Sakamoto A*, Izutsu K, Yoshida H, Abe Y, Inoue D*, Sugano K*: Simple bicarbonate buffer system for dissolution testing: floating lid method and its application to colonic drug delivery.
*J Drug Deliv Sci Tech*. 2021;63:102447. doi: 10.1016/j.jddst.2021.102447

In this study, we developed a simple and easy method to use bicarbonate buffer solutions for dissolution tests. A floating lid was newly introduced to prevent the evaporation of $CO_2$ from bicarbonate buffer solutions (the floating lid method). This method was used to evaluate the dissolution profiles of 5-aminosalicylic acid (5-ASA) colonic delivery tablets with a pH-sensitive film coating (5-ASA CDT) (Asacol® 400 mg tablet and the generic products A and B). A sodium bicarbonate solution was added to the dissolution test vessel, covered with the floating lid made of foamed styrol, and adjusted to each pH with an HCl solution (total 500 mL, 2 to 50 mM, pH 6.0 to 7.5). Without the floating lid, the pH value increased by more than one pH unit within 3.5 h. The floating lid suppressed the increase in pH to less than 0.1 pH unit at 3.5 h and 0.7 pH unit at 22 h. According to the drug product information, all 5-ASA CDTs show similar disintegration times (approximately 0.5 h) and release profiles in the diluted McIlvaine buffer (pH 7.5, 50 mM phosphate, 25 mM citrate). However, in the bicarbonate buffer (pH 7.5, 10 mM), the disintegration time was prolonged to 4.3 h for Asacol®, 8.0 h for generic A, and 7.6 h for generic B. The floating lid method would be useful not only for formulation development but also for quality control.
Keywords: bicarbonate, dissolution test, floating lid

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Yoshida H, Teruya K*, Abe Y, Furuishi T*, Kaori Fukuzawa K*, Yonemochi E*, Izutsu K: Altered media flow and tablet position as factors of how air bubbles affect dissolution of disintegrating and non-disintegrating tablets using a USP 4 flow-through cell apparatus.
*AAPS PharmSciTech*. 2021;22:227. doi: 10.1208/s12249-021-02117-4

This study investigated how air bubbles in media affect tablet dissolution in a flow-through cell system (USP 4) using disintegrating (USP prednisone) and non-disintegrating (USP salicylic acid) tablets. Cell hydrodynamics were studied using particle image velocimetry (PIV) and computational fluid dynamics (CFD). The PIV analysis showed periodic changes in the local flow corresponding to the discharge and suction of the pump cycles. The absence of prior deaeration induced small air bubbles in the media and lower maximum flow during the cycle, explaining the slower dissolution of the USP salicylic acid tablets. Bubbles, occurring during the USP prednisone tablets study, induced the transition of floating disintegrated particles towards the cell outlet, whereas the particles precipitated to form a white layer on the glass beads used in the study with prior deaeration. CFD analysis showed local flow variation in multiple positions of small (ID 12 mm) and large (ID 22.6 mm) cells, explaining the different rates of dissolution of prednisone tablet particles depending on their distribution. These results emphasize the importance of prior deaeration in dissolution studies using a flow-through system. Bubbles in the flow-through cell system affected tablet dissolution by reducing the area in contact with the media (wettability), lowering the maximum instantaneous flow (pressure buffering), and altering the position of disintegrated particles in the cell.
Keywords: flow-through cell dissolution test, dissolved gas, fluid velocity

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Yamamoto E, Takeda Y*1, Ando D, Koide T, Amano Y*2, Miyazaki S*2, Miyazaki T, Izutsu K, Kanazawa H*2, Goda Y: Detection and analysis of drug crystals in medical transdermal patches by using X-ray diffraction measurement.
*International Journal of Pharmaceutics*. 2021;605:120834. doi: 10.1016/j.ijpharm.2021.120834

A non-destructive discrimination method for crystals in solid dosage drug forms was first developed using a combination of Raman spectroscopy and X-ray micro-computed tomography (X-ray CT). Identification of the crystal form of an active pharmaceutical ingredient

（API）at the appropriate pharmaceutical dosage is crucial, as the crystal form is a determinant of the quality and performance of the final formulation. To develop a non-destructive analytical methodology for the discrimination of solid API crystals in a solid dosage form, we utilized a combination of Raman spectroscopy and X-ray CT to differentiate between ranitidine crystal polymorphs（forms 1 and 2）in tablet formulations containing three excipients. The difference in electron density correlated with the true density between ranitidine polymorphs, thereby enabling the discrimination of crystal forms and visualization of their three-dimensional spatial localization inside the tablets through X-ray CT imaging. Furthermore, X-ray CT imaging revealed that the crystal particles were of varying densities, sizes, and shapes within the same batch. These findings suggest that X-ray CT is not only an imaging tool but also a unique method for quantitative physicochemical characterization to study crystal polymorphs and solid dosage forms.

Keywords: X-ray micro-computed tomography, crystal form, polymorphs

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Yamamoto E, Tominaga N, Kan-no H, Ando D, Miyazaki T, Izutsu K: Evaluation of drug sorption on laboratory materials with Abraham solvation parameters of drugs and its prevention.
*Pharmaceutical Research*. 2021;38:2167-77. doi: 10.1007/s11095-021-03156-z

Undesired drug sorption on laboratory material surfaces reduces the performance of analytical methods and results in the generation of unreliable data. Hence, we characterized the sorption of drugs and evaluated the sorption extent using a linear free energy relationship（LFER）model with Abraham solvation parameters of drugs. Furthermore, to prevent sorption, the effects of additives, such as organic solvents and salts, were evaluated. The sorption of fifteen model drugs（concentration: 2 μM）, with various physicochemical properties, on materials in 0.2% dimethyl sulfoxide aqueous solutions was evaluated. Drug sorption extent on the materials was determined using high-performance liquid chromatography. The obtained results were analyzed using an LFER model with Abraham solvation parameters of the drugs. The effect of additives on the sorption of itraconazole, one of the most hydrophobic drugs among those tested in this study, was investigated. Sorption was dependent on the physicochemical properties of drugs, rather than the type of materials used, and additives altered the rate of drug sorption. Equations were developed to evaluate the sorption extent（nmol）of drugs to glass and polypropylene using the Abraham solvation parameters of the drugs. LFER modeling with Abraham solvation parameters of drugs enabled us to evaluate drug sorption on materials. All the additives altered the rate of drug sorption, and some organic solvents effectively prevented sorption. The developed LFER model would be useful for assessment of the sorption properties of compounds in *in vitro* evaluations in drug discovery research and various other biochemical fields.

Keywords: linear free energy relationship, physicochemical property, sorption mechanism

Yamamoto E, Kan-no H, Tomita N, Ando D, Miyazaki T, Izutsu K: Isolation of N-nitrosodimethylamine from drug substances using solid-phase extraction-liquid chromatography-tandem mass spectrometry.
*Journal of Pharmaceutical and Biomedical Analysis*. 2022;210:114561. doi: 10.1016/j.jpba.2021.114561

N-Nitrosodimethylamine（NDMA）has been detected in some drug substances and pharmaceutical products containing sartans, ranitidine and metformin, and a potential risk of NDMA contamination exists in other drug substances and their pharmaceutical products. To quantitate NDMA in various drugs having diverse physicochemical properties, a specific, sensitive, and reliable analytical method is required, in addition to methods that can be applied to a class of nitrosamines. We aimed to develop an off-line isolation method for NDMA in drug substances using SPE for quantification with LC-APCI-MS/MS. Impediments to accurate quantitation of NDMA in drug substances using LC-MS/MS and insufficient durability of the system are attributed to the extremely large amounts of active pharmaceutical ingredients（APIs）in sample solutions in comparison to the trace amount of NDMA. A reduced retention of NDMA and/or decreased separation from other substances in LC, matrix effect in MS detection,

and undesirable contamination of instruments with API and other substances may be occasionally encountered, all of which consequently result in deterioration of system performance and generation of unreliable data, even in the cases where a divert valve is configured between the column and ion source of the MS instrument. To address these problems, an off-line NDMA isolation methodology from APIs exhibiting diverse physicochemical properties, namely ranitidine hydrochloride (ranitidine), metformin hydrochloride (metformin), nizatidine, valsartan, and telmisartan, was developed. The applicability of the method was confirmed by batch analysis of metformin and ranitidine. Furthermore, contrary to previous reports, NDMA was found to be stable over a wide pH range. The proposed methodology and data from this study would contribute to the control of NDMA contamination in various drugs to realize the safe delivery of pharmaceuticals to patients.

Keywords: N-nitrosodimethylamine, solid-phase extraction, sample preparations

Kato M[*1], Yamaguchi M[*1], Morita T[*1], Watanabe N[*1], Ota S[*2], Yamamoto E: A method for purifying nanoparticles using cationic modified monoliths and aqueous elution.

*Journal of Chromatography A*. 2022;1664:462802. doi: 10.1016/j.chroma.2021.462802

Nanoparticles are widely used in the medical field for diagnosis and therapy. In particular, the use of nanoparticles containing vaccines has spread rapidly; hence, ensuring nanoparticle safety and minimizing their side effects have become important concerns worldwide. In this study, we used three types (NH2, poly-Lys, and trimethylaminopropyl) of cationic modified silica monoliths with cylindrical structures, diameters of 4.2 mm, and heights of 1.5 mm. Doxil, an anticancer nanomedicine, and exosomes, as typical nanoparticles, were separated from model leaked drugs (e.g., doxorubicin and oligonucleotides) and proteins (e.g., albumin) coexisting in nanoparticle sample solutions using these monoliths. Each nanoparticle solution (200 μL) was applied to each monolith followed by centrifugation at 9,100 g for 1 min. The ionic concentration of the elution solution was increased stepwise to determine the concentration required to elute the nanoparticles from each monolith by centrifugation. The NH2- and poly-Lys-modified monoliths separated and purified nanoparticles from leaked drugs or proteins coexisting in nanoparticle sample solutions. The nanoparticles were separated from other substances by changing the pH and concentration of the aqueous Tris buffer used as the eluent. Doxil was eluted with 500-1,000 mM Tris buffer (pH 8) when using the NH2-modified monolith, and with 200-1,000 mM Tris buffer (pH 6) when using the poly-Lys-modified monolith. Exosome was obtained using 1,000 mM Tris buffer (pH 8) and the NH2-modified monolith. The recovery efficiencies (ratio of nanoparticle content in the most abundant fraction to that in the sample solution before purification) of Doxil and exosome were 64% and 55%, respectively. Because this method can purify nanoparticles using only low-speed centrifugation for a few minutes, we expect it will be used to improve nanoparticle safety.

Keywords: cationic modified monolith, nanomedicine, purification

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宮崎玉樹，阿曽幸男，合田幸広：X線回折測定による医療用経皮吸収型製剤中の薬物結晶の検出と解析.

*Yakugaku Zasshi*. 2022;142:65-74. doi: 10.1248/yakushi.21-00160

Crystallization of active pharmaceutical ingredients (APIs) in matrix-type transdermal patches has implications for the rate of drug absorption through the skin and the adhesion strength. Therefore, the presence or absence and the degree of crystalline API are the important factors to be controlled to guarantee the quality of patches. In this study, the feasibility of laboratory-level X-ray diffractometers for the detection and analysis of crystalline APIs in transdermal patches was investigated using medical patches of tulobuterol and isosorbide dinitrate. Some of the matrix-type patches had employed a controlled drug delivery system containing intentionally crystallized API. In the measurements of such patches, a benchtop X-ray diffractometer as well as a high-resolution X-ray diffractometer for laboratories could detect several characteristic peaks of the APIs even if the patches were wrapped in an outer bag. Although, the benchtop model was inferior to the high-resolution model in

terms of peak intensity; the peak high was one-seventh to one-fifth. Further, when an isosorbide dinitrate patch, which had an unintentionally crystallized spot, was measured while wrapped in an outer bag, several peaks derived from isosorbide dinitrate were detected only at the crystallized spot. For such samples in which crystallization occurred only at specific sites, the high-resolution X-ray diffractometer was considered to be quite advantageous in terms of high detection sensitivity and a high degree of freedom of the measurement site. The results indicate that the laboratory-level X-ray diffractometer can be used to examine the crystalline state of APIs in patches inside an unopened outer bag.

Keywords: transdermal patch, drug crystal, X-ray diffraction measurement

Inoue M[*1], Yamashita K[*1], Tsuji Y[*1], Miki M[*1], Amano S[*1], Okumura T[*1], Kuge K[*1], Tone T[*1], Enomoto S[*1], Yoshimine C[*1], Morita Y[*1], Ando D, Kamada H[*2], Mikami N[*3], Tsutsumi Y[*4], Tsunoda S[*1]: Characterization of a TNFR2-selective agonistic TNF-α mutant and its derivatives as an optimal regulatory T cell expander.

*The Journal of Immunology*. 2021;206:1740-51. doi: 10.4049/jimmunol.2000871

Regulatory T cells (Tregs) are a subpopulation of lymphocytes that play a role in suppressing and regulating immune responses. Recently, it was suggested that controlling the functions and activities of Tregs might be applicable to the treatment of human diseases such as autoimmune diseases, organ transplant rejection, and graft-versus-host disease. TNF receptor type 2 (TNFR2) is a target molecule that modulates Treg functions. In this study, we investigated the role of TNFR2 signaling in the differentiation and activation of mouse Tregs. We previously reported the generation of a TNFR2-selective agonist TNF mutant, termed R2agoTNF, by using our unique cytokine modification method based on phage display. R2agoTNF activates cell signaling via mouse TNFR2. In this study, we evaluated the efficacy of R2agoTNF for the proliferation and activation of Tregs in mice. R2agoTNF expanded and activated mouse $CD4^+CD25^+$ Tregs *ex vivo*. The structural optimization of R2agoTNF by internal cross-linking or IgG-Fc fusion selectively and effectively enhanced Treg expansion *in vivo*. Furthermore, the IgG-Fc fusion protein suppressed skin-contact hypersensitivity reactions in mice. TNFR2 agonists are expected to be new Treg expanders.

Keywords: TNFR2, regulatory T cell, TNF-α mutant

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Ando D, Miyazaki T, Yamamoto E, Koide T, Izutsu K: Chemical imaging analysis of active pharmaceutical ingredient in dissolving microneedle arrays by Raman spectroscopy.

*Drug Delivery and Translational Research*. 2022;12:426-34. doi: 10.1007/s13346-021-01052-y

The purpose of this study was to develop a quality evaluation method for dissolving microneedle arrays (DMNAs) and determine the spatial distribution pattern of drugs in DMNAs. Raman spectroscopy mapping was used to visualize the drug distribution in DMNAs and drug-loaded polymer films as a model. Powder X-ray diffraction (PXRD) and high-pressure liquid chromatography were also performed to characterize DMNAs. Drug-loaded polymer films and DMNAs were prepared by drying the aqueous solutions spread on the plates or casting. PXRD analysis suggested the crystallization of diclofenac sodium (DCF) in several forms depending on its amount in the sodium hyaluronate (HA)-based films. The Raman spectra of HA and DCF showed characteristic and non-overlapping peaks at 1376 and 1579 cm-1 Raman shifts, respectively. The intensity of the characteristic peak of DCF in the DCF-loaded films increased linearly with the increasing drug content in the range of 4.8 to 16.7% (DCF, w/w). Raman imaging analysis revealed a homogenous dispersion of small DCF crystals in these films. Raman imaging indicates the distribution of DCF on the surface of the DMNA needle. This work highlights the benefit of using Raman spectroscopy mapping to reveal the spatial distribution of drugs in DMNAs.

Keywords: dissolving microneedles, quality evaluation method, Raman spectroscopy

Arai H*[1], Nagato T*[2], Koide T, Yonemochi E*[3], Yamamoto H*[4], Sugiyama H*[5]: Tablet quality-prediction model using quality material attributes: toward flexible switching between batch and continuous granulation.
*Journal of Pharmaceutical Innovation*. 2021;16:588-602. doi: 10.1007/s12247-020-09466-w

The purpose of the study was to develop a model to predict the critical quality attribute (CQA) of tablets during continuous and batch manufacturing using only critical material attributes (CMAs). Experiments were performed using ethenzamide as the active pharmaceutical ingredient processed with batch and continuous high-shear granulators. The disintegration time of tablets was defined as the CQA, and the particle-size distribution of granules and tablet hardness were defined as the CMAs. We first investigated the influence of granulation conditions on particle-size distribution during batch and continuous granulation. We then proceeded to construct the CQA estimation model by producing tables using batch and continuous granulation. The results indicated the similarity of the granulation mechanisms, as observed by the bimodality of the distributions and the significant causal factors. Principal component analysis revealed that the CQA was influenced strongly by the particle-size distribution and that the CMA-CQA correlations were similar for both processes. Finally, a model based on partial least-squares regression could be developed that could reasonably estimate the CQA using CMAs without involving any process parameters. This approach of using process-independent CQA prediction could enable flexible switching between batch and continuous manufacturing during a product life cycle, thus offering new possibilities for efficient life cycle management.

Keywords: continuous manufacturing, design space, high shear granulation

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Ohashi R*, Fujii A*, Fukui K*, Koide T, Fukami T*: Non-destructive quantitative analysis of pharmaceutical ointment by transmission Raman spectroscopy.
*European Journal of Pharmaceutical Sciences*. 2022;169:106095. doi: 10.1016/j.ejps.2021.106095

Transmission Raman spectroscopy was used to develop a non-destructive quantitative analytical model for the assay of a crystal dispersion-type ointment containing acyclovir as a model drug with a concentration of 3% w/w. The obtained Raman spectra were pre-processed by applying multiplicative scatter correction, standard normal variate, and first or second derivative by the Savitzky-Golay method to optimize the partial least squares (PLS) regression model. The optimized PLS model showed good prediction performance for 85%, 100%, and 115% label claims, with average recovery values of 100.7%, 99.3%, and 99.8%, respectively. Although the material properties and manufacturing method of acyclovir and white petrolatum were expected to be different from those of the calibration set, the mean recovery value of the commercial product was 104.2%. These results indicate that transmission Raman spectroscopy is a useful process analytical technology tool for product development and quality control of a crystal dispersion-type ointment with low drug concentration.

Keywords: transmission Raman spectroscopy, process analytical technology, ointment

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Shimura K*, Mohara M*, Aiko K*, Sakamoto T, Ono T*: Discrimination of pharmaceutical tablets based on the analysis of solid-state structures of ingredients using terahertz transmission spectroscopy with the injection-seeded parametric generation technique.
*ACS Omega*. 2021;6:40:26707-14. doi: 10.1021/acsomega.1c04121

A frequency-domain terahertz (THz) spectrometer that uses a tunable source, called an injection-seeded THz parametric generator, was applied to the analysis of solid-state structures of ingredients in pharmaceutical tablets, and its performance on discriminating pharmaceutical products was evaluated. The spectrometer has a dynamic range of 70 dB at 2 THz and is suitable for analyzing

materials such as pharmaceutical ingredients that often have characteristic absorption peaks between 0.5 and 2.5 THz. Nine ofloxacin (racemate) and four levofloxacin (levorotatory enantiomer) tablet products commercially available in Japan were used as samples. They contain 8-12 additives in addition to the API. The sample tablets were filed down to a thickness of 1.2 mm (ofloxacin tablets) and 1.6 mm (levofloxacin tablets) to obtain transmission spectra over the wide spectral range of 0.8-2.1 THz. The absorption spectra obtained from the spectrometer were preprocessed by the second derivative; then, principal component analysis (PCA) was conducted on the results. Next, quadratic discriminant analysis (DA) was conducted on the scores of the three PCA components. The accuracy of the DA for all 13 products was 96.1%. In addition to the difference in crystal forms of the active ingredient, the small differences in the formulation were clearly discriminated using the THz absorption spectra. The spectrometer combined with data analysis shows potential for applications such as identifying pharmaceutical tablets, monitoring the stability of production processes, evaluating the stability of formulations during storage, and detecting counterfeit drugs on the market.

