of two metabolites derived from *o*-toluidine and *o*-anisidine after 28-day oral administration to rats.

J Toxicol Sci. 2022;47:457-66. doi.org/10.2131/jts.47.457.

Although both *o*-toluidine and *o*-anisidine are known as aromatic amines with bladder carcinogenicity, the specific metabolites involved in carcinogenesis are still unclear. Here, we examined the toxicological effects of head-to-tail dimers of *o*-toluidine and *o*-anisidine, 2-methyl-N^4-(2-methylphenyl) benzene-1,4-diamine (MMBD) and 2-methoxy-N^4-(2-methoxyphenyl) benzene-1,4-diamine (MxMxBD), respectively, in rats. Six-week-old male F344 rats were orally administered MMBD, MxMxBD, *o*-toluidine, and *o*-anisidine at a dose of 100 mg/kg/day for 28 days. Rats administered 400 mg/kg *o*-toluidine and 600 mg/kg/day *o*-anisidine were set as high-dose groups for comparison. Histopathology and immunohistochemistry for γ-H2AX, a DNA damage biomarker, and bladder stem cell markers, including aldehyde dehydrogenase 1A1 (ALDH1A1), were performed. MMBD and MxMxBD caused different toxicities than their monomers, inducing hepatotoxicity such as vacuolar degeneration but not splenic lesions due to methemoglobinemia. Bladder lesions, including urothelial hyperplasia, were observed in the high-dose *o*-toluidine and *o*-anisidine

groups, whereas no obvious changes were induced in the low-dose groups or their dimers. Although γ-H2AX formation was significantly increased by *o*-toluidine and *o*-anisidine treatment, γ-H2AX formation did not differ among the MMBD, MxMxBD, and control groups. Notably, immunohistochemistry revealed marked increases in ALDH1A1 expression in the bladder urothelium of the MMBD and MxMxBD groups and in the *o*-toluidine and *o*-anisidine groups, suggesting that the two dimers may contribute to the bladder carcinogenic effects of *o*-toluidine and *o*-anisidine to some extent. The degrees of bladder lesions and γ-H2AX formation did not correlate with the amount of unchanged *o*-toluidine and *o*-anisidine in urine, indicating the presence of other metabolites responsible for these findings.

Keywords: aromatic amine, γ-H2AX, urinary bladder

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Yamada T, Toyoda T, Matsushita K, Akane H, Morikawa T, Cho YM, Ogawa K: Persistent γ-H2AX formation and expression of stem cell markers in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced bladder carcinogenesis in rats.

Toxicol Sci. 2022;189:51-61. doi: 10.1093/toxsci/kfac064.

We investigated γ-H2AX formation, a biomarker of DNA damage, and expression of stem cell markers (SCMs), including cytokeratin 14, aldehyde dehydrogenase 1A1 (ALDH1A1), and CD44, in the development of rat bladder tumors induced by short-term administration of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN). Histopathological examination showed that diffuse simple hyperplasia of the bladder urothelium induced by BBN recovered to the normal-appearing urothelium after withdrawal, whereas focal proliferative lesions were newly developed and subsequently progressed to benign papilloma and carcinoma. Immunohistochemical analysis revealed that BBN-induced γ-H2AX formation and ALDH1A1 and CD44 expression persisted at higher levels in the normal-appearing urothelium than those in the control group for long periods after withdrawal. Since persistent chronic inflammation was observed even after withdrawal, targeted gene expression analysis of inflammation-related factors revealed 101 genes that showed persistent high expression. Pathway analysis suggested that Stat3 and/or Myc activation may be associated with SCM expression. We focused on hepatocyte growth factor (*Hgf*), one of the genes predicted in relation to *Stat3/Myc*, and confirmed that HGF-positive cells increased by BBN persisted in the normal-appearing urothelium after withdrawal and colocalized with γ-H2AX and SCMs. These results suggested that the long-term persistence of γ-H2AX formation and SCM expression, which occurred during the early stages of bladder tumorigenesis, is not a transient response to exposure and might contribute to bladder tumorigenesis. Although further studies are needed, BBN-induced rat bladder tumors may originate from focal hyperplasia arising from SCM-positive cells via activation of the STAT3/MYC pathway after DNA damage involving γ-H2AX formation.

Keywords: carcinogenicity, γ-H2AX, urinary bladder

Hayashi TI*, Furuhama A, Yokomizo H*, Yamamoto H*: Quantitative analyses of misclassification rates in the hazard classification of environmental chemicals: Evaluation of procedures for deriving predicted no-effect concentrations in the Chemical Substance Control Law in Japan.

Risk Anal. 2023;43:686-699. doi: 10.1111/risa.13952.

The quality of chemical management depends more or less on practical procedures used to assess chemicals. This study quantitatively assessed the efficacy of a derivation procedure for calculating no-effect concentrations for screening assessment of environmental hazards under the Chemical Substance Control Law in Japan. We first evaluated the derivation procedure by applying a series of test ecotoxicity datasets to the procedure and calculating the resulting misclassification rates of the hazardous class of chemicals. In this study, a chemical was deemed to have been misclassified if its classification differed from its classification based on the full dataset (chronic toxicity data for three trophic levels), which was defined as the correct assignment. We also calculated the effects of additional uncertainty factors to decrease the variance (i.e., to improve the consistency) of the misclassification rates among cases with different data availability in the derivation procedure. The results showed that the derivation procedure resulted in very high rates of

misclassification when only particular sets of ecotoxicity data were available (e.g., only chronic toxicity data of algae were available). Our analyses also showed that the use of additional uncertainty factors improved the consistency of the misclassification rates within the derivation procedure. Our study presents a broadly applicable calculation framework for quantifying error rates in assessment procedures and serves as a case study for future development and reforms of chemical assessment processes and policies, while additional analyses using more extensive ecotoxicity data with various modes of actions are needed in the future.

Keywords: Chemical Substance Control Law, ecotoxicity, hazard classification

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Honma M, Yamada M, Yasui M, Horibata K, Sugiyama K, Masumura K: Genotoxicity assessment of food-flavoring chemicals used in Japan.

Toxicol Rep. 2022;9:1008-1012. doi: 10.1016/j.toxrep.2022.04.026.

We assessed the genotoxicity of 30 food-flavoring chemicals used in Japan that have not been investigated before. These 30 food-flavoring chemicals have representative chemical structures belonging to 18 chemical classes. The Ames and chromosomal aberration (CA) tests (*in vitro* tests) were first conducted in accordance with the "Food Additive Risk Assessment Guidelines" of the Japan Food Safety Commission. If the *in vitro* test yielded a positive result, an *in vivo* micronucleus test or a transgenic mouse gene mutation assay was performed to verify the *in vitro* test results. Of the 30 food-flavoring chemicals, 3 yielded a positive result in both Ames and CA tests. Another 11 chemicals yielded positive results in the CA test. However, none of the chemicals yielding positive *in vitro* test results yielded positive results in the *in vivo* tests. These findings indicate no genotoxicity concerns of the food-flavoring chemicals belonging to the abovementioned 18 chemical classes used in Japan unless there are other structural modifications.

Keywords: food safety commission, food flavor, genotoxicity

Sugiyama K, Kinoshita M, Grúz P, Kasamatsu T, Honma M: Bisphenol-A reduces DNA methylation after metabolic activation.

Genes Environ. 2022;44:20. doi: 10.1186/s41021-022-00249-y

Bisphenol-A (BPA) is an important environmental contaminant with adverse health effects suspected to be mediated through epigenetic mechanisms. We had reported that the FLO1-dependent flocculation of transgenic yeast expressing human DNA methyltransferase (DNMT yeast) is a useful tool in epigenotoxicology studies. In this report, we have investigated the effects of BPA in the presence of metabolic activation (S-9 mix) on the transcription level of the FLO1 gene in the DNMT yeast. In the presence of metabolic activation, BPA inhibited the intensity of green fluorescence reporter protein (GFP) driven by the FLO1 promoter. A metabolite of BPA, 4-methyl-2,4-bis(p-hydroxyphenyl) pent-1-ene (MBP), also exhibited similar inhibitory effect. Furthermore, BPA in the presence of S-9 mix had only a weak while MBP had no inhibitory effects on the expression of modified GFP reporter gene under the control of FLO1 promoter with reduced CpG motifs. Aforementioned behavior was confirmed by the inhibition of flocculation as well as FLO1 gene mRNA expression. In addition, the global DNA methylation level in the human HEK293 cells was also reduced by MBP. These results indicate that BPA metabolites have inhibitory effect on DNA methylation. Our approach offers a novel *in vitro* method for screening for chemicals that can alter the epigenome by a mechanism dependent on their metabolic activation.