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Horita K*, Akiyama K*, Sakamoto T, Takahashi K*, Satozono H*: Terahertz time-domain attenuated total reflection spectroscopy by a flow-through method for the continuous analysis of hydrous ethanol.
*J Infrared, Millimeter, and Terahertz Waves*. 2021;42:1094-104. doi: 10.1007/s10762-021-00833-3

In the pharmaceutical industry, a nondestructive and real-time monitoring method is a promising analytical technology to guarantee the quality of the manufactured products. This study develops a novel nondestructive monitoring method based on the terahertz time-domain attenuated total reflection spectroscopy (THz-ATR) system, which can be used for measuring the optical constants of liquids circulating over the surface of the ATR prism. We focused on controlling moisture content in ethanol, which is used as a solvent in the chemical reactions. In the pharmaceutical industry, even a small amount of water in the solvent may cause problems in chemical reactions during research for purposes such as drug discovery. A continuous and nondestructive monitoring method for the moisture content in the solvent is essential because general moisture measurements are destructively. Our results demonstrate that the moisture content of ethanol can be determined by monitoring the refractive index of the liquid in the THz range. In other words, a simple method that only monitors the THz absorption is sometimes inadequate for ethanol. The proposed system can successfully evaluate the ethanol concentration or moisture content continuously and nondestructively. This method is a promising solution for the real-time measurements of chemical reactions for quality control in pharmaceutical manufacturing.

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Takechi-Haraya Y, Ohgita T*[1], Kotani M*[1], Kono H*[1], Saito C*[2], Tamagaki-Asahina H*[1], Nishitsuji K*[3], Uchimura K*[4], Sato T*[1], Kawano R*[2], Sakai-Kato K*[5], Izutsu K, Saito H*[1]: Effect of hydrophobic moment on membrane interaction and cell penetration of apolipoprotein E-derived arginine-rich amphipathic $\alpha$-helical peptides.
*Scientific Reports*. 2022;12:4959. doi: 10.1038/s41598-022-08876-9

We previously developed an amphipathic arginine-rich peptide, A2-17, which has high ability to directly penetrate across cell membranes. To understand the mechanism of the efficient cell-penetrating ability of the A2-17 peptide, we designed three structural isomers of A2-17 having different values of the hydrophobic moment and compared their membrane interaction and direct cell penetration. Confocal fluorescence microscopy revealed that cell penetration efficiency of peptides tends to increase with their hydrophobic moment, in which A2-17 L14R/R15L, an A2-17 isomer with the highest hydrophobic moment, predominantly remains on plasma cell membranes. Consistently, Trp fluorescence analysis indicated the deepest insertion of A2-17 L14R/R15L into lipid membranes among all A2-17 isomers. Electrophysiological analysis showed that the duration and charge flux of peptide-induced pores in lipid membranes were prominent for A2-17 L14R/R15L, indicating the formation of stable membrane pores. Indeed, the A2-17 L14R/R15L peptide exhibited

the strongest membrane damage to CHO-K1 cells. Atomic force microscopy quantitatively defined the peptide-induced membrane perturbation as the decrease in the stiffness of lipid vesicles, which was correlated with the hydrophobic moment of all A2-17 isomers. These results indicate that optimal membrane perturbation by amphipathic A2-17 peptide is critical for its efficient penetration into cells without inducing stabilized membrane pores.

Keywords: arginine-rich peptide, membrane perturbation, atomic force microscopy

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Suzuki T, Hashii N, Tada M, Ishii-Watabe A: The influence of antibody engineering on Fc conformation and Fc receptor binding properties: Analysis of FcRn-binding engineered antibodies and an Fc fusion protein.
*mAbs*. 2021;13(1):1923366. doi: 10.1080/19420862.2021.1923366

Therapeutic immunoglobulin G (IgG) antibodies have comparatively long half-lives because the neonatal Fc receptor (FcRn) binds to the IgG Fc at acidic pH in the endosome and protects IgG from degradation. To further prolong the half-lives, amino acid-substituted antibodies having high affinity to FcRn are being developed, and one such therapeutic antibody (ravulizumab) has been approved. In this study, we investigated the binding property to FcγR and the conformation of seven FcRn affinity-modulated adalimumab variants to clarify the impact of the amino acid substitutions on the function and conformation of IgG Fc. The amino acid substitutions in T254-P261 caused a change in deuterium uptake into some regions of Fc in HDX-MS analysis, but those at T311, M432 and N438 did not cause such a change. The conformations around F245-L255 (FLFPPKPKDTL) were particularly influenced by the amino acid substitution in M256-P261, and the conformational changes of this region were correlated with the decrease of the affinity to FcγRIIIa. Additionally, we investigated the conformational difference of Fc between a Fc fusion protein (etanercept) and a native IgG (adalimumab). Although the Fc fusion proteins were expected to have similar FcRn affinity to IgGs, the affinity of etanercept to FcRn was lower than that of adalimumab, and its half-life was shorter than those of the IgG antibodies. Differences in deuterium uptakes were observed in the two regions where they were also detected in the adalimumab variants, and the conformational differences appeared to be an important factor for the low FcRn affinity of etanercept.

Keywords: FcRn, FcγR, conformation of Fc

柴田寛子，原園景，後藤かの子*，鳴瀬諒子*，石井明子：日本薬局方注射剤の不溶性微粒子試験法　第２法　顕微鏡粒子計数法に関する検討.
*医薬品医療機器レギュラトリーサイエンス*. 2021;52(5):378-387.

日本薬局方一般試験法＜6.07＞注射剤の不溶性微粒子試験法にて規定されている第２法顕微鏡粒子計数法（顕微鏡法）に着目し，主にフィルターの種類と照射方法の違いが計測結果に及ぼす影響を評価した．また，第１法である光遮蔽法や標準化が期待されているフローイメージング法によって測定される粒子数と顕微鏡法によって測定される粒子数の関係について考察した．本研究により，第２法でタンパク質に由来する不溶性微粒子を測定する際の留意点や問題点の一部を明らかにした．

Keywords：注射剤，不溶性微粒子，顕微鏡法

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*eLife*. 2021;10:e70357. doi: 10.7554/eLife.70357

Sequential mannose trimming of N-glycan, from M9 to M8B and then to oligosaccharides exposing the α1,6-linked mannosyl residue (M7A, M6, and M5), facilitates endoplasmic reticulum-associated degradation of misfolded glycoproteins (gpERAD). We previously showed that EDEM2 stably disulfide-bonded to the thioredoxin domain-containing protein TXNDC11 is responsible for the first step (George et al., 2020). Here, we show that EDEM3 and EDEM1

are responsible for the second step. Incubation of pyridylamine-labeled M8B with purified EDEM3 alone produced M7（M7A and M7C）, M6, and M5. EDEM1 showed a similar tendency, although much lower amounts of M6 and M5 were produced. Thus, EDEM3 is a major α1,2-mannosidase for the second step from M8B. Both EDEM3 and EDEM1 trimmed M8B from a glycoprotein efficiently. Our confirmation of the Golgi localization of MAN1B indicates that no other α1,2-mannosidase is required for gpERAD. Accordingly, we have established the entire route of oligosaccharide processing and the enzymes responsible.

Keywords: endoplasmic reticulum-associated degradation, ER degradation enhancing α-mannosidase-like protein, mannose trimming

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Shinnakasu R*1, Sakakibara S*2, Yamamoto H*1, Wang PH*1, Moriyama S*3, Sax N*4, Ono C*5, 6, Yamanaka A*7, 8, Adachi Y*3, Onodera T*3, Sato T*9, Shinkai M*9, Suzuki R*10, Matsuura Y*5, 6, Hashii N, Takahashi Y*3, Inoue T*1, Yamashita K*4, Kurosaki T*1, 6, 11: Glycan engineering of the SARS-CoV-2 receptor-binding domain elicits cross-neutralizing antibodies for SARS-related viruses.
*J Exp Med*. 2021;218(12):e20211003. doi: 10.1084/jem.20211003

Broadly protective vaccines against SARS-related coronaviruses that may cause future outbreaks are urgently needed. The SARS-CoV-2 spike receptor-binding domain（RBD）comprises two regions, the core-RBD and the receptor-binding motif（RBM）; the former is structurally conserved between SARS-CoV-2 and SARS-CoV. Here, in order to elicit humoral responses to the more conserved core-RBD, we introduced N-linked glycans onto RBM surfaces of the SARS-CoV-2 RBD and used them as immunogens in a mouse model. We found that glycan addition elicited higher proportions of the core-RBD-specific germinal center（GC）B cells and antibody responses, thereby manifesting significant neutralizing activity for SARS-CoV, SARS-CoV-2, and the bat WIV1-CoV. These results have implications for the design of SARS-like virus vaccines.

Keywords: glycan engineering, SARS-CoV-2 spike receptor-binding domain, SARS-like virus vaccine

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Shibata H, Harazono A, Kiyoshi M, Ishii-Watabe A: Quantitative Evaluation of Insoluble Particulate Matters in Therapeutic Protein Injections Using Light Obscuration and Flow Imaging Methods.
*J Pharm Sci*. 2021;S0022-3549(21):00533-5. doi: 10.1016/j.xphs.2021.09.047

Flow imaging（FI）has emerged as a powerful tool to evaluate insoluble particles derived from protein aggregates as an orthogonal method to light obscuration（LO）. However, few reports directly compare the FI and LO method in the size and number of protein particles in commercially available therapeutic protein injections. In this study, we measured the number of insoluble particles in several therapeutic protein injections using both FI and LO, and characterized these particles to compare the

analytical performance of the methods. The particle counts measured using FI were much higher than those measured using LO, and the difference depended on the products or features of particles. Some products contained a large number of transparent and elongated particles, which could escape detection using LO. Our results also suggested that the LO method underestimates the size and number of silicone oil droplets in prefilled syringe products compared to the FI method. The count of particles ≥10 μm in size in one product measured using FI exceeded the criteria (6000 counts per container) defined in the compendial particulate matter test using the LO method. Thus precaution should be taken when setting the acceptance criteria of specification tests using the FI method.

Keywords: flow imaging, insoluble particulate matter test, light obscuration

Aoyama M, Tada M, Yokoo H, Demizu Y, Ishii-Watabe A: Fcγ Receptor-Dependent Internalization and Off-Target Cytotoxicity of Antibody-Drug Conjugate Aggregates.

*Pharm Res*. 2022;39(1):89-103. doi: 10.1007/s11095-021-03158-x

Purpose: Antibody-drug conjugates (ADCs), which are monoclonal antibodies (mAbs) conjugated with highly toxic payloads, achieve high tumor killing efficacy due to the specific delivery of payloads in accordance with mAbs' function. On the other hand, the conjugation of payloads often increases the hydrophobicity of mAbs, resulting in reduced stability and increased aggregation. It is considered that mAb aggregates have potential risk for activating Fcγ receptors (FcγRs) on immune cells, and are internalized into cells via FcγRs. Based on the mechanism of action of ADCs, the internalization of ADCs into target-negative cells may cause the off-target toxicity. However, the impacts of aggregation on the safety of ADCs including off-target cytotoxicity have been unclear. In this study, we investigated the cytotoxicity of ADC aggregates in target-negative cells.

Methods: The ADC aggregates were generated by stirring stress or thermal stress. The off-target cytotoxicity of ADC aggregates was evaluated in several target-negative cell lines, and FcγR-activation properties of ADC aggregates were characterized using a reporter cell assay.

Results: Aggregation of ADCs enhanced the off-target cytotoxicity in several target-negative cell lines compared with non-stressed ADCs. Notably, ADC aggregates with FcγR-activation properties showed dramatically enhanced cytotoxicity in FcγR-expressing cells. The FcγR-mediated off-target cytotoxicity of ADC aggregates was reduced by using a FcγR-blocking antibody or Fc-engineering for silencing Fc-mediated effector functions.

Conclusions: These results indicated that FcγRs play an important role for internalization of ADC aggregates into non-target cells, and the aggregation of ADCs increases the potential risk for off-target toxicity.

Keywords: antibody-drug conjugate, Fcγ receptor, aggregation

Shibata H, Nishimura K, Maeda T*, Honma M, Goda Y, Ishii-Watabe A, Saito Y: Evaluation of the analytical performance of anti-SARS-CoV-2 antibody test kits distributed or developed in Japan.

*Bioanalysis*. 2022 Mar 2. doi: 10.4155/bio-2021-0254

Background: With the spread of COVID-19, anti-SARS-CoV-2 antibody tests have been utilized. Herein we evaluated the analytical performance of anti-SARS-CoV-2 antibody test kits using a new reference standard prepared from COVID-19 patient sera. Methods: Fifty-seven kits in total (16 immunochromatography types, 11 ELISA types and 30 types for automated analyzers) were examined. By measuring serially diluted reference standards, the maximum dilution factor showing a positive result and its precision were investigated. Results: The measured cut-off titers varied largely depending on the antibody kit; however, the variability was small, with the titers obtained by each kit being within twofold in most cases. Conclusion: The current results suggest that a suitable kit should be selected depending on the intended purpose.

Keywords: anti-SARS-CoV-2 antibody test, antibody titer, cut-off value

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Y [*2], Shibata H, Isaka R[*1], Zhang TQ[*1], Haga Y[*1], Higashisaka K[*1], Tsujino H [*1], Ishida T [*2], Ishii-Watabe A, Tsutsumi Y [*1]: Subvisible Particles Derived by Dropping Stress Enhance Anti-PEG Antibody Production and Clearance of PEGylated Proteins in Mice.

*J Pharm Sci.* 2022 Jan 31:S0022-3549(22)00033-8. doi: 10.1016/j.xphs.2022.01.023

Bioconjugation with polyethylene glycol (PEG) is important for protein drug development as it has improved biological stability. In contrast, proteins including PEGylated ones are susceptible to physicochemical stresses. Particularly, protein drugs in solution may form aggregates or subvisible particles if they are exposed to dropping stress during transportation. However, many PEGylation studies have focused on its usefulness, such as the extension of half-life in blood, and changes in the physical properties or biological responses of PEGylated proteins under dropping stress remain unexplored. Here, we prepared four PEGylated ovalbumin (PEG-OVA) molecules conjugated with different lengths (5 or 20 kDa) and numbers (large [L] or small [S]) of PEG, analyzed the formation of subvisible particles under dropping stress, and examined their impact on antibody production and clearance. Under dropping stress, the aggregated particle concentration of 20 kDa PEG-OVA (S) and (L) solutions was approximately 3-fold that of the OVA solution. Moreover, administration of 20 kDa PEG-OVA with dropping stress induced anti-PEG antibody production and clearance of PEG-OVA. As a mechanism, dropping stress could enhance the uptake of 20 kDa PEG-OVA (L) by macrophages. These findings could provide insights into proper transportation conditions to ensure the quality of PEGylated protein drugs.

Keywords: PEGylation, subvisible particle, anti-PEG antibody

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Suzuki R[*], Kasuya Y[*], Sano A[*], Tomita J[*], Maruyama T, Kitamura M[*]: Comparison of Various Commercially Available Cinnamon Barks using NMR Metabolomics and the Quantification of Coumarin by Quantitative NMR Methods

*J. Nat. Med.* 2022;76:87-93. doi: 10.1007/s11418-021-01554-6

Cinnamon bark is an important spice worldwide. In this study, the chemical diversity of various commercially available cinnamon barks that differed in their production areas and utility applications (culinary spice or medicines) were investigated by the use of $^{1}$H NMR metabolomics. Our results indicated that principle component analysis (PCA) and hierarchical cluster analysis (HCA) of the $^{1}$H NMR spectra of the cinnamon bark methanolic extracts including the deduction of their species by nucleotide sequence analysis enabled differentiation of the cinnamon barks according to their species, production areas and utility applications. The constituents of Vietnam cinnamon were found to differ significantly from the other samples investigated based on PCA score plots and HCA constellation dendrograms. Coumarin was found to be a key compound for the discrimination of Vietnamese cinnamon by multivariate analysis of the $^{1}$H NMR spectral data and direct comparison of the $^{1}$H NMR spectra. In addition, coumarin was quantified using quantitative NMR methods. As a result, coumarin was contained in Vietnamese cinnamon at a higher level compared to other cinnamons. This study indicated that $^{1}$H NMR metabolomics could deduce spices, utility, and producing area of commercially available cinnamon barks. Furthermore, combining quantitative $^{1}$H NMR methods with $^{1}$H NMR metabolomics enable quantification of coumarin in cinnamon bark on a single measurement.

Keywords: cinnamon bark, $^{1}$H NMR metabolomics, coumarin

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Wang Z[*1], Okutsu K[*2], Yoshizaki Y[*1,2], Futagami T[*1,2], Tamaki H[*1,2], Maruyama T, Toume K[*3], Komatsu K[*3], Takamine K[*1,2]: Effects of microbial fermentation on enzyme activity and volatile properties of Massa Medicata Fermentata

*Traditional & Kampo Medicines* 2022;9:10-17. doi: 10.1002/tkm2.1303

**Aim:** *Massa Medicata Fermentata* (MMF) is a crude drug used in East Asia to treat anorexia and dyspepsia. It is prepared from wheat and several herbs through microbial fermentation using *Aspergillus*

sp. and *Rhizopus* sp. There is great difference in the quality of commercial MMF, and the microbes of MMF are suggested to affect its quality. We investigated the effects of microbial fermentation on the quality of MMF.

**Methods:** Raw materials of MMF were mixed according to the ratio listed in the National Standard for Chinese Patent Drugs, and MMF was prepared using pure cultures of *Aspergillus oryzae* or *Rhizopus oryzae*. Digestive enzyme activities (α-amylase, protease, and lipase) and volatile compounds were measured using an analytical kit and GC-MS, respectively.

**Results:** Enzyme activity increased in MMF. MMF prepared with *A. oryzae* (MMF-A) showed higher α-amylase and lipase activities than that prepared with *R. oryzae* (MMF-R). Protease activity was marginally higher in MMF-R than in MMF-A. GC-MS analysis revealed that terpenoids decreased with fermentation; however, 2,3-butanediol, acetoin, and guaiacol were detected in MMF only. C8 compounds such as 1-octen-3-ol were higher in MMF-A than MMF-R; however, aromatic compounds such as 4-vinylguaiacol and pyrazines were higher in MMF-R than MMF-A.