Keywords: bisphenol-A, DNA methylation, metabolic activation

Horibata K, Takasawa H[*1], Hojo M[*2], Taquahashi Y, Shigano M[*1], Yokota S, Kobayashi N, Sugiyama K, Honma M, Hamada S[*3]: *In vivo* genotoxicity assessment of a multiwalled carbon nanotube in a mouse *ex vivo* culture.

Genes Environ. 2022;44:24. doi: 10.1186/s41021-022-00253-2

Background: Multiwalled carbon nanotubes (MWCNTs) are suspected lung carcinogens because their shape and size are similar to asbestos. Various MWCNT types are manufactured; however, only

MWNT-7 is classified into Group 2B by The International Agency for Research on Cancer. MWNT-7's carcinogenicity is strongly related to inflammatory reactions. On the other hand, inconsistent results on MWNT-7 genotoxicity have been reported. We previously observed no significant differences in both *Pig-a* (blood) and *gpt* (lung) mutant frequencies between MWNT-7-intratracheally treated and negative control rats. In this study, to investigate *in vivo* MWNT-7 genotoxicity on various endpoints, we attempted to develop a lung micronucleus assay through *ex vivo* culture targeting the cellular fraction of Clara cells and alveolar Type II (AT-II) cells, known as the initiating cells of lung cancer. Using this system, we analyzed the *in vivo* MWNT-7 genotoxicity induced by both whole-body inhalation exposure and intratracheal instillation. We also conducted an erythrocyte micronucleus assay using the samples obtained from animals under intratracheal instillation to investigate the tissue specificity of MWNT-7 induced genotoxicities.

Results: We detected a significant increase in the incidence of micronucleated cells derived from the cellular fraction of Clara cells and AT-II cells in both MWNT-7-treated and positive control groups compared to the negative control group under both whole-body inhalation exposures and intratracheal instillation. Additionally, the erythrocyte micronucleus assay detected a significant increase in the incidence of micronucleated reticulocytes only in the positive control group.

Conclusions: Our findings indicated that MWNT-7 was genotoxic in the lungs directly exposed by both the body inhalation and intratracheal instillation but not in the hematopoietic tissue.

Keywords: MWNT-7, *in vivo* genotoxicity, lung micronucleus assay

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Liu W*1, Yasui M, Sassa A*2, You X*1, Wan J*1, Cao Y*1, Xi J*1, Zhang X*1, Honma M, Luan Y*1: FTO regulates the DNA damage response via effects on cell-cycle progression.

Mutat Res Genet Toxicol Environ Mutagen. 2023;887:503608. doi: 10.1016/j.mrgentox.2023.503608

The fat mass and obesity-associated protein FTO is an "eraser" of N6-methyladenosine, the most abundant mRNA modification. FTO plays important roles in tumorigenesis. However, its activities have not been fully elucidated and its possible involvement in DNA damage - the early driving event in tumorigenesis - remains poorly characterized. Here, we have investigated the role of FTO in the DNA damage response (DDR) and its underlying mechanisms. We demonstrate that FTO responds to various DNA damage stimuli. FTO is overexpressed in mice following exposure to the promutagens aristolochic acid I and benzo[a]pyrene. Knockout of the FTO gene in TK6 cells, via CRISPR/Cas9, increased genotoxicity induced by DNA damage stimuli (micronucleus and TK mutation assays). Cisplatin- and diepoxybutane-induced micronucleus frequencies and methyl methanesulfonate- and azathioprine-induced TK mutant frequencies were also higher in FTO KO cells. We investigated the potential roles of FTO in DDR. RNA sequencing and enrichment analysis revealed that FTO deletion disrupted the p38 MAPK pathway and inhibited the activation of nucleotide excision repair and cell-cycle-related pathways following cisplatin (DNA intrastrand cross-links) treatment. These effects were confirmed by western blotting and qRT-PCR. FTO deletion impaired cell-cycle arrest at the G2/M phase following cisplatin and diepoxybutane treatment (flow cytometry analysis). Our findings demonstrated that FTO is involved in several aspects of DDR, acting, at least in part, by impairing cell cycle progression.