**Conclusion:** Microbial fermentation contributes to increased enzyme activity and changes in MMF volatiles. These properties of MMF were considerably affected by the microbes used, and it is proposed in this study that it is important to have microbial control in the production of commercial MMF.

Keywords: *Massa Medicata Fermentata*, GC-MS, *Aspergillus oryzae*

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Masada S, Hosoe J, Arai R, Demizu Y, Hakamatsuka T, Goda Y, Uchiyama N: Miroestrol quantification in *Pueraria mirifica* crude drugs and products by single-reference UPLC/PDA/MS using relative molar sensitivities to kwakhurin.
*Chem. Pharm. Bull.* 2021;69,573-580. doi: 10.1248/cpb.c21-00160

Owing to occasional health damages caused by health food products derived from Pueraria mirifica (PM), the Japanese government has designated PM as an "ingredient calling for special attention". Miroestrol is a specific isoflavone isolated from PM and possesses very strong estrogenic activity enough to induces side effects in small amount. Therefore, routine analyses for miroestrol quantification is recommended to control the safety and quality of PM products. However, miroestrol content in PM is quite low, and commercial reagent for its detection is rarely available. In this study, we developed a quantitative analysis method for miroestrol in PM without using its analytical standard by using the relative molar sensitivity (RMS) of miroestrol to kwakhurin, another PM-specific isoflavone, as a reference standard. The RMS value was obtained by an offline combination of 1H-quantitative NMR spectroscopy and a LC/PDA and miroestrol content was determined by single-reference LC/PDA using RMS. Furthermore, we investigated miroestrol content in commercially available PM crude drugs and products, and the RMS method was compared with the conventional calibration curve method in terms of performance. The rate of concordance of miroestrol contents determined by two method was 89 - 101%. The results revealed that our developed LC/PDA/MS method with RMS using kwakhurin as a reference standard was accurate for routine monitoring of miroestrol content in PM crude drugs and products to control their quality.

Keywords: *Pueraria mirifica*, relative molar sensitivity, quantification

Uchiyama N, Hosoe J, Sugimoto N, Ishizuki K, Koide T, Murabayashi M*1, Miyashita N*2, Kobayashi K*2, Fujimine Y*3, Yokose T*3, Ofuji K*4, Shimizu H*4, Hasebe T*5, Asai Y*5, Ena E*5, Kikuchi J*6, Kiyota K*6, Fujita K*6, Makino Y*7, Yasobu N*8, Iwamoto Y*9, Miura T*9, Mizui K*9, Asakura K*10, Suematsu T*11, Muto H*11, Kohama A*12, Goto T*13, Yasuda M*13, Ueda T*14, Goda Y: Purity determination of cyclophosphamide hydrate by quantitative $^{31}$P-NMR and method validation.

*Chem. Pharm. Bull.* 2021;69:630-638. doi: org/10.1248/cpb.c21-00109

Recently, quantitative NMR (qNMR), especially 1H-qNMR, has been widely used to determine the absolute quantitative value of organic molecules. We previously reported an optimal and reproducible sample preparation method for $^{1}$H-qNMR. In the present study, we focused on a $^{31}$P-qNMR absolute determination method. An organophosphorus compound, cyclophosphamide hydrate (CP), listed in the Japanese Pharmacopeia 17th edition was selected as the target compound, and the $^{31}$P-qNMR and $^{1}$H-qNMR results were compared under three conditions with potassium dihydrogen phosphate ($KH_2PO_4$) or *O*-phosphorylethanolamine (PEA) as the reference standard for $^{31}$P-qNMR and DSS-$d_6$ as the standard for $^{1}$H-qNMR. Condition 1: separate sample containing CP and $KH_2PO_4$ for $^{31}$P-qNMR or CP and DSS-$d_6$ for $^{1}$H-qNMR. Condition 2: mixed sample containing CP, DSS-$d_6$, and $KH_2PO_4$. Condition 3: mixed sample containing CP, DSS-$d_6$, and PEA. As conditions 1 and 3 provided good results, validation studies at multiple laboratories were further conducted. The purities of CP determined under condition 1 by $^{1}$H-qNMR at 11 laboratories and $^{31}$P-qNMR at 10 laboratories were 99.76 ± 0.43% and 99.75 ± 0.53%, respectively, and those determined under condition 3 at five laboratories were 99.66 ± 0.08% and 99.61 ± 0.53%, respectively. These data suggested that the CP purities determined by $^{31}$P-qNMR are in good agreement with those determined by the established $^{1}$H-qNMR method. Since the $^{31}$P-qNMR signals are less complicated than the $^{1}$H-qNMR signals, $^{31}$P-qNMR would be useful for the absolute quantification of compounds that do not have a simple and separate $^{1}$H-qNMR signal, such as a singlet or doublet, although further investigation with other compounds is needed.

Keywords: quantitative $^{31}$P-NMR, cyclophosphamide hydrate, absolute purity

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Tsujimoto T*1, Arai R, Yoshitomi T*2, Yamamoto Y*3, Ozeki Y*1, Hakamatsuka T, Uchiyama N: UHPLC/MS and NMR-based metabolomic analysis of dried water extract of citrus-type crude drugs. *Chem. Pharm. Bull.* 2021;69:741-746. doi: org/10.1248/cpb.c21-00180

Citrus-type crude drugs (CCDs) are commonly used to formulate decoctions in Kampo formula (traditional Japanese medicine). Our previous study reported metabolomic analyses for differentiation of the methanol extracts of Citrus-type crude drugs (CCDs) using ultra-HPLC (UHPLC)/MS, and 13C- and 1H-NMR. The present study expanded the scope of its application by analyzing four CCD water extracts (Kijitsu, Tohi, Chimpi, and Kippi); these CCDs are usually used as decoction ingredients in the Kampo formula. A principal component analysis score plot of processed UPLC/MS and NMR analysis data indicated that the CCD water extracts could be classified into three groups. The loading plots showed that naringin and neohesperidin were the distinguishing components. Three primary metabolites, α-glucose, β-glucose, and sucrose were identified as distinguishing compounds by NMR spectroscopy. During the preparation of CCD dry extracts, some compounds volatilized or decomposed. Consequently, fewer compounds were detected than in our previous studies using methanol extract. However, these results suggested that the combined NMR- and LC/MS based metabolomics can discriminate crude drugs in dried water extracts of CCDs.

Keywords: metabolomics, citrus, dried water extract

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田中誠司，新井玲子，細江潤子，政田さやか，袴塚高志，内山奈穂子：ヒハツ，ヒハツモドキ，コショウ関連製品の流通実態調査

*日本食品化学学会誌* 2021;28(2):71-81. doi: 10.18891/jjfcs.28.2_71

In April 2015, "Foods with Functional Claims (FFCs)" was established as a new category of voluntary labeling in health food products sold in Japan. Several products comprising piperine as a functional substance, which is found in plants such as pepper, long pepper, and Java long pepper, have been categorized as FFCs. Health food products such as dietary supplements and spices derived from the above-mentioned peppers have also been sold commercially. Although piperine has been reported to improve blood flow and induce other positive effects, some reports showed that it exhibits acute toxicity values that are equivalent to those of powerful drugs. Therefore, ensuring the quality of piperine-containing food products is important. In this study, a quantitative analysis using HPLC was conducted for 28 piperine-containing products belonging to three product categories (FFCs, health food products, and spices) with a commercially available piperine reagent that was determined its absolute purity via quantitative NMR analysis. Four health food products and seven spices contained piperine at daily intake levels fifty times greater than those of FFCs. Conversely, piperine was not detected in two spice products; these products might have been made using different ingredients than those on the labels. We will continue to pay attention to the market trends of piperine-containing food products.

Keywords: Foods with Functional Claims, health food product, piperine

Tanaka S, Uchiyama N, Goda T*, Iida T*, Horie S*, Masada S, Arai R, Yamamoto E, Hakamatsuka T, Okuda H, Goda Y: A simple and rapid method to simultaneously analyze ciclesonide and its impurities in a ciclesonide metered-dose inhaler using on-line supercritical fluid extraction/supercritical fluid chromatography/quadrupole time-of-flight mass spectrometry.

*J. Pharm. Biomed. Anal.* 2021;204:114253. doi: 10.1016/j.jpba.2021.114253

A simple and rapid on-line SFE/SFC/quadrupole TOF-MS method to simultaneously analyze active pharmaceutical ingredients and impurities from metered-dose inhalers (MDIs) was developed using ciclesonide MDI (CIC-MDI) as an example. CIC-MDI, as drug Alvesco®, has been approved for the treatment of bronchial asthma, and its major impurities are listed in the European Pharmacopoeia and in the supplementary package inserts of Alvesco® (called as "Pharmaceutical interview form" in Japan). In the developed method, CIC-MDI was manually sprayed only once on a glass disc prior to the SFE/SFC/quadrupole TOF-MS. In the SFE, CIC and its impurities and other impurities having various polarities and hydrophobicity, were extracted in 3.5 min and subsequently separated on a CHIRALPAK IE-3 column to be detected by quadrupole TOF-MS in 6.5 min. This method would be applicable to the analysis of other inhalable pharmaceutical products whose sample preparation requires complicated procedures, as well as to the analysis of general pharmaceutical products for profiling impurities.

Keywords: on-line SFE/SFC/quadrupole TOF-MS, ciclesonide metered-dose inhaler, impurity test

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*生薬学雑誌* 2021;752:63-75.

Rokujo (鹿茸, Antler Velvet, Cornu Cervi Pantotrichum) is stated in "The Japanese Standards for non-Pharmacopoeial crude drugs 2018" as "The young unossified antler of male *Cervus nippon* Temminck, *C. elaphus* Linne, *C. canadensis* Erxleben or congeners (*Cervidae*)." The mature antler is called Rokkaku and has a different medicinal effect from that of Rokujo. The young antler of Tonakai (*Rangifer tarandus* Linne, also known as Junroku 馴鹿) of the same Cervidae family may be misused as a raw material of Rokujo because their antlers have been used as a crude drug in some regions other than Japan. The first step in assuring the quality of crude drugs is to use the correct raw materials. To guarantee the quality of the raw materials, it is important to establish a test method that uses objective criteria to evaluate product quality. In this study to differentiate Rokujo, we attempted not only morphological observation, but also catalase reaction test that can easily and quickly

discriminate blood present tissues. Furthermore, because these antlers possess a slight autofluorescence, we also attempted their fluorescence microscope observation and measurement their fluorescence fingerprints. The combination of these tests revealed that one market product of Rokujo used young antlers of *R. tarandus* as a raw material. So, we concluded that the proper combination of these tests is the best way to discriminate between Rokujo, Rokkaku, and young antler of Tonakai. Additionally, this is a good method that can be applied even to powder samples.

Keywords: Antler Velvet, microscopic examination, fluorescence

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Sawamoto A*1, Kanazaki A*1, Nakanishi M*2, Amakura Y*2, Yoshimura M*2, Uchiyama N, Hakamatsuka T, Okuyama S*1, Nakajima M*1: Cynandione A causes a dynamic change in SIRT1 nuclear trafficking via PKA signaling and beige adipocyte differentiation in 3T3-L1 cells.
*European J. Pharmocol.* 2021;909:174382. doi: 10.1016/j.ejphar.2021.174382

Inducible brown-like adipocytes, also known as beige adipocytes, dissipate energy through thermogenesis. Although recent reports suggest that silent information regulator 2 homolog 1 (SIRT1) promotes beige adipocyte differentiation (beiging), the activation mechanism of SIRT1 remains unknown. Here, we report that cynandione A (CA), a major component of Cynanchum wilfordii, causes dynamic changes in SIRT1 nuclear trafficking via protein kinase cAMP-dependent (PKA) signaling and induces the beiging process in adipocyte lineage cells. SIRT1 is located in both the cytoplasm and the nucleus of 3T3-L1 cells. Using cell fractionation and RNA interference experiments, we found that the translocation of SIRT1 from the cytoplasm to the nucleus was enhanced after CA treatment and was followed by upregulation of beige adipocyte-related gene expression. Moreover, we found that CA-induced SIRT1 nuclear trafficking is dependent on the PKA signaling pathway. These results suggest a novel mechanism of CA by which PKA signaling promotes SIRT1 nuclear trafficking, which permits the docking of SIRT1 to its nuclear substrates, leading to beiging in 3T3-L1 cells.

Keywords: SIRT1, nuclear trafficking, beige adipocytes

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*Tetrahedron Letters.* 2021;83:153423. doi: 10.1016/j.tetlet.2021.153423

A new trimeric monoterpenoid indole alkaloid, divaricamine A (**1**) consisting of a vobasine-vobasineiboga type skeleton, was isolated from the root of Tabernaemontana divaricata. The structure including absolute stereochemistry was elucidated on the basis of 2D NMR data and CD spectrum. Divaricamine A (**1**) showed potent anti-malarial activity.

Keywords: divaricamine A, indole alkaloid, malaria

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Hirasawa Y*1, Tanaka T*1, Hirasawa S*1, Wong CP*2, Uchiyama N, Kaneda T*1, Goda Y, Morita H*1: Cliniatines A-C, new Amaryllidaceae Alkaloids from Clivia miniata, inhibiting Acetylcholinesterase.
*J. Nat. Med.* 2022;76:171-177. doi: 10.1007/s11418-021-01570-6

Cliniatines A-C (1-3), three new Amaryllidaceae alkaloids, consisting of 2,6-dimetylpyridine and lycorine-type and/or galanthamine-type were isolated

from Clivia miniata (Lindl.) Bosse. The structures and absolute configurations of **1-3** were elucidated based on spectroscopic data and chemical correlation. Cliniatines A-C showed moderate inhibitory activity against acetylcholinesterase.

Keywords: amaryllidaceae alkaloid, cliniatine A, acetylcholinesterase

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飯田基雄，花尻（木倉）瑠理：キラル溶媒和剤を用いたNMRによる覚醒剤及び覚醒剤原料の立体識別法の検討

*YAKUGAKU ZASSHI* 2021;141:1041-1048. doi: 10.1248/yakushi.21-00090

Some controlled substances, such as stimulants and narcotics, have asymmetric carbons in their molecules. Because the enantiomers do not always show the same pharmacological effects, and there are substances with different controls due to differences in their stereochemistry, a simple and unambiguous method for assessment of the composition of enantiomers is necessary. In this study, to develop a simple and rapid stereoscopic identification method for methamphetamine and its raw materials (ephedrine and pseudoephedrine), the $^{1}$H-NMR method was studied using three commercially available chiral solvating agents (CSAs); 1,1'-bi(2-naphthol) (BINOL), 2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE) and α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA). In addition, the accuracy of the optical purity, which was measured using samples mixed with enantiomers in various ratios, was investigated. The NMR peaks of the enantiomers were separated by adding (*R*)- or (*S*)-form of BINOL, TFAE or MTPA to the chloroform-*d* solution of methamphetamine, ephedrine or pseudoephedrine. A sufficient discrimination of enantiomers was obtained by adding about 10 equal amounts of each CSA to the solutions. With regard to the optical purity, it was possible to determine accurately the mixing of small amounts of enantiomers of about 5% even if the NMR peaks did not reach the baseline separation, when impurity peaks do not overlap. This method will be one of the useful techniques for the rapid and simple discrimination of enantiomers of illegal methamphetamine and its raw materials.

Keywords: methamphetamine, chiral solvating agent, NMR

Morita I*, Kiguchi Y*, Oyama H*, Takeuchi A*, Tode C*, Tanaka R, Ogata J, Kikura-Hanajiri R, Kobayashi N*: Derivatization-assisted enzyme-linked immunosorbent assay for identifying hallucinogenic mushrooms with enhanced sensitivity.

*Anal. Methods* 2021;13:3954-3962. doi: 10.1039/D1AY01157J

A sensitive immunochemical method for identifying hallucinogenic mushrooms (magic mushrooms) is required for regulating their illicit use. We have previously generated a monoclonal antibody (mAb) that targets psilocin (Psi), the major psychoactive compound in hallucinogenic mushrooms, and developed an enzyme-linked immunosorbent assay (ELISA). However, this ELISA failed to achieve the expected low-picomole-range sensitivity, as a result of insufficient affinity of the mAb to Psi. It is recognized that haptenic antigens with a larger molecular mass tend to induce antibodies with higher affinities. Thus, we herein report a "derivatization-assisted ELISA," in which the "real analyte" Psi was determined as a "surrogate analyte," the *tert*-butyldimethylsilyl ether analog thereof (TBS/Psi) having a 1.6-fold greater molecular mass (*Mr* 318.53) than Psi. A novel mAb against TBS/Psi, prepared by immunizing mice with a TBS/Psi-albumin conjugate showed a 69-fold higher affinity to TBS/Psi residues ($K_a$ = 3.6 × $10^7$ $M^{-1}$ as IgG) than that of our previous mAb against Psi. This mAb consequently enabled a competitive ELISA for measuring TBS/Psi with the desired sensitivity: the dose-response curve midpoint (12.1 pmol per assay) was >100-fold lower than that of the previous ELISA for determining Psi. Extracts of dried mushroom powders were mixed with TBS triflate for 30 min at room temperature, converting Psi into TBS/Psi in approximately 50% yield. The reaction mixture was then subjected to an ELISA using the anti-TBS/Psi mAb to determine TBS/Psi. *Psilocybe cubensis*, a species of hallucinogenic mushrooms, gave rise to positive signals, indicating the presence of Psi therein

in the expected quantity, while no detectable response was observed for four kinds of edible mushrooms available in the markets.

Keywords: magic mushrooms, psilocin, derivatization-assisted ELISA

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Hirai T, Sato A[*1,2], Koizumi N[*1], Kurioka Y[*1], Suzuki Y[*1], Kano J[*1], Yamakawa M[*1], Nomura T[*1], Fujii M[*3], Sakurai F[*4], Mizuguchi H[*4], Watanabe Y[*5], Utoguchi N[*1]: The infectivity of progeny adenovirus in the presence of neutralizing antibody.

*J Gen Virol*. 2021;102:001590. doi: 10.1099/jgv.0.001590.

Human adenoviruses (Ads), common pathogens that cause upper respiratory and gastrointestinal infections, are blocked by neutralizing antibodies (nAbs). However, Ads are not fully eliminated even in hosts with nAbs. In this study, we assessed the infectivity of progeny Ad serotype 5 (Ad5) in the presence of nAb. The infectivity of Ad5 was evaluated according to the expression of the Ad genome and reporter gene. Infection by wild-type Ad5 and Ad5 vector continued to increase until 3 days after infection even in the presence of nAb. We established an assay for determining the infection levels of progeny Ad5 using a sorting system with magnetic beads and observed little difference in progeny Ad5 counts in the presence and absence of nAb 1 day after infection. Moreover, progeny Ad5 in the presence of nAb more effectively infected coxsackievirus and adenovirus receptor (CAR)-positive cells than CAR-negative cells. We investigated the function of fiber proteins, which are the binding partners of CAR, during secondary infection, observing that fibre proteins spread from infected cells to adjacent cells in a CAR-dependent manner. In conclusion, this study revealed that progeny Ad5 could infect cells even in the presence of nAb, differing from the common features of the Ad5 infection cycle. Our findings may be useful for developing new therapeutic agents against Ad infection.