Keywords: nucleotide excision repair, p38, MAPK

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Tanabe S, Quader S*1, Ono R, Cabral H*2, Aoyagi K*3, Hirose A, Perkins EJ*4, Yokozaki H*5, Sasaki H*3: Regulation of Epithelial-Mesenchymal Transition Pathway and Artificial Intelligence-Based Modeling for Pathway Activity Prediction.

Onco. 2023;3(1):13-25. doi: 10.3390/onco3010002

Because activity of the epithelial-mesenchymal transition (EMT) is involved in anti-cancer drug resistance, cancer malignancy, and shares some characteristics with cancer stem cells (CSCs), we used artificial intelligence (AI) modeling to identify the cancer-related activity of the EMT-related pathway in datasets of gene expression. We generated images of gene expression overlayed onto molecular pathways with Ingenuity Pathway Analysis (IPA). A dataset of 50 activated and 50 inactivated pathway images of EMT regulation in the development pathway was then modeled by the DataRobot Automated Machine Learning platform. The most accurate models were based on the Elastic-Net Classifier algorithm. The model was validated with 10 additional activated and 10 additional inactivated pathway images. The generated models had false-positive and false-negative results. These images had significant features of opposite labels, and the original data were related to Parkinson's disease. This approach reliably identified cancer phenotypes and treatments where EMT regulation in the development pathway was activated or inactivated thereby identifying conditions where therapeutics might be applied or developed. As there are a wide variety of cancer phenotypes and CSC targets that provide novel insights into the mechanism of CSCs' drug resistance and cancer metastasis, our approach holds promise for modeling and simulating cellular phenotype transition, as well as predicting molecular-induced responses.

Keywords: artificial intelligence, epithelial-mesenchymal transition, molecular pathway network

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Kawashima A, Inoue K, Ushida K, Kai K, Suzuki H, Matsumoto M, Masumura K, Hirose A: Derivation of human health hazard assessment values for toluene under the Japanese Chemical Substances Control Law.

Fundam Toxicol Sci. 2022;9(4):123-133. doi: 10.2131/fts.9.123

Toluene had been designated as a priority assessment chemical substance under the Japanese Chemical Substances Control Law (CSCL), and as a result of prioritization, a detailed human health hazard assessment was conducted under Assessment II. We evaluated its general, reproductive, and developmental toxicities, as well as its genotoxicity and carcinogenicity, based on the hazard information provided by domestic and international risk assessment organizations, and the following hazard assessment values for oral and inhalation exposure are proposed. The hazard assessment value of 0.223 mg/kg/day for oral exposure was calculated from a no-observed-adverse-effect level (NOAEL) of 312 mg/kg/day (equal to an average daily dose of 223 mg/kg/day) based on liver and kidney weight increases in a 13-week oral toxicity study in rats by using an uncertainty factor (UF) of 1,000 (interspecies variation: 10, intraspecies variation: 10, and short test period: 10). The hazard assessment value of 0.1 ppm (0.383 mg/m3) for inhalation exposure was calculated from a NOAEL of 45 ppm (equal to a continuous exposure level of 10.7 ppm) based on toxic effects on the central nervous system found in epidemiological investigations of occupational exposure by using a UF of 100 (intraspecies variation: 10 and severe effect: 10).

Keywords: toluene (CAS No. 108-88-3), Chemical Substance Control Law (CSCL), Assessment Ⅱ for human health effects

Murayama N*1, Yamada T, Yamazoe Y*2: Application of CYP1A2-Template system to understand metabolic processes in the safety assessment.

Food Safety. 2022;10:129-139. doi: 10.14252/foodsafetyfscj.D-22-00008

Cytochrome P450 (CYP)-mediated metabolisms of four chemicals have been investigated to understand their unresolved phenomena of their metabolisms using human CYP-Template systems developed in our previous studies (Drug Metab Pharmacokinet 2019, 2021, 2022). Simulation experiments of a topoisomerase-targeting agent, amonafide, offered a possible new inhibitory-mechanism as Trigger-residue inactivation on human CYP1A2 Template. N-Acetylamonafide as well as amonafide would

inactivate CYP1A2 through the interference of Trigger-residue movement with their dimethylaminoethyl parts. The mechanism was also supported on the inhibition/inactivation of two other drugs, DSP-1053 and binimetinib. Both the drugs, after other CYP-mediated slight structural alterations, were expected to interact with Trigger-residue for the intense inhibition on CYP1A2 Template. Possible formation of reactive intermediates of amonafide and 3-methylindole was also examined on CYP1A2 Template. Placements of amonafide suggested the scare N-oxidation of the arylamine part due to the Trigger-residue interaction. Placements of 3-methylindole suggested the formation of a reactive intermediate, 3-methyleneindolenine, rather selectively on rodent CYP1A2 than on human CYP1A2, in consistent with the experimental data. These results suggest that CYP Template systems developed are effective tools to warn an appearance of unstable reactive intermediates. Our CYP-Template systems would support confident judgements in safety assessments through offering the mechanistic understandings of the metabolism.

Keywords: metabolic process, ligand-enzyme interaction, CYP1A2 Template system

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Yamada T, Kawamura T, Tsujii S, Miura M, Ohata H, Katsutani K, Matsumoto M, Hirose A: Formation and evaluation of mechanism-based chemical categories for regulatory read-across assessment of repeated-dose toxicity: a case of hemolytic anemia.
Regul Toxicol Pharmacol. 2022;136:105275. doi: 10.1016/j.yrtph.2022.105275

The aim of this study is to define chemical categories that can be applied to regulatory read-across assessments for repeated-dose toxicity, by classifying toxic substances based on their structures and mechanism of actions (MoAs). Hemolytic anemia, which often appears primarily, was examined as an example. An integrated database was constructed by collecting publicly available datasets on repeated-dose toxicity, in which 423 out of a total of 1518 chemicals were identified as capable of inducing hemolytic anemia. Subsequently, by grouping these chemicals based on their chemical structures and plausible MoAs on hemolytic substances, we identified the following categories: (i) anilines, (ii) nitrobenzenes, (iii) nitroanilines, (iv) dinitroanilines, (v) ethylene glycol alkyl ethers, (vi) hydroquinones, (vii) oximes, and (viii) hydrazines. In these categories, the toxicant and the measurable key events leading to hematotoxicity were identified, thereby allowing us to justify the categories and to discriminate the category substances. Moreover, toxicokinetics seems to critically affect the hemolytic levels of the category substances. Overall, the categories were validated through a comprehensive analysis of the collected information, while the utility was demonstrated by conducting a case study on the selected category. Further endeavors with this approach would attain categories for other organ toxicity endpoints.

Keywords: category approach, mode of action, repeated-dose toxicity

Yamada T, Katsutani K, Maruyama T, Kawamura T, Yamazaki H*1, Murayama N*1, Tong W*2, Yamazoe Y*3, Hirose A: Combined risk assessment of food-derived coumarin with *in silico* approaches.
*Food Safe*ty. 2022;10:73-82. doi: 10.14252/foodsafetyfscj.D-21-00015

Hepatotoxicity associated with food-derived coumarin occurs occasionally in humans. We have, herein, assessed the data of existing clinical and nonclinical studies as well as those of *in silico* models for humans in order to shed more light on this association. The average intakes of food-derived coumarin are estimated to be 1-3 mg/day, while a ten-times higher level is expected in the worst-case scenarios. These levels are close to or above the tolerable daily intake suggested by a chronic study in dogs. The human internal exposure levels were estimated by a physiologically-based pharmacokinetic model with the use of virtual doses of coumarin in the amounts expected to derive from foods. Our results suggest that: (i) coumarin can be cleared rapidly *via* 7-hydroxylation in humans, and (ii) the plasma levels of coumarin and of its metabolite, *o*-hydroxyphenylacetic acid associated with hepatotoxicity, are considerably lower than those yielding hepatotoxicity in rats. Pharmacokinetic data suggest a low or negligible concern regarding a

coumarin-induced hepatotoxicity in humans exposed to an average intake from foods. Detoxification of coumarin through the 7-hydroxylation, however, might vary among individuals due to genetic polymorphisms in CYP2A6 enzyme. In addition, the CYP1A2- and CYP2E1-mediated activation of coumarin can fluctuate as a result of induction caused by environmental factors. Furthermore, the daily consumption of food-contained coumarin was implicated in the potential risk of hepatotoxicity by the drug-induced liver injury score model developed by the US Food and Drug Administration. These results support the idea of the existence of human subpopulations that are highly sensitive to coumarin; therefore, a more precise risk assessment is needed. The present study also highlights the usefulness of *in silico* approaches of pharmacokinetics with the liver injury score model as battery components of a risk assessment.