Keywords: adenovirus, immune evasion, neutralizing antibody

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Hirai T, Kono K, Sawada R, Kuroda T, Yasuda S, Matsuyama S, Matsuyama A[*1], Koizumi N[*2], Utoguchi N[*2], Mizuguchi H[*3], Sato Y: A selective cytotoxic adenovirus vector for concentration of pluripotent stem cells in human pluripotent stem cell-derived neural progenitor cells.

*Sci Rep*. 2021;11:11407. doi: 10.1038/s41598-021-90928-7.

Highly sensitive detection of residual undifferentiated pluripotent stem cells is essential for the quality and safety of cell-processed therapeutic products derived from human induced pluripotent stem cells (hiPSCs). We previously reported the generation of an adenovirus (Ad) vector and adeno-associated virus vectors that possess a suicide gene, inducible Caspase 9 (iCasp9), which makes it possible to sensitively detect undifferentiated hiPSCs in cultures of hiPSC-derived cardiomyocytes. In this study, we investigated whether these vectors also allow for detection of undifferentiated hiPSCs in preparations of hiPSC-derived neural progenitor cells (hiPSC-NPCs), which have been expected to treat neurological disorders. To detect undifferentiated hiPSCs, the expression of pluripotent stem cell markers was determined by immunostaining and flow cytometry. Using immortalized NPCs as a model, the Ad vector was identified to be the most efficient among the vectors tested in detecting undifferentiated hiPSCs. Moreover, we found that the Ad vector killed most hiPSC-NPCs in an iCasp9-dependent manner, enabling flow cytometry to detect undifferentiated hiPSCs intermingled at a lower concentration (0.002%) than reported previously (0.1%). These data indicate that the Ad vector selectively eliminates hiPSC-NPCs, thus allowing for sensitive detection of hiPSCs. This cytotoxic viral vector could contribute to ensuring the quality and safety of hiPSCs-NPCs for therapeutic use.

Keywords: induced pluripotent stem cells, neural progenitor cells, viral vector

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Kono K, Kataoka K, Yuan Y*, Yusa K*, Uchida K*, Sato Y: Infectivity assessment of porcine endogenous retrovirus using high-throughput sequencing technologies.
*Biologicals*. 2021;71:1-8. doi: 10.1016/j.biologicals.2021.05.001.

Xenogenic cell-based therapeutic products are expected to alleviate the chronic shortage of human donor organs. For example, porcine islet cell products are currently under development for the treatment of human diabetes. As porcine cells possess endogenous retrovirus (PERV), which can replicate in human cells *in vitro*, the potential transmission of PERV has raised concerns in the case of products that use living pig cells as raw materials. Although several PERV sequences exist in the porcine genome, not all have the ability to infect human cells. Therefore, polymerase chain reaction analysis, which amplifies a portion of the target gene, may not accurately assess the infection risk. Here, we determined porcine genome sequences and evaluated the infectivity of PERVs using high-throughput sequencing technologies. RNA sequencing was performed on both PERV-infected human cells and porcine cells, and reads mapped to PERV sequences were examined. The normalized number of the reads mapped to PERV regions was able to predict the infectivity of PERVs, indicating that it would be useful for evaluation of the PERV infection risk prior to transplantation of porcine products.
Keywords: porcine endogenous retrovirus, high-throughput sequencing, xenotransplantation

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Matsuzaka Y*, Kusakawa S, Uesawa Y*, Sato Y, Satoh M*: Deep learning-based *in vitro* detection method for cellular impurities in human cell-processed therapeutic products.
*Applied Sciences*. 2021;11:9755. doi: 10.3390/app11209755.

Automated detection of impurities is in demand for evaluating the quality and safety of human cell-processed therapeutic products in regenerative medicine. Deep learning (DL) is a powerful method for classifying and recognizing images in cell biology, diagnostic medicine, and other fields because it automatically extracts the features from complex cell morphologies. In the present study, we construct prediction models that recognize cancer-cell contamination in continuous long-term (four-day) cell cultures. After dividing the whole dataset into Early- and Late-stage cell images, we found that Late-stage images improved the DL performance. The performance was further improved by optimizing the DL hyperparameters (batch size and learning rate). These findings are first report for the implement of DL-based systems in disease cell-type classification of human cell-processed therapeutic products (hCTPs), that are expected to enable the rapid, automatic classification of induced pluripotent stem cells and other cell treatments for life-threatening or chronic diseases.
Keywords: human cell-processed therapeutic products, cellular impurities, deep learning

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Naresh Amin K*1, Rajagru P*2, Sarkar K*1, Ganesh MR*1, Suzuki T, Ali D*3, Kunka Mohanram R*1.: Pharmacological Activation of Nrf2 by rosolic acid attenuates endoplasmic reticulum stress in endothelial cells.
*Oxid Med Cell Longev*. 2021:8:2732435. doi: 10.1155/2021/2732435

We found that Rosolic acid (RA) treatment dose-dependently activates Nrf2 in endothelial cells using the enzyme fragment complementation assay. The contribution of Nrf2 in RA-mediated defense mechanism in endothelial cells was established by knockout studies using Nrf2-CRISPR/Cas9. The treatment with RA to ER stress-induced endothelial cells exhibited activation of Nrf2, as demonstrated by Nrf2 translocation and reduction of ER stress markers. We found that the Nrf2 knockout sensitized the endothelial cells against ER stress, and further, RA failed to mediate its cytoprotective effect. Proteomic studies using LC-MS/MS revealed that among the

1370 proteins detected, we found 296 differentially regulated proteins in ER stress-induced endothelial cells, and RA administration ameliorated 71 proteins towards the control levels. Of note, the ER stress in endothelial cells was attenuated by the treatment with the RA, suggesting the role of the Nrf2 activator in the pathological conditions of ER stress-associated diseases.
Keywords: Nrf2, rosolic acid, proteomics

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Tanaka T*1, Takata N*1, Sakurai Y*1, Yoshida T, Inoue T, Tamagawa S*2, Nakai Y*2, Tange K*2, Yoshioka H*2, Maeki M*3, Tokeshi M*3, Akita H*1: Delivery of Oligonucleotides Using a Self-Degradable Lipid-Like Material.
*Pharmaceutics*. 2021;13:544. doi: 10.3390/pharmaceutics13040544

The world-first success of lipid nanoparticle (LNP)-based siRNA therapeutics (ONPATTRO®) promises to accelerate developments in siRNA therapeutics/gene therapy using LNP-type drug delivery systems (DDS). In this study, we explore the optimal composition of an LNP containing a self-degradable material (ssPalmO-Phe) for the delivery of oligonucleotides. siRNA or antisense oligonucleotides (ASO) were encapsulated in LNP with different lipid compositions. The hepatic knockdown efficiency of the target genes and liver toxicity were evaluated. The optimal compositions for the siRNA were different from those for ASO, and different from those for mRNA that were reported in a previous study. Extracellular stability, endosomal escape and cellular uptake appear to be the key processes for the successful delivery of mRNA, siRNA and ASO, respectively. Moreover, the compositions of the LNPs likely contribute to their toxicity. The lipid composition of the LNP needs to be optimized depending on the type of nucleic acids under consideration if the applications of LNPs are to be further expanded.
Keywords: antisense oligonucleotide, lipid nanoparticle, siRNA

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Tokugawa M*1, Inoue Y*1, Ishiuchi K*1, Matsuno M*2, Ri M*1, Itoh Y, Miyajima C*1, Morishita D*1, Ohoka N, Iida S*1, Mizukami H*2, Makino T*1, Hayashi H*1: Periplocin and cardiac glycosides suppress the unfolded protein response.
*Scientific reports*. 2021;11:9528. doi: 10.1038/s41598-021-89074-x

The unfolded protein response (UPR) controls protein homeostasis through transcriptional and translational regulation. However, dysregulated UPR signaling has been associated with the pathogenesis of many human diseases. Therefore, the compounds modulating UPR may provide molecular insights for these pathologies in the context of UPR. Here, we screened small-molecule compounds that suppress UPR, using a library of Myanmar wild plant extracts. The screening system to track X-box binding protein 1 (XBP1) splicing activity revealed that the ethanol extract of the Periploca calophylla stem inhibited the inositol-requiring enzyme 1 (IRE1)-XBP1 pathway. We isolated and identified periplocin as a potent inhibitor of the IRE1-XBP1 axis. Periplocin also suppressed other UPR axes, protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Examining the structure-activity relationship of periplocin revealed that cardiac glycosides also inhibited UPR. Moreover, periplocin suppressed the constitutive activation of XBP1 and exerted cytotoxic effects in the human multiple myeloma cell lines, AMO1 and RPMI8226. These results reveal a novel suppressive effect of periplocin or the other cardiac glycosides on UPR regulation, suggesting that these compounds will contribute to our understanding of the pathological or physiological importance of UPR.
Keywords: IRE-1, unfolded protein response, XBP1

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Yunoki M*1, Urayama T*1, Aoyama S*2, Okaniwa N*2, Kaoru Sakai*1, Uchida E, Ikuta K*3, Yamaguchi T*4: Polyethyleneimine-modified resins effectively remove porcine circovirus and cellular prion protein.

*Journal of Virological Methods* 2021;294:114181. doi:10.1016/j.jviromet.2021.114181

Polyethyleneimine (PEI) possesses various molecular weights (MWs), structures, and virus capture capacities. However, whether PEI can capture porcine circovirus (PCV) and animal cell-derived prion protein (PrPC) that may contaminate source materials is unclear. Therefore, we conducted a feasibility study to assess the effectiveness of PEI in removing PCV and PrPC as a model of pathogenic prions. The removal performance of PCV was evaluated by quantitative PCR using PEIs with various MWs, structures, and ion exchange capacities in Tris (pH 7.5) and acetate (pH 5.5) buffers under neutral (pH 7.5) to acidic (pH 5.5) conditions. Removal performances of PrPC were also evaluated by western blotting using PEIs with various MWs and structures. Tris buffer did not affect the ability of PEI-modified resins to remove PCV, whereas acetate buffer affected removal performances, except those of PEI-10K-Br and PEI-70K-Br, which showed high ion-exchange capacities. PrPC was captured by PEIs with high MWs, especially PEI-70K-Br, which was the most effective. The results of this feasibility study suggested that PEI-modified resin could remove PCV and PrPC. PEI-70K-Br with an ion-exchange capacity of at least 0.3 meq/mL appears suitable as a PEI molecule for pathogen capture or removal of PCV or PrPC from biological materials.

Keywords: porcine circovirus, prion, removal

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Yasuhara H*, Yoshida T, Sasaki K, Obika S*, Inoue T: Reduction of off-target effects of gapmer antisense oligonucleotides by oligonucleotide extension.
*Molecular Diagnosis & Therapy*. 2022;26:117. doi: 10.1007/s40291-021-00573-z

Aim: Antisense oligonucleotide (ASO) has the potential to induce off-target effects by inadvertent binding of ASOs to unintended RNAs that have a sequence similar to the target RNA. In the present study, we focused on the association between oligonucleotide length and off-target effects. Oligonucleotide extension is assumed to have bilateral effects on hybridization-dependent changes in gene expression, i.e., one is the decrease of off-target effects based on the reduced number of off-target candidate genes with perfect matches, and the other is the increase of off-target effects based on the increased binding affinity between the ASO and the complementary RNAs that leads to better tolerability for mismatches. Methods: To determine the effects of oligonucleotide extension of gapmer ASOs on off-target effects, an extensive microarray analysis was performed using human cells treated with a 14-mer gapmer ASO and the extended 18-mer derivatives with the same core 14-mer region. Results and discussion: Our data indicated that change in gene expression in the cells treated with 18-mer ASOs was significantly smaller than those with a 14-mer ASO, showing the decrease of off-target effects by oligonucleotide extension.

Keywords: antisense oligonucleotide, gapmer, off-target effects

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Nagasaka M*, Inoue Y*, Yoshida M*, Miyajima C*, Morishita D*, Tokugawa M*, Nakamoto H*, Sugano M*, Ohoka N, Hayashi H*: The deubiquitinating enzyme USP17 regulates c-Myc levels and controls cell proliferation and glycolysis.
*FEBS Letters*. 2022;596:465-78. doi: 10.1002/1873-3468.14296

The c-Myc oncoprotein is frequently overexpressed in human cancers and is essential for cancer cell proliferation. The dysregulation of ubiquitin-proteasome-mediated degradation is one of the contributing factors to the upregulated expression of c-Myc in human cancers. We herein identified USP17 as a novel deubiquitinating enzyme that regulates c-Myc levels and controls cell proliferation and glycolysis. The overexpression of USP17 stabilized the c-Myc protein by promoting its deubiquitination. In contrast, the knockdown of USP17 promoted c-Myc degradation and reduced c-Myc levels. The knockdown of USP17 also suppressed cell proliferation and glycolysis. Collectively, the present results reveal a novel role for USP17 in the regulation of c-Myc

stability and suggest its potential as a therapeutic target for cancer treatment.

Keywords: USP17, c-Myc, deubiquitination

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Nomura Y, Fukui C, Yamamura J[*1], Kuromatsu H[*1], Naito T[*2], Takahashi Y[*2], Haishima Y: Evaluation of pyrogens remaining on reusable medical devices after washing and sterilization.

*The Japanese journal of medical instrumentation*. 91:323-331, 2021. doi :10.4286/jjmi.91.323

In this study, we determined the most appropriate method to evaluate the amount of pyrogens, including bacterial endotoxins, remaining on reusable medical devices after washing and sterilization, and investigated an improved sterilization method to reduce the contamination level of pyrogens. Scissors used for surgery were provided from six hospitals after cleaning with a washer disinfector, and then sterilized with an autoclave or low-temperature ozone/hydrogen peroxide ($O_3/H_2O_2$) mixed gas exposure. The amount of pyrogens remaining on the scissors was evaluated through direct human cell-based pyrogen tests (HCPTs) and Limulus amebocyte lysate (LAL) coagulation assays. A direct HCPT revealed that an average of 0.977-20.7 EU/device of pyrogens remained on the autoclaved scissors, and this amount decreased to 0.177-5.16 EU/device after the sterilization with $O_3/H_2O_2$ mixed gas. Conversely, with the LAL assay, no endotoxins were detected in two samples and only 0.0107-0.894 EU/device of endotoxins were detected on other sample sets, regardless of the type of sterilization method used. These results suggest that $O_3/H_2O_2$ mixed gas sterilization is very effective in reducing the contamination level of pyrogens remaining on reusable medical devices and that the HCPT is more accurate than the LAL assay by which false negative or low quantitative results were obtained in the evaluation of residual endotoxins.

Keywords: gas sterilization, residual endotoxin, pyrogen inactivation

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Nomura Y, Yamamura J[*1], Fukui C, Fujimaki H[*2], Sakamoto K[*1], Matsuo K[*1], Kuromatsu H[*1], Kikuchi Y, Haishima Y: Performance evaluation of bactericidal effect and endotoxin inactivation by low-temperature ozone/hydrogen peroxide mixed gas exposure.

*JBMR Part B*, 109:1807-1816, 2021. doi: 10.1002/jbm.b.34840

This study evaluated the performance of a new $O_3/H_2O_2$ mixed gas sterilization instrument for killing microorganisms and inactivating bacterial endotoxin at low temperatures. Sterility assurance level was achieved by an over 6-log reduction of Geobacillus stearothermophilus ATCC 12980, and the decimal reduction value was 0.77 min in sterilization mode. A reduction of over 3 logs in Limulus amebocyte lysate coagulation activity of purified endotoxin from Escherichia coli was observed after treatment in endotoxin-inactivation mode. The same inactivation ability was observed when treating dried bacterial cells. Biomaterials made of polymer or metal did not exhibit cytotoxicity after gas exposure at $O_3$ concentrations below 200 ppm. As the results of human cell-based pyrogen testing, significant amounts of endotoxin that were over the limit for medical devices contacting cerebrospinal fluid (2.15 EU/device) were detected on scissors washed with a washer-disinfector and sterilized with ethylene oxide or autoclaving. In contrast, endotoxin decreased to 0.29 ± 0.05 EU/device after $O_3/H_2O_2$ mixed gas sterilization in endotoxin-inactivation mode. Compared to conventional gas sterilization methods, $O_3/H_2O_2$ mixed gas has high sterilization ability and a strong capacity to inactivate endotoxin. It is expected that this sterilization technology will improve the safety of reusable medical devices and utensils for regenerative medicine.

Keywords: gas sterilization, endotoxin inactivation, reusable medical device

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Hongprasit A*, Okamoto Y, Toida T*, Ogra Y*: Comparison of quantification of selenocyanate and thiocyanate in cultured mammalian cells between HPLC-fluorescence detector and HPLC-inductively

coupled plasma mass spectrometer.
*J. Chromatogr. B*, 1181 122924-122924 (2021). doi: 10.1016/j.jchromb.2021.122924

The simultaneous detection of cyanide (CN), thiocyanate (SCN), and selenocyanate (SeCN) by a HPLC-fluorescence detector (FLD) with the post-column König reaction was recently reported. SCN and SeCN are also detectable by HPLC-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) because sulfur and selenium can be detected, respectively, without any pre- or post-treatment. ICP-MS has high sensitivity for selenium and sulfur detection and is robust to sample matrices. In this study, we compared HPLC-FLD with the post-column König reaction and HPLC-ICP-MS in terms of SCN and SeCN detection sensitivity and linearity. The limit of detection (LOD) for SCN indicated that HPLC-FLD with the post-column König reaction was 354 times more sensitive than HPLC-ICP-MS. Likewise, the LOD for SeCN indicated that HPLC-FLD was 51 times more sensitive than HPLC-ICP-MS. These results demonstrated that HPLC-FLD was a more suitable technique for SeCN and SCN detection than HPLC-ICP-MS. We previously reported that SeCN was generated in selenite-exposed mammalian cells to detoxify excess selenite. HPLC-FLD with the post-column König reaction enabled good separation and detection for quantifying SCN and SeCN in mammalian cell lines exposed to selenite. The intracellular SCN and SeCN concentrations determined by this technique suggested differences in the metabolic capacity for selenite to form SeCN among the cell lines. In addition, since the amount of intracellular SCN and SeCN were significantly decreased by pretreatment of myeloperoxidase (MPO) inhibitors, SCN and SeCN were resulted from the interaction of sulfur and selenium with endogenous CN, respectively, generated with MPO.
Keywords: ICP-MS, König reaction, Post-column

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Nagasaki Y[*1], Abe M[*2], Onishi S[*1], Okamoto Y, Toida T[*2] and Higashi K[*1]: Structure and immunomodulating activity of glycogen derived from honeybee larvae (Apis mellifera).
*Biol. Pharm. Bull.*, 44 (8), 1156-1159 (2021). doi: 10.1248/bpb.b21-00239

Honeybee larvae have been recognized as nutrient-rich food in many countries. Although glycogen, a storage form of glucose in animals, is synthesized in honeybee larvae, there is no information on the structure of glycan and its biological activity. In this study, we successfully extracted glycogen from honeybee larvae using hot water extraction and investigated the structure and biological activity of glycan. It was found that the molecular weight of glycogen from honeybee larvae is higher than that of glycogen from bovine liver and oysters. In addition, treatment of RAW264.7 cells with glycogen from honeybee larvae resulted in a much higher production of tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6 than treatment with glycogen from either bovine liver or oysters. These results suggest that the high molecular weight glycogen from honeybee larvae is a functional food ingredient with immunomodulatory activity.
Keywords: honeybee larvae, glycogen, immunomodulatory activity

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Okamoto Y, Higashi K[*1] & Toida T[*2]: A novel preparation method for a proteoglycan in a matrix with collagen from salmon (*Oncorhynchus keta*) nasal cartilage and its affinity to L-selectin.
*Jpn. J Food Chem.*, 28 (1), 9-15 (2021). doi: 10.18891/jjfcs.28.1_9

An intact proteoglycan was extracted from salmon (*Oncorhynchus keta*) nasal cartilage containing type II collagen and prepared using a novel extraction procedure in water containing a sugar fatty acid ester as an edible detergent. This isolation step suppressed the degradation of the proteoglycan and simultaneously afforded a proteoglycan-type II collagen matrix. The extracted proteoglycan was purified, and its properties were compared with those prepared via different extraction procedures using gel permeation chromatography and polyacrylamide gel electrophoresis. Furthermore, the interaction between the purified proteoglycan and human L-selectin was analyzed using a bio-layer interferometry biosensor assay; the proteoglycan demonstrated strong binding to L-selectin.