Keywords: coumarin, hepatotoxicity, individual susceptibilities

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Fujita M*1, Nakashima N*1, Wanibuchi S*1, Yamamoto Y*1, Kojima H, Ono A*2, Kasahara T*1: Assessment of commercial polymers with and without reactive groups using amino acid derivative reactivity assay based on both molar concentration approach and gravimetric approach.
J Appl Toxicol. 2023;43(3):446-457. doi: 10.1002/jat.4395

The amino acid derivative reactivity assay (ADRA), an alternative method for testing skin sensitization, has been established based on the molar concentration approach. However, the additional development of gravimetric concentration and fluorescence detection methods has expanded its range of application to mixtures, which cannot be evaluated using the conventional testing method, the direct peptide reactivity assay (DPRA). Although polymers are generally treated as mixtures, there have been no reports of actual polymer evaluations using alternative methods owing to their insolubility. Therefore, in this study, we evaluated skin sensitization potential of polymers, which is difficult to predict, using ADRA. As polymers have molecular weights ranging from several thousand to more than several tens of thousand Daltons, they are unlikely to cause skin sensitization due to their extremely low penetration into the skin, according to the 500-Da rule. However, if highly reactive functional groups remain at the ends or side chains of polymers, relatively low-molecular-weight polymer components may penetrate the skin to cause sensitization. Polymers can be roughly classified into three major types based on the features of their constituent monomers; we investigated the sensitization capacity of each type of polymer. Polymers with alert sensitization structures at their ends were classified as skin sensitizers, whereas those with no residual reactive groups were classified as nonsensitizers. Although polymers with a glycidyl group need to be evaluated carefully, we concluded that ADRA (0.5 mg/ml) is generally sufficient for polymer hazard assessment.

Keywords: ADRA-FL, ADRA-UV, skin sensitization

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Kojima H, Nakada T*1, Yagami A*2, Todo H*3, Nishimura J*4, Yagi M*4, Yamamoto K*4, Sugiyama M*5, Ikarashi Y, Sakaguchi H*6, Yamaguchi M*6, Hirota M*6, Aizawa S*6, Nakagawa S*6, Hagino S*6, Hatao M*6: A step-by-step approach for assessing acute oral toxicity without animal testing for additives of quasi-drugs and cosmetic ingredients.
Curr Res Toxicol. 2022;4:100100. doi: 10.1016/j.crtox.2022.100100

Animal testing of cosmetic ingredients and products has been banned in the European Union since 2013. However, in Japan, the application of new quasi-drugs requires the generation of data on acute oral toxicity through animal testing. A weight of evidence approach for assessing oral toxicity was challenged. This approach used a combination of safety data, including a neutral red uptake cytotoxicity assay using BALB/c3T3 cells (3T3-NRU cytotoxicity assay), which can assess the acute oral toxicity of quasi-drugs or cosmetic ingredients. We conclude that the step-by-step approach can be used to assess test substances that cause low acute oral toxicity, such as the median

lethal dose (LD 50) > 2000 mg/kg, thereby avoiding animal testing.

Keywords: acute oral toxicity, 3T3-NRU, cytotoxicity assay

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J Appl Toxicol. 2023;43(6):874-886. doi: 10.1002/jat.4431