Keywords: Salmon nasal cartilage, proteoglycan, sugar fatty acid ester

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Okamoto Y, Nunome M, Kondo M, Kitayama I, Suzuki Y & Akiyama H: Quantification of progesterone in beef with marbling using liquid chromatography-tandem mass spectrometry with stable isotope-labelled standards.
*Food Additives & Contaminants: Part A*, 38 (3), 409-417 (2021). doi: 10.1080/19440049.2020.1869326

Progesterone (P4) is contained naturally in animal tissue, and it is also used as a veterinary drug in cattle for treatment purposes. To assess the risk from P4 residues in beef derived from treated cattle, it is essential to quantify the P4 contained naturally in cattle tissue (endogenous P4). Therefore, we performed a method validation for the quantification of endogenous P4 (method quantification limit = 0.06 ng $g^{-1}$) by using isotope-labelled P4s, and investigated the P4 contents in Japanese beef (n= 112; 0.07 to 121 ng $g^{-1}$). The P4 contents in cattle muscle ranged from 0.07 to 54.3 ng $g^{-1}$ in males, and from 0.27 to 121 ng $g^{-1}$ in females. Our investigation also indicated that the developed method using both $^{13}C$- and deuterium-labelled P4 standards could be used to certify the recovery of P4 from cattle muscle containing various amounts of intramuscular fat, and enabled the determination of the P4 content in all Japanese beef samples that exceeded the method quantification limit.
Keywords: Progesterone, beef, liquid chromatography-tandem mass spectrometry

Miyajima A, Kawakami T, Komoriya K, Kato R, Usami M*1, Isama K*2: Potency shift in immunomodulatory activities of zinc oxide (ZnO) nanoparticles in THP-1 cells is associated with cytotoxicity.
*Fundam. Toxicol. Sci.* 2021:8:205-213. doi: 10.2131/fts.8.205.

Two zinc oxide nanoparticles (ZnO NPs) with different physicochemical properties (ZnO(α) and ZnO(Σ)) were examined in THP-1 cells to investigate their effects on cellular immunomodulation and cytotoxicity. THP-1 cells were cultured in the presence of ZnO(α) or ZnO(Σ) for 48 hr, and the expression of proinflammatory cytokines and immune cell surface antigens was examined. ZnO(α) and ZnO(Σ) reduced cell viability in a concentration- and time-dependent manner, with the latter being more potent. ZnO(α) and ZnO(Σ) increased the expression of CD54, IL-8, and TNF-α to the same extent between 24 and 48 hr. While ZnO(Σ) was more potent at effective concentrations, this potency was comparable between ZnO(α) and ZnO(Σ) when normalized to their cytotoxic concentrations (LC50, LC25, or LC5). It was considered that there was a potency shift that is associated with cytotoxicity and physicochemical properties, in immunomodulatory activities in THP-1 cells between ZnO NPs.
Keywords: Zinc oxide, THP-1, Nanoparticle

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Sakoda H, Uematsu M, Okamoto Y, Haishima Y: *In vitro* evaluation of delamination resistance of PEEK and CFR-PEEK.
*Proceedings of the Institution of Mechanical Engineers, Part H.* 2022;235:279-285. doi: 10.1177/09544119211042992

Poly-ether-ether-ketone (PEEK) and carbon fiber reinforced PEEK as orthopaedic implant materials exhibit excellent material properties. Although delamination of PEEK materials has been reported in knee joint wear research, the delamination resistance behavior still remains unclear. In this study, the delamination resistance of PEEK materials was investigated; these materials were compared to ultra-high molecular weight polyethylene (UHMWPE). The results of a ball-on-flat type delamination test indicated that the PEEK materials underwent delamination considerably earlier than UHMWPE, and the contact area of the PEEK materials was smaller than that of UHMWPE. Moreover, the indentation modulus, hardness, and coefficient of friction were higher for PEEK materials than for UHMWPE. The lower tendency of PEEK materials to undergo deformation to mitigate the stress concentration at low conformity contact conditions contributed to their inferior delamination resistance compared to that of UHMWPE. The delamination resistance of the PEEK materials

was equivalent to that of degraded UHMWPE, which highlights the risk of delamination of PEEK implants in a clinical context. Consequently, when using PEEK materials as an implant component loaded at a low conformity contact condition, the material selection and component design must be carefully considered. Overall, the results of this study can help guide the future development of PEEK-based implants.
Keywords: elastic modulus, hardness, friction

Moriwaki[*1] T, Okamoto Y, Yamaga H[*2], Fujisaki K[*1], Uematsu M, Sakoda H, Haishima Y: *In vitro* measurement of contact pressure applied to a model vessel wall during balloon dilation by using a film-type sensor.
*Journal of Neuroendovascular Therapy*. 2022;16:192-197. doi: 10.5797/jnet.oa.2021-0068
Objective: As an important evaluation index of vascular damage, the study aims to clarify the value of contact pressure applied to blood vessels and how it changes with respect to balloon pressure during balloon dilation.
Methods: The contact pressure was evaluated through an *in vitro* measurement system using a model tube with almost the same elastic modulus as the blood vessel wall and our film-type pressure sensor. A poly (vinyl alcohol) hydrogel tube with almost the same elastic modulus was fabricated as the model vessel. The film-type sensor was inserted between the balloon catheter and the model vessel, and the balloon was dilated.
Results: The contact pressure applied to the blood vessel was less than 10% of the balloon pressure, and the increase in contact pressure was less than 1% of the increase in balloon pressure (8 to 14 atm). Moreover, the contact pressure and its increase were larger in the model with a high elastic modulus.
Conclusion: The contact pressure to expand the soft vessel model was not high, and the balloon pressure almost appeared to act on the expansion of the balloon itself. Our experiment using variable stiffness vessel models containing film-type sensors showed that the contact pressure acting on the vessel wall tended to increase as the wall became harder, even when the nominal diameter of the balloon was almost identical to the vessel. Our results can be clinically interpreted: when a vessel is stiff, the high-pressure inflation may rupture it even if its nominal diameter is identical to the diameter of the vessel.
Keywords: balloon, catheter, pressure

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安田将大[*1]，植月啓太[*2]，迫田秀行，富田直秀[*1]：折れ線モデルを用いた球状ポリエチレン粒子の生体反応性評価.
*臨床バイオメカニクス* 2021;42:239-243.
粒子状物質の生体反応性の測定は，人工関節の寿命予測等に用いられる重要な試験法であるが，未だその定量評価方法は確立していない。本研究では，上下倒置培養法にて球状ポリエチレン粒子とマウスマクロファージ様細胞株RAW264を接触させ，TNF-α産生量を測定した。飽和を含む非線形な挙動を示す，粒子添加量とサイトカイン産生量の関係を折れ線モデルへフィッティングしたところ，決定係数は高値を示し，低用量域の範囲が客観的に決定された。また，粒径のn乗に比例する用量（n＝2のとき表面積用量，n＝3のとき体積用量）を定義し，低用量域における挙動が最も近いと判断される用量次元をフィッティング時の決定係数から算出したところ，n＝2.1を得，過去の研究と矛盾しない，より厳密な結果を得た2.1次元用量に対する低用量域の直線の傾きは，本実験系において粒子の比重に依らない材料固有の生体反応性評価指標として活用できる可能性がある。
Keywords: biomaterials, bio-reactivity, artificial joint

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迫田秀行，岡本吉弘，菅野伸彦*：力学特性評価に基づく超高分子量ポリエチレン製コンポーネントの劣化評価.
*臨床バイオメカニクス* 2021;42:245-250.
人工関節の耐久性に影響を与える超高分子量ポリエチレン（UHMWPE）製コンポーネントの酸化劣化は，フーリエ変換赤外分光光度計（FTIR）で測定した酸化度で評価されることが多い．しかし，酸化度は材料破壊に直結する力学特性の劣化の程度と必ずしも一致しない上に，測定の空間分解能も低い．本研究では，力学特性により直接劣化を評価する方法の可能性について，酸化劣化を生じた抜去コンポーネントを用いて検討した．抜去したアニーリング処理を施された高度架橋UHMWPE人工股関節ライナー1例を対象とし，ダイナミック超微

小硬度計で弾性率及び硬度の分布を，FTIR測定により酸化度の分布を，それぞれ求めた．その結果，酸化度が３を超えた測定点でのみ，弾性率と硬度の有意な上昇が認められた．今回用いた方法の検出感度は，酸化度による方法より低く，力学特性測定を劣化評価に応用するためには，さらなる検討が必要と思われた．

Keywords: highly crosslinked UHMWPE, indentation test, FTIR

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Oshima N, Tahara M, Sakai S, Ikarashi Y: Analysis of volatile organic compounds emitted from bedding products.

*BPB Reports* 2021;4:182-92. doi: 10.1248/bpbreports.4.6_182

In this study, we analyzed the volatile organic compounds (VOCs) emitted from a sample of bedding products. These items are intended for long-term use indoors and therefore will be present for long periods of time in the breathing zone of household occupants. Forty bedding products (20 pillows and 20 mattresses) were obtained from the Japanese domestic market for analysis. We have pioneered the measurement of VOCs from bedding products using the sampling bag method, and our measurements showed that a variety of VOCs were emitted from the items. In the pillow sample, polyethylene pillows emitted the most aliphatic hydrocarbons, while buckwheat hull pillows emitted fewer chemicals overall. All pillows emitted tetradecane, toluene, and xylene. VOCs emissions from the mattresses tended to be higher than from the pillows. The mattresses emitted 2-ethyl-1-hexanoic acid frequently and at high concentrations. To further understand the effects of indoor air pollution, it is necessary to continue research into testing the emissions from bedding products and other household items.

Keywords: indoor air, sampling bag method, volatile organic compounds

Oshima N, Saito M*, Niino M*, Hiraishi Y*, Ueki K*, Okoshi K*, Hakamatsuka T, Hada N*: Elucidation of chemical interactions between crude drugs using quantitative thin-layer chromatography analysis.

*Molecules* 2022;27:593. doi: 10.3390/molecules27030593

To elucidate the interactions between crude drugs in Kampo medicines (traditional Japanese medicines), it is important to determine the content of the constituents in a cost-effective and simple manner. In this study, we quantified the constituents in crude drug extracts using thin-layer chromatography (TLC), an inexpensive and simple analytical method, to elucidate the chemical interactions between crude drugs. We focused on five crude drugs, for which quantitative high-performance liquid chromatography (HPLC) methods are stipulated in the Japanese Pharmacopoeia XVIII (JP XVIII) and compared the analytical data of HPLC and TLC, confirming that the TLC results corresponded with the HPLC data and satisfied the criteria of JP XVIII. (*Z*)-ligustilide, a major constituent in Japanese Angelica Root, for which a method of quantification has not been stipulated in JP XVIII, was also quantitatively analyzed using HPLC and TLC. Furthermore, Japanese Angelica Root was combined with 26 crude drugs to observe the variation in the (*Z*)-ligustilide content from each combination by TLC. The results revealed that combinations with Phellodendron Bark, Citrus Unshiu Peel, Scutellaria Root, Coptis Rhizome, Gardenia Fruit, and Peony Root increased the (*Z*)-ligustilide content. Quantifying the constituents in crude drug extracts using the inexpensive and simple TLC method can contribute to elucidating interactions between crude drugs in Kampo medicines, as proposed by the herbal-pair theory.

Keywords: thin-layer chromatography, herbal-pair theory, chemical interaction

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小林憲弘，土屋裕子，五十嵐良明：イプロジオンの水中での分解性と検査法の検討．

*水道協会雑誌* 2021;90(11):11-22.

イプロジオンの水中での分解性について，模擬実験を行って評価するとともに，GC-MSおよびLC-MS/MSによる原体と代謝物（分解物）の同時分析法について検討した．イプロジオンは水中で即座に分解して，分解した原体のほぼ全量が代謝物に変換されたことから，水道水質検査では原体と代謝物の両方を分析し，その濃度を合計することが適切であると考えられた．また，精製水および水道水添加回収試験の結果，GC-MSによる分析は濃度によってガイドラインの目標を満たさなかったが，

LC-MS/MSによる分析は良好な結果であったことから，アセトニトリルで固相抽出後，LC-MS/MSで測定する方法が適していると考えられた．
Keywords：加水分解，水道水，農薬

小林憲弘，高木総吉[*1]，木下輝昭[*2]，仲野富美[*3]，古川浩司[*4]，粕谷智浩[*5]，松巾宗平[*6]，寺中郁夫[*7]，山本剛[*8]，米久保淳[*9]，田中誠也[*10]，丹羽宏之[*11]，会田祐司[*12]，髙原玲華[*13]，齊藤香織[*14]，五十嵐良明：液体クロマトグラフィー質量分析による水道水中の陰イオン一斉分析法の検討と妥当性評価．
*水環境学会誌* 2022;45(2):51-66. doi: 10.2965/jswe.45.51

水道水質基準や目標値が設定されている６種類の陰イオンを一斉分析可能なLC/MSあるいはLC/MS/MS分析条件を検討した．さらに，本研究で確立した分析条件が様々な種類の水道水や機種に適用できるかどうか検証するために，15機関で分析法のバリデーション試験を実施した．水道水を用いた添加回収試験の結果，臭素酸，塩素酸，亜塩素酸，過塩素酸の４物質は，それぞれ12機関以上が良好な分析精度が得られたことから，本分析法は水道水に含まれるこれら４種類の陰イオンを高精度に一斉分析可能と考えられる．ただし，チオ硫酸ナトリウムによる亜塩素酸の分解が見られたことから，亜塩素酸を分析する場合にはEDAで脱塩素処理を行う必要がある．また，物質によって検量線の直線性が確保できる範囲が大きく異なったことから，これらの陰イオンの分析においては検量線の妥当性が確保できる範囲を確認した上で適切な検量線を作成する必要がある．
Keywords:陰イオン，水道水，液体クロマトグラフィー質量分析（LC/MS）

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小林憲弘，土屋裕子，五十嵐良明：GC/MSターゲットスクリーニング分析法による水道水中農薬の定量精度の評価．
*環境科学会誌* 2022;35(2):34-48. doi: 10.11353/sesj.35.88

水質検査の対象農薬としてリストアップされている172農薬を対象として，GC/MSターゲットスクリーニング分析用の検量線データベースをメーカーが異なる２台の装置で合計７回作成し，各農薬の検量線の傾きや，それらの検量線から得られる定量値を相互に比較した．各農薬の検量線の傾きを比較した結果，いずれの装置でも各農薬と保持時間が最も近い内標を用いて作成した検量線が，複数回の測定で最も再現性が良かった．また，装置の移設前後および移設後に繰り返し測定して作成した検量線を比較した結果，移設後に繰り返し測定して作成した検量線の方が良好な再現性が得られたことから，検出感度等の装置状態を一定に保つことで，良好な定量精度が得られることが分かった．各農薬の検量線の定量下限における定量値を比較した結果，同一の装置で作成した検量線データベースを用いた場合は，ほとんどの農薬が５倍以内の定量誤差で測定できることが分かった．これらの結果から，水道水に含まれる農薬を広く検索し，検出農薬の目標値の超過を評価するための手法として，GC/MSターゲットスクリーニング分析法は有用と考えられた．定量誤差の要因として装置感度の変化が考えられたことから，ターゲットスクリーニング分析法を水道水試料に適用する際には，検量線データベース作成時と同様に良好な装置感度を保つことが重要であると考えられた．
Keywords：GC/MS，水道水，農薬

西以和貴[*1]，上村仁[*1]，大嶋智子[*2]，菅谷なえ子[*3]，印南佳織[*4]，田畑佳世[*5]，河上強志：有害物質を含有する家庭用品の規制に関する法律（有害物質含有家庭用品規制法）における繊維製品中防虫加工剤試験法改定に係る検討．
*薬学雑誌* 2021;141:1031-40. doi: 10.1248/yakushi.21-00058

In Japan, the use of mothproofing agents [dieldrin and 4,6-dichloro-7-(2,4,5-trichlorophenoxy)-2-trifluoromethylbenzimidazole (DTTB)] in textiles is regulated by the Act on the Control of Household Products Containing Harmful Substances. Since official analytical methods for these agents have been in place for approximately 40 years, we developed an improved method in a previous study. In the present study, we validated this method. Accordingly, six institutions analyzed the sample prepared at 3 μg/g (1/10 of the

regulation value）and 30 μg/g（the regulation value）. The high accuracy of the results for these samples in almost all the cases（accuracy: 70%-120%, repeatability: <10%, reproducibility: <15%）, confirming the validity of the method. In addition, we examined three samples that were distributed before the introduction of the regulation. The analysis results for these samples showed little variation between institutions, indicating that our method is also applicable to actual samples. Meanwhile, the quantitative value was clearly lower in one institution than in the others. We presumed that the enhanced effect of the sample matrix（matrix effect）on the internal standards in GC-MS analysis was the main cause for this trend. Therefore, we examined the analytical method using polyethylene glycol 300（PEG）as an analyte protectant. As PEG minimized the GC-MS response difference between the standard solution and the matrix-containing solution, GC-MS analysis with PEG would be useful for matrix effect measurements in this method.

Keywords: dieldrin and DTTB, GC-MS, textile

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Suzuki K*1, Futamura K*1, Kawakami T, Numata M*2, Sasaki K*2, Matsunaga K*1, Yagami A*1: Contact dermatitis caused by a disposable paper napkin containing colophony.
*Contact Dermatitis* 2021;85:377-9. doi: 10.1111/cod.13864

A 46-year-old woman without atopic dermatitis or pollinosis, but with a history of contact dermatitis from the use of a compress and a cosmetic glue for the eyelids, wiped her mouth with a disposable paper napkin after eating some hot dog at a restaurant. Eight months later, she wiped her mouth with a paper napkin after eating paella and shrimp salad at another restaurant. A few hours later, she felt a tingling sensation in her lips, after which they became swollen. We performed patch testing using a paper napkin, sanitary pad, eye cosmetics, Japanese baseline series, urushiol and mercuric chloride, cosmetic series, dye series, and resin series. Based on these results, we concluded that colophonium was the causative allergen in this case. This is the first case of contact dermatitis on the lips caused by colophonium in disposable paper napkins. Our case report suggests that in cases of lip swelling after eating, it is important to consider both the possibility of food allergies and allergy to paper napkins that may have contacted the lips during eating.

Keywords: allergic contact dermatitis, colophonium, napkin

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Kawakami T, Tahara M, Ikarashi Y: Presence of Solvent Orange 60 and Solvent Red 179 in Eyeglass Frames and Temple Tips in Japan.
*Dermatitis* 2021;32:e138-40. doi: 10.1097/DER.0000000000000794

Allergic contact dermatitis（ACD）caused by Solvent Orange 60（SO60）, a perinone-type dye found in the plastic frames and temple tips of eyeglasses, goggles, and other articles, has been reported in Japan and Northern Europe. SO60 reportedly showed a strong positive reaction, and the use of SO60 in eyeglass frames and temple tips has been voluntarily restricted in Japan. However, recently, ACD due to SO60 in eyeglass frames and temple tips has been increasing again.1 Thus, we surveyed presence of SO60 in eyeglass frames and temple tips that could be purchased in Japan. Out of the 56 samples, SO60 was detected in 14 samples（13 products）and its concentrations were between 6.1 and 1,600 μg/g. SR179 was detected in 2 samples（2 products）that did not contain SO60 and its concentrations were 8.1 and 31 μg/g. All of these 15 products, except for two, were made in China. This study confirmed that SO60 is again being used in eyeglass frames and temple tips that are currently distributed in Japan. Thus, consumers might be again exposed to risks of ACD due to a strong contact sensitizer SO60. In addition, ACD has been also reportedly caused by SO60 in goggles and helmets, and most recently in protective gloves in other countries. Therefore, we recommend that the use of SO60 should be avoided in products that come into prolonged contact with the skin, not

only eyeglass frames and temple tips.
Keywords: allergic contact dermatitis, Solvent Orange 60 and Solvent Red 179, eyeglass frames

Kawakami T, Tahara M, Ikarashi Y: Analysis of isothiazolinone preservatives in household deodorizers and air fresheners through solid-phase extraction and liquid chromatography-tandem mass spectrometry.
*J Liq Chromatogr Relat Technol* 2021;44:564-9. doi: 10.1080/10826076.2021.1990944

Isothiazolinone preservatives are known to cause contact dermatitis. Although they are used in household deodorizers and air fresheners, the actual extent of their use remains unclear. In this study, we developed a method to simultaneously analyze five isothiazolinones (2-methyl-4-isothiazilin-3-one: MI, 5-choro-2-methyl-4-isothiazolin-3-one: CMI, benzisothiazolin-3-one: BIT, 2-*n*-octyl-4-isothiazolin-3-one: OIT, 4,5-dichloro-*n*-octyl-4-isothiazolin-3-one: 2Cl-OIT) in spray-type household deodorizers and air fresheners. The samples were analyzed through solid-phase extraction and liquid chromatography-tandem mass spectrometry. Three solid-phase extraction cartridges were examined, and good results were obtained for the OASIS HLB Plus LP cartridge. The recoveries and standard deviations for isothiazolinone preservatives extracted using this cartridge were 72-99% and 1.6-6.0%, respectively. In addition, the limit of detection and limit of quantification were as follow: 0.012 and 0.037 μg/mL for MI, 0.029 and 0.089 μg/mL for CMI, 0.032 and 0.098 μg/mL for BIT, 0.013 and 0.040 μg/mL for OIT, and 0.015 and 0.047 μg/mL for 2Cl-OIT. Among the 51 analyzed products, only 10 were detected with isothiazolinone compounds. MI and CMI were detected in five products at concentration levels of 0.31-22 μg/mL and 0.77-95 μg/mL, respectively, while BIT was detected in the other five products at 2.7-101 μg/mL.
Keywords: isothaizolinone preservatives, solid-phase extraction, deodorizer and air fresher

Fujita M[*1], Yamamoto Y[*1], Wanibuchi S[*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 4 mM test chemical solution: Results of ring-study implementation from five participating laboratories.
*J Appl Toxicol* 2022;42:318-33. doi: 10.1002/jat.4268

Amino acid derivative reactivity assay (ADRA) for skin sensitization was adopted as an alternative method in the 2019 OECD Guideline for the Testing of Chemicals (OECD TG 442C). The molar ratio of the nucleophilic reagent to the test chemicals in the reaction solution was set to 1:50. Imamura et al. reported that changing this molar ratio from 1:50 to 1:200 reduced in false negatives and improved prediction accuracy. Hence, a ring study using ADRA with 4 mM of a test chemical solution (ADRA, 4mM) was conducted at five different laboratories to verify within- and between-laboratory reproducibilities (WLR and BLR, respectively). In this study, we investigated the WLR and BLR using 14 test chemicals grouped into three classes: (1) eight proficiency substances, (2) four test chemicals that showed false negatives in the ADRA with 1 mM test chemical solution (ADRA, 1 mM), but correctly positive in ADRA (4 mM), and (3) current positive control (phenylacetaldehyde) and a new additional positive control (squaric acid diethyl ester). The results showed 100% reproducibility and 100% accuracy for skin sensitization. Hence, it is clear that the ADRA (4 mM) is an excellent test method in contrast to the currently used ADRA (1 mM). We plan to resubmit the ADRA (4 mM) test method to the OECD Test Guideline Group in the near future so that OECD TG 442C could be revised for the convenience and benefit of many ADRA users.
Keywords: ADRA (amino acid derivative reactivity assay), OECD TG 442C, ring-study

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大嶋智子*，角谷直哉*，山口之彦*，河上強志：繊維製品に含まれる防炎加工剤のビス（2,3-ジブロモプロピル）ホスフェイト及びトリス（2,3-ジブロモプロピル）ホスフェイトのGC-MS分析法.

*薬学雑誌* 2022;142:279-87. doi: 10.1248/yakushi.21-00197

The use of flame retardants, namely bis (2,3-dibromopropyl) phosphate (BDBPP) and tris (2,3-dibromopropyl) phosphate (TDBPP), in textile products such as curtains, carpets and sleeping clothes is banned in Japan under the 'Act on the Control of Household Products Containing Harmful Substances'. Herein, we developed a GC-MS based method to quantify these compounds with greater accuracy and safety than the current official method. For accurate and sensitive quantification, deuterated compounds, BDBPP-$d_{10}$ and TDBPP-$d_{15}$, were used as surrogate standards. In consideration of the safety of the analyst, certain solvents and reagents used for the pretreatment that are carcinogenic or have a risk of explosion were replaced. For the extraction step, benzene was replaced by ethyl acetate, and for the methyl derivatization step, the reagent was changed from a self-prepared solution of diazomethane in ether to a solution of trimethylsilyl diazomethane in hexane, a safe and easy-to-use commercially available reagent. The calibration curves were liner in the range of 0.5-8.0 μg/mL for both methylated BDBPP (BDBPP-Me) and TDBPP. The detection limit was 0.05 μg/g for BDBPP-Me and 0.3 μg/g for TDBPP, which is sufficiently low compared to the current detection limits of 10 μg/g for BDBPP-Me and 8 μg/g for TDBPP. The recoveries in various curtain material were 66-108% and relative standard deviations were 1.2-10.2% when 5 μg BDBPP and TDBPP were added to 0.5 g of samples. Thus, the developed method is applicable to textile products of various materials.

Keywords: BDBPP and TDBPP, textile, GC-MS

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Yamashita R*1, Takahashi Y*1,2, Takashima K*1,2, Okano H*1,2, Ojiro R*1,2, Tang Q*1,2, Kikuchi S*1,2, Kobayashi M*1, Ogawa B*1, Jin M*3, Kubota R, Ikarashi Y, Yoshida T*1,2, Shibutani M*1,2,4: Induction of cellular senescence as a late effect and BDNF-TrkB signaling-mediated ameliorating effect on disruption of hippocampal neurogenesis after developmental exposure to lead acetate in rats.

*Toxicology* 2021;456:152782. doi: 10.1016/j.tox.2021.152782

Lead (Pb) exposure causes cognitive deficits in children. The present study investigated the effect of developmental exposure to Pb acetate (PbAc) on postnatal hippocampal neurogenesis. Pregnant rats were administered drinking water containing 0, 2000, or 4000 ppm PbAc from gestational day 6 until day 21 post-delivery (weaning), and offspring were maintained without PbAc exposure until adulthood on postnatal day (PND) 77. There was a dose-related accumulation of Pb in the offspring brain at weaning, while Pb was mainly excreted in adulthood. In the hippocampus, metallothionein I/II immunoreactive (+) glia were increased through adulthood as a neuroprotective response to accumulated Pb, accompanied by increased astrocyte and microglia numbers in adulthood, suggesting sustained neural damage. Gene expression changes suggested elevated oxidative stress at weaning and suppression of the antioxidant system in adulthood, as well as continued neuroinflammatory responses. At weaning, granule cell apoptosis was increased and numbers of type-3 neural progenitor cells (NPCs) were decreased. By contrast, type-2a and type-2b NPCs were increased, suggesting suppressed differentiation to type-3 NPCs. In adulthood, there were increased numbers of immature granule cells. In the hilus of the dentate gyrus, somatostatin+ interneurons were increased at weaning, while calbindin-D-29K+ interneurons were increased throughout adulthood, suggesting a strengthened interneuron regulatory system against the suppressed differentiation at weaning. In the dentate gyrus, Bdnf, Ntrk2, and Chrna7 gene expression were upregulated and numbers of hilar TrkB+ interneurons increased at weaning. These findings suggest activation of BDNF-TrkB signaling to increase somatostatin+ interneurons and promote cholinergic signaling, thus increasing later production of immature granule cells. In adulthood, Pcna and Apex1 gene expression were downregulated and Chek1 and cyclin-dependent kinase inhibitor expression were upregulated. Furthermore, there was an increase in γ-H2AX+ SGZ cells, suggesting induction of cellular senescence of SGZ cells due to Pb genotoxicity.

Keywords: cellular senescence, genotoxicity, lead acetate

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岡部　亮*，久保田晶子*，根本了，青柳光敏*：LC-MS/MSを用いた畜産物中のアルベンダゾール代謝物の分析法.

*食品衛生学雑誌* 2021;62(4):113-118. doi:https://doi.org/10.3358/shokueishi.62.113

A method for determining albendazole metabolite (metabolite I) in livestock products using LC-MS/MS was proposed. Livestock samples were hydrolyzed with 6 mol/L HCl at 110℃ for an hour and defatted with ethyl acetate and n-hexane (1 : 1, v/v) mixture. Metabolite I was extracted with acetonitrile from the sample, and the extracts were salted out under basic conditions, allowing the acetonitrile layer to separate. The acetonitrile solution was cleaned up using a cartridge column packed with divinylbenzene-*N*-vinylpyrolidone copolymer bearing sulfo groups. The HPLC separation was conducted on an Inertsil ODS-4 column with a gradient formed from water containing 0.05%(v/v) formic acid and acetonitrile containing 0.05%(v/v) formic acid. To detect metabolite I, tandem mass spectrometry with positive ion electrospray ionization was used. Truenesses (n=5) of metabolite I from cattle meat, fat, liver, and milk spiked at the maximum residue limits or the 0.01 mg/kg were in the range from 83.6 to 97.9%, and the relative standard deviations were from 1.6 to 6.1%.

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

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Saito-Shida S, Kashiwabara N, Nemoto S, Akiyama H: Determination of 8α-hydroxymutilin as a marker residue for tiamulin in swine tissue by liquid chromatography-tandem mass spectrometry.

*Food Analytical Methods* 2021;14:845-855. doi:https://doi.org/10.1007/s12161-020-01950-w

Tiamulin is a semi-synthetic derivative of the natural antibiotic pleuromutilin and is widely used as a veterinary drug for swine. Herein, we report the development of a sensitive and reliable method for determining 8α-hydroxymutilin as a marker residue for tiamulin in swine tissue using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method consists of sample extraction with acetone, defatting by acetonitrile/hexane partitioning, hydrolysis of the tiamulin metabolites to 8α-hydroxymutilin under alkaline conditions, liquid-liquid extraction with ethyl acetate, cleanup using a primary secondary amine cartridge, and LC-MS/MS analysis. The developed method was validated for 8α-hydroxymutilin in swine muscle, fat, and liver at two levels, namely 0.01 mg/kg and the maximum residue limits established in Japan (i.e., 0.1 mg/kg for swine muscle and fat, and 0.6 mg/kg for liver). The trueness ranged from 82 to 89%, and the relative standard deviations ranged from 1 to 3%. No chromatographic interference was observed near the retention time of 8α-hydroxymutilin, and matrix effects were negligible for all matrices, suggesting that the cleanup protocol was effective. The calibration curve was linear in the 0.005-0.5 μg/mL range, with a coefficient of determination greater than 0.997. The developed method enabled accurate quantification using solvent-based calibration without compensating for matrix effects and losses during sample preparation. The limit of detection of the method was 0.0005 mg/kg for each matrix. The developed method is suitable for regulatory-purpose analysis of 8α-hydroxymutilin as a marker residue for tiamulin as defined by the European Union and several other countries.

Keywords: tiamulin, 8α-hydroxymutilin, LC-MS/MS

Saito-Shida S, Kashiwabara N, Nemoto S, Akiyama H: Development of an LC-MS/MS-based method for determination of acetochlor and its metabolites in crops.

*Journal of Food Composition and Analysis* 2022;108:104454. doi:https://doi.org/10.1016/j.jfca.2022.104454

A reliable quantitative method was developed to determine acetochlor residues in soybeans and

sweet corn by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Acetochlor and its metabolites were analyzed as the sum of compounds converted to 2-ethyl-6-methylaniline (EMA) and 2-(1-hydroxyethyl)-6-methylaniline (HEMA) during hydrolysis, expressed as acetochlor equivalent, according to the residual definition used by the United States and Japan. The method involved sample homogenization in methanol, heating at 120℃ under strongly basic conditions (methanol/50% (w/w) sodium hydroxide solution (1:1, v/v)) in a glass vial to convert acetochlor and its metabolites to EMA or HEMA, cleanup using a strong anion-exchange polymer-based cartridge, and subsequent analysis using LC-MS/MS. The developed method was validated for acetochlor as an EMA-producing compound and 2-[(ethoxymethyl){2-(1-hydroxyethyl)-6-methylphenyl}amino]-2-oxoacetic acid (EHO) as a HEMA-producing compound in soybeans and sweet corn. Excellent analytical performances were observed for acetochlor and EHO, with the average recoveries of 82%-92% and relative standard deviations of 1%-3%. No interference was observed near the retention times of EMA and HEMA, which indicated high selectivity. Overall, the developed method is viable for regulatory analysis of acetochlor residues.

Keywords: acetochlor, 2-ethyl-6-methylaniline, 2-(1-hydroxyethyl)-6-methylaniline

Nabeshi H, Imamura M, Tsutsumi T, Maeda T, Hachisuka A, Akiyama H: Radiocesium Concentration in Commercially-Available Foods Produced in Japan: 2017-2019.

*Food Safety* 2022;10(1):1-12. doi: https://doi.org/10.14252/foodsafetyfscj.D-21-00011

We investigated the concentration of radioactive cesium (r-Cs: 134Cs and 137Cs) in commercially-available foods to confirm the effectiveness of pre-shipment radioactive material inspections mainly conducted by local governments. We focused on selected production areas and foods with high probability of r-Cs detection. To this end, we evaluated 715, 685, and 683 samples using scintillation spectrometer and high-purity germanium γ-spectrometer in fiscal years 2017, 2018, and 2019, respectively. The results accounted for 9 samples (1.3%), 10 samples (1.5%), and 5 samples (0.7%) for each fiscal year exceeded the standard limit of radioactive material (100 Bq/kg as r-Cs concentration for general foods). Although we selected and evaluated foods with high probability of r-Cs detection, percentage of samples exceeding the standard limit in each fiscal year was very low, less than 2% to be exact. This suggests that food management system, including pre-shipment inspections, were effectively functioning. In addition, samples exceeding the standard limit were bound to edible wild plants and wild mushrooms, and log-cultivated mushrooms. The former is consider to be difficult for cultivation/feeding control, and the latter was known to be parts of foods greatly affected by radioactive materials. This suggests that the concentration of r-Cs in these items remains at relatively high levels. In contrast, r-Cs was not detected in items with controalble cultivation/feeding. Based on these observations, it is better to be inspected on more difficult-to-cotrol cultivation/feeding items, in order to achieve further streamlining and improving of inspection efficiency. Our results indicate that r-Cs concentration in commercially-available foods of easy-to cultivation/feeding control, such as general vegetables, fruits, and meat, have been well-controlled in Japan, however, difficult-to-cultivation/feeding control items need to be more paid attention to r-Cs concentrations.

Keywords: concentration of radioactive cesium, Fukushima Daiichi Nuclear Power Plant Accident, commercially-available foods

Shiono K, Tsutsumi T, Nabeshi H, Ikeda A*, Yokoyama J*, Akiyama H: Simple and rapid determination of biogenic amines in fish and fish products by liquid chromatography-tandem mass spectrometry using 2,4,6-triethyl-3,5-dimethyl pyrylium trifluoromethanesulfonate as a derivatization reagent.

*Journal of Chromatography A* 2021;1643:462046. doi: https://doi.org/10.1016/j.chroma.2021.462046

A simple and rapid analytical method was developed for determination of four biogenic amines [histamine (Him), cadaverine (Cad), tyramine (Tym), 2-phenylethylamine (Pea)] in fish and fish products. This method uses a new derivatization reagent, 2,4,6-triethyl-3,5-dimethyl pyrylium trifluoromethanesulfonate (Py-Tag). The

four biogenic amines in the samples were extracted with trichloroacetic acid. The diluted extract was derivatized with Py-Tag (15 min at 50℃) and then subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). The limits of quantification for the method were 2 mg/kg for Him, Tym, and Pea and 10 mg/kg for Cad. The matrix effects derived from the tested fish and fish products were negligible in the LC-MS/MS analysis. The impact of the sample matrices on the Py-Tag derivatization was also negligible. The trueness and repeatability of the method were assessed by performing replicate analyses (n = 5) of five samples of fish and fish products, each spiked with the four biogenic amines at three different concentration levels. Analysis of the samples found 87%-104% of the spiked concentrations and the relative standard deviations were <6.1%. A reference sample and quality control canned fish samples were analyzed by the method, and the concentrations of the Him were within acceptable limits. The developed method was successfully used to determine concentrations of the four biogenic amines in 48 fish and fish products on the Japanese market. The developed method does not require cleanup using a solid-phase extraction column or similar, and the derivatization reaction time was only 15 min. The results suggested that the present method is reliable and suitable for rapid analysis of the four biogenic amines in fish and fish products.