The aim of this study is to validate an *in vitro* skin irritation test (SIT) using three-dimensional reconstructed human epidermal (RhE) skin equivalents prepared by layer-by-layer (LbL) method (LbL-3D Skin) in a series of interlaboratory studies. The goal of these validation studies is to evaluate the ability of this *in vitro* test to reliably discriminate skin irritant from nonirritant chemicals, as defined by OECD and UN GHS. This me-too validation study is to assess the within- and between-laboratory reproducibility, as well as the predictive capacity, of the LbL-3D Skin SIT in accordance with performance standards for OECD TG 439. The developed skin model, LbL-3D Skin had a highly differentiated epidermis and dermis, similar to the validated reference methods (VRM) and native human skin. The quality parameters (cell survival in controls, tissue integrity, and barrier function) were similar to VRM and in accordance with OECD TG 439. The LbL-3D Skin SIT validation study was performed by three participating laboratories and consisted of three independent tests using 20 reference chemicals. The results obtained with the LbL-3D Skin demonstrated high within-laboratory and between-laboratory reproducibility, as well as high accuracy for use as a stand-alone assay to distinguish skin irritants from nonirritants. The predictive potency of LbL-3D Skin SIT using total 54 test chemicals were comparable to those in other RhE models in OECD TG 439. The validation study demonstrated that LbL-3D Skin has proven to be a robust and reliable method for predicting skin irritation.

Keywords: reconstructed skin equivalents, skin irritation, validation

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Kimura Y*1, Yasuno R*2, Iwaki T*3, Fujimura C*1, Ohmiya Y*2, Nakajima Y*3, Omori T*4, Corsini E*5, Inoue T*6, Rogen EL*7, Kojima H, Aiba S*1: An international validation study of the interleukin-2 luciferase leukocyte toxicity test (IL-2 Luc LTT) to evaluate potential immunosuppressive chemicals and its performance after use with the interleukin-2 luciferase assay (IL-2 Luc assay).

Toxicol In Vitro. 2022;88:105535. doi: 10.1016/j.tiv.2022.105535

We previously reported that the IL-2 Luc LTT can detect immunosuppressive effects of drugs that are attributed to their antimitotic activity. Here, we report an official validation study of the IL-2 Luc LTT. In the Phase I study that evaluated five coded chemicals, the within-laboratory reproducibility of three independent laboratories was 100.0%. In the combined results of the Phase I and II studies that evaluated 20 coded chemicals, the between-laboratory reproducibility was 92.0%. When compared with the reference data based on the previously-reported immunotoxicological information, the predictivity of the combined Phase I and II studies was 76.0% for Lab A and 72.0% for Labs

B and C. In contrast, in the study in which the lead laboratory examined 37 non-pharmaceutical chemicals, the predictivity of the IL-2 Luc LTT and the IL-2 Luc assay was 48.6% and 64.9%, respectively, whereas that of the combined assays was 74.3%. It is clear that an integrated approach combining multiple assays is necessary for the development of *in vitro* immunosuppression testing. These data suggest that the IL-2 Luc LTT alone is not sufficient as a component of the integrated approach, but the combination of the IL-2 Luc assay and IL-2 Luc LTT is promising.

Keywords: IL-2, immunotoxicity test, antimitotic activity

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Fukuhara K*1, Nakanishi I*2, Imai K*1, Mizuno M*1, Matsumoto K*3, Ohno A: DTPA-Bound Planar Catechin with Potent Antioxidant Activity Triggered by Fe^{3+} Coordination.
Antioxidants (Basel), 2023;12(2):225. doi: 10.3390/antiox12020225.

In diseases related to oxidative stress, accumulation of metal ions at the site of pathogenesis results in the generation of reactive oxygen species (ROS) through the reductive activation of oxygen molecules catalyzed by the metal ions. If these metals can be removed and the generated ROS can be strongly scavenged, such diseases can be prevented and treated. Planar catechins exhibit stronger radical scavenging activity than natural catechins and can efficiently scavenge hydroxyl radicals generated by the Fenton reaction without showing pro-oxidant effects, even in the presence of iron ions. Hence, in the current study, we designed a compound in which diethylenetriaminepentaacetic acid (DTPA), a metal chelator, was bound to a planar catechin with enhanced radical scavenging activity by immobilizing the steric structure of a natural catechin to be planar. This compound showed almost no radical scavenging activity due to intramolecular hydrogen bonding of DTPA with the planar catechins; however, when coordinated with Fe^{3+}, it showed more potent radical scavenging activity than planar catechins. Owing to its potent antioxidant activity triggered by metal coordination and its inhibition of ROS generation by trapping metal ions, this compound might exert excellent preventive and therapeutic effects against oxidative stress-related diseases.

Keywords: Fe^{3+}, antioxidant activity, catechin

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