Keywords: biogenic amines, rapid determination, LC-MS/MS

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Tsutsumi T, Adachi R, Akiyama H: Evaluation of GC-MS/MS analysis of organochlorine pesticides using the Helium Saver injector

*Jpn. J. Food Chem. Safety* 2021;28(1):33-38. doi: https://doi.org/10.18891/jjfcs.28.1_33

The Helium (He) Saver injector can dramatically reduce consumption of He gas in GC-MS(/MS) analysis in comparison to a conventional split/splitless (SSL) injector using helium carrier gas. The He Saver injector was evaluated in comparison with SSL injector using organochlorine pesticides including their metabolites (10 analytes). The standard solutions (1 ng/mL and 100 ng/mL) of the analytes were analyzed 5 times each by GC-MS/MS using the two injectors. Retention times, peak shapes and peak areas of the analytes were compared between the two injectors. The retention times obtained by the He Saver injector were in good agreement with those obtained by the SSL injector. The selected reaction monitoring chromatograms from both the injectors showed no remarkable differences in peak profiles. The average peak areas using the He Saver injector were close to those obtained using the SSL injector (94-115% of the SSL injector). Although there were statistically significant differences of the peak areas in three of the ten analytes using a two-sided t-test ($p<0.05$), these differences were not considered to be important in a practical analysis. Peak area ratios for the analytes (qualifier ions/quantifier ions) using the He Saver injector were also close to those using the SSL injector (94- 104% of the SSL injector). Overall results indicate that the He Saver injector can be used for as an alternative for a conventional SSL injector and contribute to a reduction of helium consumption in GC-MS(/MS) analysis.

Keywords: organochlorine pesticides, GC-MS/MS, Helium Saver injector

Cai X*[1,2], Taguchi T, Wang H*[2], Yuki M*[3], Tanaka M*[3], Gong K*[1], Xu J*[1], Zhao Y*[1], Ichinose K*[3], Li A*[1]: Identification of a C-Glycosyltransferase Involved in Medermycin Biosynthesis.

*ACS Chem. Biol.* 2021;16;1059-1069. doi: 10.1021/acschembio.1c00227

*C*-Glycosylation in the biosynthesis of bioactive natural products is quite unique, which has not been studied well. Medermycin, as an antitumor agent in the family of pyranonaphthoquinone antibiotics, is featured with unique *C*-glycosylation. Here, a new *C*-glycosyltransferase (*C*-GT) Med-8 was identified to be essential for the biosynthesis of medermycin, as the first example of *C*-GT to recognize a rare deoxyaminosugar (angolosamine). *med*-8 and six genes (*med*-14, -15, -16, -17, -18, and -20 located in the medermycin biosynthetic gene cluster) predicted for the biosynthesis of angolosamine were proved to be functional and sufficient for *C*-glycosylation. A *C*-glycosylation cassette composed of these seven genes could convert a proposed substrate into a *C*-glycosylated product. In conclusion, these genes

involved in the *C*-glycosylation of medermycin were functionally identified and biosynthetically engineered, and they provided the possibility of producing new *C*-glycosylated compounds.

Keywords: *C*-glycosylation, medermycin, biosynthesis

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石川和樹*，八木諒人*，田口貴章，橋元誠*，馬場本絵未*，市瀬浩志*：サンシシ抽出物のTLC分析中に観察される青色呈色物質の同定 －生薬化学実習における学修効果向上を目指した基礎研究結果の応用－．*生薬学雑誌* 2021;75;76-82.

'Sanshishi (SS)' is the dried fruit of *Gardenia jasminoides* Ellis and is an important herbal medicine in Kampo. Geniposide, an iridoid glycoside, which is the active component of Kampo, is an important compound not only in pharmacy but also in industry as a raw material for blue pigment. A simple and rapid procedure was developed for the isolation of geniposide by the treatment of water extracts of SS with an absorbent, DIAION® HP-20 followed by silica gel chromatography (SGC). In the TLC analysis of samples during the SGC purification, a characteristic blue spot was observed near the geniposide on the TLC plate by spraying dilute sulfuric acid followed by heating. This compound was isolated, and NMR analysis elucidated its structure as gardenoside, whose additional hydroxyl group was suggested to provide the property of acid-induced blue pigmentation. The findings were applied to the chemical laboratory practice (LP) of pharmacognosy for pharmaceutical students, and the students with and without the structural information about blue pigmentation on TLC plate were assessed. Analysis of student reports indicated an increase in the number of students describing mechanistic consideration on pigmentation, leading to the improved overall quality of LP results in the group with the structural information. This study serves as a model case to show that the application of new findings from basic research to student LP is useful in improving the learning effectiveness in pharmaceutical education.

Keywords: *Gardenia jasminoides* Ellis, geniposide, pharmaceutical education

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Kumamoto T*1, Kainuma M*2, Takahashi A*2, Matsuo Y*2, Katakawa K*2, Taguchi T, Ichinose K*2: Total Synthesis of 6-Deoxydihydrokalafungin, a Key Biosynthetic Precursor of Actinorhodin, and Its Epimer.
*Molecules* 2021;26;6397. doi: 10.3390/molecules26216397

In this article, we report the total synthesis of 6-deoxydihydrokalafungin (DDHK), a key biosynthetic intermediate of a dimeric benzoisochromanequinone antibiotic, actinorhodin (ACT), and its epimer, *epi*-DDHK. Tricyclic hemiacetal with 3-siloxyethyl group was subjected to $Et_3SiH$ reduction to establish the 1,3-*cis* stereochemistry in the benzoisochromane, and a subsequent oxidation/deprotection sequence then afforded *epi*-DDHK. A bicyclic acetal was subjected to $AlH_3$ reduction to deliver the desired 1,3-*trans* isomer in an approximately 3:1 ratio, which was subjected to a similar sequence to that used for the 1,3-*cis* isomer that successfully afforded DDHK. A semisynthetic approach from (*S*)-DNPA, an isolable biosynthetic precursor of ACT, was also examined to afford DDHK and its epimer, which are identical to the synthetic products.

Keywords: benzoisochromane, diastereoselective reduction, actinorhodin

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Akiyama H*1, Takagi A*2, Inoue K*3, Suzuki Y, Ito R*1, Wakui N*1, Asai M, Sugiura J*4: Evaluation of risk communication program for pesticide residues.
*Food Hyg Safe Sci*, 62:187-192(2021). doi: 10.3358/shokueishi.62.187

To promote and raise the awareness of accurate knowledge on pesticide residues, the symposium program on risk communication on pesticide residues was held by the broadcasted online style. The risk communication program was statistically evaluated using a pre- and post-program online questionnaire survey. We had the questionnaire answers of the 105 valid participants. The analysis of post-program questionnaires shows that the risk communication program was effective in terms of

levels of understanding and interest. Pre-program risk perception or awareness of safety assessments was significantly and positively correlated with awareness for establishing standard values of pesticide residues by the analysis of pre-program questionnaires. Risk perception after the program was significantly higher than before the program, suggesting that risk perception increased due to the program by analysis the same question between pre- and post-program questionnaires. Multiple regression analysis suggests that the participants with higher pre-program awareness of safety assessments or pre-program awareness for establishing standard values appeared to have higher levels of understanding and post-program risk perception.

Keywords: nondetects, left-censored data, Bayesian model

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Suzuki Y, Kondo M, Harimoto M, Kitayama I, Akiyama H*: Dietary exposure to arsenic species in Japan in 2019 using a total diet study based on composite sample with market basket approach at the national level.
*J Food Compost Anal*, 104384(2022). doi: 10.1016/j.jfca.2022.104384.

To estimate the mean dietary exposure to total As (tAs) and some arsenic species [inorganic arsenic (iAs), methylarsonic acid (MMAs) and dimethylarsinic acid (DMAs), and arsenobetaine (AsB)] across the entire Japanese population (≥1 year old), a national total diet study (TDS) based on composite sample with a market-basket approach was conducted in Japan from 10 regions throughout Japan in 2019. Mean dietary exposure to iAs of 0.251 μg kg−1 day−1 showed a slightly lower value compared to the health-based guideline value of 0.30 μg kg−1 day−1. The calculated margin of exposure of 1.2-31.9 were lower or similar to the uncertainty factors of 30. Therefore, the possibility of a risk to Japanese people cannot be excluded. This study indicated that consumption of rice and rice products contributed to 64% of the dietary exposure to iAs. Furthermore, results showed that dietary exposure to tAs had been increasing gradually since 2004, while that to iAs remained steady since 2014. These data indicate the importance of continuing to survey the dietary exposure to total As and As species.

Keywords: inorganic arsenic, dietary exposure, total diet study

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Ohmori K*1, Akaboshi C*2, Sato E*2, Mano J*3, Kondo K, Akiyama H, Nakamura K: Detectability of papaya, tomato, apple and banana DNA in dried fruit products processed with food additive sulfites.
*Japanese Journal of Food Chemistry and Safety*, 28(3), 107-116(2021). doi: https://doi.org/10.18891/jjfcs.28.3_107

Deoxyribonucleic acids (DNAs) in dried fruit products were examined for detectability using real-time polymerase chain reaction (PCR). Endogenous genes with low copy numbers in *Carica papaya* L. (papaya), *Solanum lycopersicum* L. (tomato) and *Malus domestica* (apple) genomic DNAs, i.e., Chymopapain, LAT52 and Apo 5, respectively, were targeted for detection in dried fruit products that were processed with and without food additive sulfites as a bleaching agent, preservative or antioxidant. A total of 13/14 dried papaya, 8/8 dried tomato and 3/3 dried apple products that were processed with sulfites were not detected under a Cq value of 40 in a duplicate real-time PCR test. Despite their undetectability, endogenous 18S rDNA with high copy numbers in the genomic DNA of these fruits was detected at approximately the same amplicon size. Furthermore, BAN, a single-copy endogenous gene found in all dried *Musa acuminata* (banana) products, was detected using a 50 ng DNA template at a Cq value of 22.33-35.80 regardless of whether the fruit was processed with or without sulfites. Although the dried fruit products that were processed with sulfites may contain DNAs, the yields of extracted and purified DNAs were reduced to the degree that not all endogenous genes could be detected reliably using real-time PCR. This may affect the reliability of real-time PCR testing for detecting specific ingredients in dried fruit products,

such as genetically modified fruit and food allergens.
Keywords: dried fruit, DNA, detection

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増本直子，西﨑雄三，中島馨，杉本直樹，佐藤恭子：相対モル感度に基づくシングルリファレンスGC法およびHPLC法によるカラシ抽出物およびセイヨウワサビ抽出物中のイソチオシアン酸アリルの定量．
*食品衛生学雑誌* 2021;62:73-8. doi:10.3358/shokueishi.62.73

既存添加物であるカラシ抽出物およびセイヨウワサビ抽出物の品質確認試験として，市販のイソチオシアン酸アリル（AITC）試薬を標品とするGC-FID法が公定法としてあるが，AITC標品に不純物の存在が確認されていた．そこで，AITCとは別の高純度なシングルリファレンス（SR）を標品とし，より正確にAITCを定量するGC-FID法およびLC-RID（示差屈折率検出器）法を検討した．それぞれのクロマトグラフィー条件下におけるAITC/SRの相対モル感度（RMS）を，定量$^{1}$H-NMR（qNMR）を用いて正確に決定した．このRMSを用いたSR GC-FID法およびSR LC-RID法から算出された製品中のAITC含量は，製品に対して直接qNMR法で算出されたAITC含量と２％以内で一致した．SR法は，従来法よりも正確なAITC含量の算出が可能である．
Keywords：シングルリファレンス，相対モル感度，カラシ抽出物

Sasaki N*1, Nemoto K*1, Nishizaki Y, Sugimoto N, Tasaki K*1, Watanabe A*1, Goto F*1, Higuchi A*1, Morgan E*2, Hikage T*3, Nishihara M*1:
Identification and characterization of xanthone biosynthetic genes contributing to the vivid red coloration of red - flowered gentian.
*The Plant Journal*, 2021;107(6):1711-23. doi: 10.1111/tpj.15412

Cultivated Japanese gentians traditionally produce vivid blue flowers because of the accumulation of delphinidin-based polyacylated anthocyanins. However, recent breeding programs developed several red-flowered cultivars, but the underlying mechanism for this red coloration was unknown. Thus, we characterized the pigments responsible for the red coloration in these cultivars. A high-performance liquid chromatography with photodiode array analysis revealed the presence of phenolic compounds, including flavones and xanthones, as well as the accumulation of colored cyanidin-based anthocyanins. The chemical structures of two xanthone compounds contributing to the coloration of red-flowered gentian petals were determined by mass spectrometry and nuclear magnetic resonance spectroscopy. The compounds were identified as norathyriol 6-*O*-glucoside (i.e., tripteroside designated as Xt1) and a previously unreported norathyriol-6-*O*-(6'-*O*-malonyl)-glucoside (designated Xt2). The copigmentation effects of these compounds on cyanidin 3-*O*-glucoside were detected *in vitro*. Additionally, an RNA sequencing analysis was performed to identify the cDNAs encoding the enzymes involved in the biosynthesis of these xanthones. Recombinant proteins encoded by the candidate genes were produced in a wheat germ cell-free protein expression system and assayed. We determined that a UDP-glucose-dependent glucosyltransferase (StrGT9) catalyzes the transfer of a glucose moiety to norathyriol, a xanthone aglycone, to produce Xt1, which is converted to Xt2 by a malonyltransferase (StrAT2). An analysis of the progeny lines suggested that the accumulation of Xt2 contributes to the vivid red coloration of gentian flowers. Our data indicate that StrGT9 and StrAT2 help mediate xanthone biosynthesis and contribute to the coloration of red-flowered gentians via copigmentation effects.
Keywords: copigmentation, gential, glucosyltranseferase

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Tsutumiuchi K*1, Toyoshima T*1, Hasegawa F*1, Terasawa R*1, Honda W*1, Sakakibara M*1, Ishida Y*1, Ikai Y*1, Ishibashi R*2, Furuya K*2, Morimoto T*2, Ishizuki K, Nishizaki Y, Masumoto N, Sugimoto N, Sato K, Oka H*3:
Molecular Structure of Gardenia Blue Pigments by Reaction of Genipin with Benzylamine and Amino Acids.
*J. Agric. Food Chem.*, 2021;69:3904-11. doi: 10.1021/acs.jafc.0c07948

Genipin was reacted with benzylamine and several amino acids to prepare gardenia blue (GB). The time-course of GB formation with benzylamine was monitored by high-performance liquid chromatography (HPLC), liquid chromatography time-of-flight mass spectrometry (LC-TOFMS), and $^{1}H$ and $^{13}C$ NMR measurements. In this experiment, we determined the molecular structures of some intermediates using accurate masses and additional NMR techniques such as heteronuclear multiple bond correlation (HMBC). GBs with amino acids (GB-AAs) were characterized by both liquid and solid-state NMR measurements. Interestingly, many significant peaks appeared in the solid-state NMR spectra, although the $^{13}C$ NMR spectra from solution samples did not show any distinct peaks. Therefore, we determined that GB-AAs had an alternating copolymer structure composed of methyne and 5*H*-2-pyrindine, which was substituted by amino acids at N atom and linked with methyne at 5 and 7 positions. To confirm this molecular structure, the pyrolysis gas chromatography-mass spectrometry (GC-MS) measurement of GB-AAs was carried out, and 5*H*-2-pyrindine and its methyl derivatives were formed as main pyrolysis products from the polymer chains.

Keywords: gardenia blue, solid-state NMR, pyrolysis GC-MS

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Takahashi M*, Morimoto K*, Nishizaki Y, Masumoto N, Sugimoto N, Sato K, Inoue K*: Study on the Synthesis of Methylated Reference and Their Application in the Quantity of Curcuminoids Using Single Reference Liquid Chromatography Based on Relative Molar Sensitivity.

*Chem. Pharm. Bull.*, 2022;70:25-31. doi: 10.1248/cpb.c21-00621

We report on the recommendation of the simple and versatility of methylated reference (MR) to improve applications in the single reference (SR)-LC based on relative molar sensitivity (RMS). Three curcuminoids (Curs) such as curcumin, demethoxycurcumin and bisdemethoxycurcumin in turmeric products were determined using authentic standards and methylated curcumin. In addition, high-speed countercurrent chromatography (HSCCC) purification is necessary to separate Curs for indicating the RMS. For HSCCC separation, a biphasic solvent system was used to obtain these fractions, which were then subjected to 1H quantitative NMR to determine their contents in each test solution. Using these solutions, the RMS of Curs are calculated from slopes ratios of calibration curves (three ranges from 0-100 μmol/L, $r^2 > 0.998$). The averaged RMS of Curs were 8.92 (relative standard deviation (RSD), 1.17%), 8.97 (2.18%), and 9.61 (0.77%), respectively. Cur concentrations in turmeric products can be determined using RMS, peak area, and MR content added in these samples. This proposed method, which is based on chemical methylation and the SR-LC assay has been successfully applied for the simple and reliable estimation of Curs in turmeric products.

Keywords: relative molar sensitivity (RMS), single-reference HPLC, curcumin

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Abe Y, Yamaguchi M, Ohno H*, Kataoka Y, Mutsuga M, Sato K: Validation of the testing method for the determination of dibutyltin compounds in food utensils, containers, and packaging products made from polyvinyl chloride using gas chromatograph-mass spectrometry with nitrogen as a carrier gas.

*Jpn. J. Food Chem. Safety*, 2021;28:16-22 doi:10.18891/jjfcs.28.1_16

We validated an alternative testing method for the determination of dibutyltin (DBT) compounds in food utensils, containers, and packaging products made from polyvinyl chloride using gas chromatograph-mass spectrometry with nitrogen ($N_2$) as a carrier gas. The retention times, mass spectra, ion intensities, and signal-to-noise-ratios (*S/N*) of a DBT derivative were compared using both helium and $N_2$ as the carrier gas. The retention times were almost equal under the same flow-rate condition, as were the mass spectra. In contrast, the ion intensity with the $N_2$ carrier gas decreased to around 3/4, and the *S/N* decreased significantly to 1/10. This might be due to the increase in background noise level. We validated the performance in terms of a limit testing method

that assess its suitability by comparing the peak area values of the DBT derivative in the test solution and a standard solution at a concentration corresponding with the acceptance criteria and a quantitative testing method with $N_2$ carrier. All parameters corresponding to the trueness, repeatability, and reproducibility as intermediate precision, satisfied the target values in both cases, indicating that both approaches demonstrate good performance as a testing method.

Keywords: GC-MS, nitrogen carrier gas, dibutyltin compound

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Yoshioka T[*1], Itagaki Y[*1], Abe Y, Kawahara N[*2], Goda Y, Ozeki Y[*1], Yamada A[*1]: NaCl dependent production of coniferin in *Alluaudiopsis marnieriana* suspension cultured cells.

*Plant Biotechnology*, 2021;38:183-6 doi: 10.5511/plantbiotechnology.21.0102a

A stable salt-tolerant cell-suspension culture of *Alluaudiopsis marnieriana* was established, and intracellular compounds that accumulated under salt-stress conditions were investigated. HPLC/MS, and NMR analyses indicated that enhanced accumulation of coniferin was found during the growth phase in medium containing 150 mM NaCl. Coniferin or its derivatives may play an important role in salt-tolerance mechanisms in this plant.

Keywords: *Alluaudiopsis marnieriana*, coniferin, salt stress

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Asakura H, Nakayama T, Yamamoto S, Izawa K[*1], Kawase J[*2], Torii Y[*3], Murakami S[*3]: Long-term grow-out affects *Campylobacter jejuni* colonization fitness in coincidence with altered microbiota and lipid composition in the cecum of laying hens.

*Frontiers in Veterinary Science*. 2021;8:675570. doi: 10.3389/fvets.2021.675570

*Campylobacter jejuni* is one of the leading causes of gastrointestinal illness worldwide and is mainly transmitted from chicken through the food chain. Previous studies have provided increasing evidence that this pathogen can colonize and replicate in broiler chicken during its breeding; however, its temporal kinetics in laying hen are poorly understood. Considering the possible interaction between *C. jejuni* and gut microbiota, the current study was conducted to address the temporal dynamics of *C. jejuni* in the cecum of laying hen over 40 weeks, with possible alteration of the gut microbiota and fatty acid (FA) components. Following oral infection with *C. jejuni* 81-176, inocula were stably recovered from ceca for up to 8 weeks post-infection (p.i.). From 16 weeks p.i., most birds became negative for *C. jejuni* and remained negative up to 40 weeks p.i. 16S rRNA gene sequencing analyses revealed that most of the altered relative rRNA gene abundances occurred in the order *Clostridiales*, in which increased relative rRNA gene abundances were observed at >16 weeks p.i. in the families *Clostridiaceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Peptococcaceae*. Lipidome analyses revealed increased levels of sterols associated with bile acid metabolisms in the cecum at 16 and/or 24 weeks p.i. compared with those detected at 8 weeks p.i., suggesting that altered microbiota and bile acid metabolism might underlie the decreased colonization fitness of *C. jejuni* in the gut of laying hens.

Keywords: *Campylobacter jejuni*, chicken gut microbiota, lipidome

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Asakura H, Yamamoto S, Sasaki Y, Okada Y, Katabami[*1], Fujimori A[*2], Munakata K[*3], Shiraki Y[*4], Nishibu H[*5], Hisamoto C[*6], Kawase J[*7], Ojima Y[*8], Kiyoshima A[*9], Shiroma K[*10]: Bacterial distribution and community structure in beef cattle liver and bile at slaughter.

*Journal of Food Protection*. 2022;85:424-34. doi: 10.4315/JFP-21-288

In this study, the distribution of hygienic indicator bacteria in cattle livers and bile was examined at slaughterhouses. One hundred twenty-seven cattle livers with gallbladders were carefully eviscerated from carcasses at 10 slaughterhouses. Microbiological

examination revealed that nine bile samples (7.1% prevalence) and 19 liver parenchyma samples (15.0% prevalence) were positive for *Enterobacteriaceae* (EB) with means ± standard deviations of 3.68 ± 4.63 log CFU/mL and 1.59 ± 2.47 log CFU/g, respectively; thus, bacterial contamination was apparent even at the postevisceration stage. Subsequently, 70 cattle livers were obtained at the postprocessing and storage stage from 7 of the 10 slaughterhouses. Microbiological analysis revealed significantly higher levels of EB in the liver parenchyma (3.00 ± 3.89 log CFU/g, P = 0.011) than those at the postevisceration stage, suggesting that bacterial dissemination and/or replication occurred in the liver parenchyma during processing and storage. According to 16S rRNA ion semiconductor sequencing analysis of representative samples from 12 cattle, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were dominant in both the parenchyma and bile in which EB and *Escherichia coli* were predominant among livers with higher EB levels. These results suggest that bile plays a role as a vehicle for bacterial transmission to the liver parenchyma. This study is the first to evaluate bacterial distribution and community structure in the liver and biliary microecosystem of cattle at slaughter. Our data support the use of EB testing of bile to screen cattle livers contaminated with high levels of fecal indicator bacteria.

Keywords: bacterial distribution, beef cattle liver, microbial community

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Asakura H, Sakata J*, Sasaki Y, Kawatsu K*: Development and evaluation of fluorescence immunochromatography for rapid and sensitive detection of thermophilic *Campylobacter*.
*Food Safety (Tokyo)*. 2021;9:81-7. doi: 10.14252/foodsafetyfscj.D-21-00006

*Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are leading causes of foodborne gastroenteritis in Japan. Epidemiological surveillance has provided evidence that poultry meat is one of the main reservoirs for human campylobacteriosis, and therefore, improvement in process hygiene at slaughter is required to reduce the number of human infections. This study thus aimed to develop fluorescent immunochromatography strips for rapid and sensitive detection of thermophilic *Campylobacter* on poultry carcasses at slaughter. To establish the required detection levels, we first determined the numbers of *C. jejuni* and *C. coli* on poultry carcasses at one large-scale poultry slaughterhouse in Japan, resulting in the detection of *Campylobacter* at 1.97 ± 0.24 log CFU/25 g of neck skin during the post-chilling process by using ISO 10272-2:2017. Our developed *Campylobacter* fluorescence immunochromatography (FIC) assay exhibited a 50% limit of detection of 3.51 log CFU or 4.34 log CFU for *C. jejuni* NCTC 11168 or *C. coli* JCM 2529, respectively. Inclusive and exclusive tests resulted in good agreement. The practical usefulness of this test toward poultry carcasses should be evaluated in future studies, perhaps concentration of the target microorganisms prior to the testing might be helpful to further enhance sensitivity. Nevertheless, our data suggest the potential of FIC for rapid and sensitive detection of thermophilic *Campylobacter* for monitoring the process hygiene of poultry carcasses at slaughter.

Keywords: *Campylobacter*, fluorescence immunochromatography (FIC), process hygiene

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Morita S*1, Sato S*1, Maruyama S*1, Nagasaka M*1, Murakami K*1, Inada K*1, Uchiumi M*1, Yokoyama E*2, Asakura H, Sugiyama H*3, Takai S*4, Maeda K*3, Kabeya H*1: Whole-genome sequence analysis of Shiga toxin-producing *Escherichia coli* O157 strains isolated from wild deer and boar in Japan.
*The Journal of Veterinary Medical Science*. 2021;83:1860-8. doi: 10.1292/jvms.21-0454

The prevalence of Shiga toxin-producing *Escherichia*

*coli* O157 (STEC O157) strains in wild deer and boar in Japan was investigated. STEC O157 strains were isolated from 1.9% (9/474) of the wild deer and 0.7% (3/426) of the wild boar examined. Pulsed-field gel electrophoresis (PFGE) analysis classified the wild deer and boar strains into five and three PFGE patterns, respectively. The PFGE pattern of one wild boar strain was similar to that of a cattle strain that had been isolated from a farm in the same area the wild boar was caught, suggesting that a STEC O157 strain may have been transmitted between wild boar and cattle. Clade analysis indicated that, although most of the strains were classified in clade 12, two strains were classified in clade 7. Whole-genome sequence (WGS) analysis indicated that all the strains carried *mdfA*, a drug resistance gene for macrolide antibiotics, and also pathogenicity-related genes similar to those in the Sakai strain. In conclusion, our study emphasized the importance of food hygiene in processing meat from Japanese wild animals for human consumption.

Keywords: Shiga toxin-producing *Escherichia coli* (STEC), wild boar and deer, whole genome sequencing (WGS)

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*Comparative Immunology, Microbiology and Infectious Diseases*. 2022;82:101766. doi: 10.1016/j.cimid.2022.101766

As a part of risk analysis for consumption of meat from wild animals, the prevalence of *Campylobacter* spp. in wild deer and boar in Japan was investigated. *C. hyointestinalis* subsp. *hyointestinalis* (*C. hyointestinalis*) was isolated from 2.8% (7/253) of the wild deer and 22.1% (71/321) of the wild boar examined. All 23 wild deer isolates and 141 (72.7%) wild boar isolates carried both *chcdt-I* and *chcdt-II* genes. The remaining 53 (27.3%) wild boar isolates had only the *chcdt-II* gene. By whole-genome sequence analysis, we detected 38 to 40 virulence- and survival-associated genes (motility, chemotactic, adhesion, invasion, toxin, glycosylation, iron uptake, drug resistance, and stress response), which had been identified in *C. jejuni* and *C. coli*. In conclusion, our study highlights *C. hyointestinalis* as a possible cause of food-borne disease in humans and emphasizes the importance of food hygiene in the processing of wild meats for human consumption.

Keywords: *Campylobacter*, game meats, wild boar and deer

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Minh DV*, Kakiuchi R*, Obi T*, Asakura H, Chuma T*: The incidence of *Campylobacter* contamination levels through chicken-sashimi processing steps in a small-scale poultry processing plant applying the external stripping method.

*The Journal of Veterinary Medical Science*. 2022;84:414-9. doi: 10.1292/jvms.21-0486

This study aimed to analyze the incidence of *Campylobacter* in a small-scale chicken meat processing plant producing "chicken-sashimi", and determine the effectiveness of surface burning as a treatment during processing. The most probable number (MPN) method was used to analyze the load of *Campylobacter* in 48 samples from four different processing steps (de-feathering, chilling, surface burning, and final-products; 12 samples each). We found the highest load of isolated bacteria in chicken skin after de-feathering. *Campylobacter* was not detected after the surface burning step despite a large load of bacteria present in the cecum content. *Campylobacter* was absent in the final products. Adequate surface burning can avoid *Campylobacter* contamination of chicken sashimi in the processing plant by applying the external stripping method.

Keywords: *Campylobacter*, chicken sashimi, surface burning

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倉宏：国内の*Campylobacter jejuni*血清型別に対応した改良Penner PCR型別法.
*日本食品微生物学会雑誌* 2021;38:123-8. doi: 10.5803/jsfm.38.123

Penner血清型別法は*Campylobacter jejuni*の莢膜多糖（capsule polysaccharide; CPS）の抗原性を基に47種類に分類できるため，国内では疫学マーカーとして有益とされる．一方，近年では市販キットによる型別率の低下が顕著となっている状況を踏まえ，本研究ではPenner血清型別法の代替法としてのPCR法の改良を行い，型別能に関する検討を行った．Penner血清型別参照株27株に対して改良PCR法は特異性を示したほか，国内散発事例由来で血清型が判明した228株のうち，225株（96.5%）が対応する遺伝子型に型別された．また，市販血清で型別不能と判定された178株を同法に供した結果，166株（93.3%）がいずれかの遺伝子型に型別され，*C. jejuni*に対する同法の有用性が確認された．

Keywords：*Campylobacter jejuni*，Penner血清型別法，PCR型別法

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山本詩織，長谷川めぐみ*，岩渕絵里子*，朝倉宏：低温環境下における*Listeria monocytogenes*のバイオフィルム特性.
*Bacterial Adherence & Biofilm*. 2021;34:57-9.

冷蔵温度下における*Listeria monocytogenes* 1/2aのバイオフィルム特性について検討したところ，37℃に比べ5℃下でバイオフィルム形成能が有意に低下し，5℃下では付着関連遺伝子である*dltA*が増加傾向を示すことが判った．当該遺伝子はリポタイコ酸のD-アラニル化を促進し，菌体表層の電荷変動に関連する可能性が報告されている．菌体表層構造について調べた結果，バイオフィルム形成菌体の菌体疎水性は37℃下では有意に増加したものの，5℃下では変化が認められず，疎水性がバイオフィルム形成の程度を示す形質と考えられた．以上より，*L. monocytogenes*の5℃下における低いバイオフィルム形成性はリポタイコ酸のD-アラニル化促進に伴う結果と考えられた．

Keywords: *Listeria monocytogenes*, biofilm, low temperature

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Sasaki Y, Kakizawa H*1, Baba Y*1, Ito T*1, Haremaki Y*2, Yonemichi M*2, Ikeda T*3, Kuroda M*4, Ohya K, Hara-Kudo Y, Asai T*5, Asakura H: Antimicrobial resistance in *Salmonella* isolated from food workers and chicken products in Japan.
*Antibiotics (Basel)*. 2021;10:1541. doi: 10.3390/antibiotics10121541

*Salmonella* is an enteric bacterial pathogen that causes foodborne illness in humans. Third-generation cephalosporin (TGC) resistance in *Salmonella* remains a global concern. Food workers may represent a reservoir of *Salmonella*, thus potentially contaminating food products. Therefore, we aimed to investigate the prevalence of *Salmonella* in food workers and characterize the isolates by serotyping and antimicrobial susceptibility testing. *Salmonella* was isolated from 583 (0.079%) of 740,635 stool samples collected from food workers between January and December 2018, and then serotyped into 76 *Salmonella* enterica serovars and 22 untypeable *Salmonella* strains. High rates of antimicrobial resistance were observed for streptomycin (51.1%), tetracycline (33.1%), and kanamycin (18.4%). Although isolates were susceptible to ciprofloxacin, 12 (2.1%) strains were resistant to the TGC cefotaxime, all of which harbored β-lactamase. Moreover, 1.3% (4/309) of *Salmonella* strains isolated from chicken products were resistant to cefotaxime and harbored $bla_{\text{CMY-2}}$ or $bla_{\text{TEM-52B}}$. Thus, food workers may acquire TGC-resistant *Salmonella* after the ingestion of contaminated chicken products and further contaminate food products.

Keywords: *Salmonella*, antimicrobial resistance, food worker

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佐々木貴正，米満研三*1，百瀬愛佳，上間匡，朝倉宏，浅井鉄夫*2：ブロイラー種鶏場のサルモネラ汚染状況.
*鶏病研究会報* 2021;57:22-6.

鶏肉生産者2社（AおよびB）の協力の下でブロイラー

種鶏場におけるサルモネラ汚染状況を調査した．2019年12月～2020年６月の間に32種鶏場で新鮮盲腸便を採取したところ，サルモネラは５種鶏場（15.6%）から分離された．A社では，サルモネラ陽性３種鶏場から*Salmonella* Manhattan（２株）と*S.* Derby（１株）が分離された．B社では，サルモネラ陽性２種鶏場から*S.* Schwarzengrund（２株）が分離された．次に，B社のコマーシャルブロイラーの鶏肉のサルモネラ汚染について調査した．18製品中６製品（33.3%）からサルモネラが分離され，すべて*S.* Schwarzengrundであった．これら６株中２株は，種鶏場から分離された２株と同様にカナマイシン耐性あるいは供試薬すべてに感受性であった．以上の結果は，鶏肉製品から分離されるサルモネラの中に種鶏場に由来する株が含まれている可能性があることを示唆している．鶏肉のサルモネラ汚染を低減するには，ブロイラー養鶏場に加え，種鶏場のサルモネラ汚染低減も考慮する必要があろう．

Keywords：種鶏場，サルモネラ，鶏肉

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佐々木貴正，米満研三*1，百瀬愛佳，上間匡，朝倉宏，五十君靜信*2：食鳥処理場における鶏肉製品のカンピロバクター交差汚染とフルオロキノロン耐性．
*鶏病研究会報* 2021;57:112-7.

食鳥処理場における鶏肉のカンピロバクターによる交差汚染およびブロイラー群のフルオロキノロン耐性カンピロバクターの感染状況に関する科学データの収集・分析を目的として，１食鳥処理場の16作業日の各日において，最初に食鳥処理されるブロイラー群および次に食鳥処理されるブロイラー群の盲腸内容物および鶏肉製品を採取し，カンピロバクター分離試験を実施した．分離株はすべて*Campylobacter jejuni*で，32群中18群（56.3%）の盲腸内容物および32製品中16製品（50.0%）から分離された．16汚染製品中13製品は，感染群から加工されたものであったが，残りの３製品は非感染群から加工されたものであったこと，分離株の性状が直前に食鳥処理された感染群の盲腸内容由来株と同一であったことから交差汚染が生じたと推定された．ただし，交差汚染製品における汚染菌数は，多くの場合，感染群由来製品の１/10以下になると考えられた．調査群に対してフルオロキノロン系抗菌薬が使用されていないにも関わらず，盲腸内容物の44%（８/18）からフルオロキノロン耐性株が検出された．さらに，調査群由来株の代表株およびその製品由来株の中で最も多かったのは，multilocus sequence typingにおいてST9681であった．これらの結果は，この耐性株はフルオロキノロン選択圧がない環境下でも維持・拡散できることを示唆している．

Keywords：カンピロバクター，薬剤耐性，交差汚染

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中山達哉，山口貴弘*1，陳内理生*2，河原隆二*1，朝倉宏，久米田裕子*3，長谷篤*4：輸入水産食品におけるプラスミド伝播が推定されるセフェム系およびカルバペネム系プラスミド性薬剤耐性菌の汚染状況．
*日本食品微生物学会誌* 2021;38:67-77. doi: 10.5803/jsfm.38.67

Fifteen fishery products retailed in Japan, imported from Southeast and South Asia during January to March 2020, were examined to detect AmpC/ESBL-producing *Escherichia coli*, or antibiotic-resistant *Vibrio* spp. A total of 172 strains were finally isolated from 15 samples. Our data indicated the frequent contamination of imported fishery materials with AmpC/ESBL and carbapenemase-producing bacteria, with possible transferability of CTX-M or NDM-1 genes to intestinal *E. coli* in humans.

Keywords: imported fisheries products, AmpC/ESBL-producing bacteria, carbapenemase-producing *Enterobacter cloacae* complex

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Nakayama T*1, Yamaguchi T*2, Jinnai M*3, Yamamoto S, Li HT*4, Ngo PT*4, Tran DNM*4, Nguyen OTH*4, Hoang PH*4, Nguyen PD*4, Dang CV*4, Kumeda Y*5, Hase A*6: Untargeted phylogenetic group III of multi-drug-resistant *Bacillus cereus* isolated using fraser medium from retail chickens in Ho Chi Minh City.
*Current Microbiology*. 2021;78:3115-23. doi: 10.1007/s00284-021-02562-1

The prevalence of food-borne bacteria in developing countries is less well understood than in developed countries. The ISO11290-1 isolation method is commonly used to study *Listeria* contamination in chicken; however, all isolates are identified as untargeted *Bacillus cereus*. This study aimed to

determine the classification, antibiotic susceptibility, and virulence genes of *B. cereus* isolated from retail chickens in Vietnam. Bacterial isolation using the ISO11290-1 method yielded 12 strains of *B. cereus* from seven out of 60 chickens. For determining bacterial diversity, *panC* and multilocus sequence typing (MLST) analyses were performed. *PanC* analysis showed that all seven strains belong to the phylogenetic group III, to which the highest risk of foodborne illnesses was associated. MLST analysis showed that most strains contained a ST205 complex; further, all strains were found to be resistant to ampicillin, ciprofloxacin, and tetracycline. Virulence genes were also investigated. *ces*, a cereulide-related gene, was detected in 50% of the isolated strains, followed by *cytK*, *nheA*, and *hblA* enterotoxins in 41.7%, 16.7%, and 25% of the strains, respectively. In conclusion, *B. cereus* may be erroneously detected when attempting to detect *Listeria* in food using the ISO11290-1 method. Further study of the prevalence of *B. cereus* in Vietnamese food is needed to improve food safety.

Keywords: *Bacillus cereus*, chicken, Vietnam

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Ikehara T*, Chikanishi K*, Oshiro N: Specification of the okadaic acid equivalent for okadaic acid, dinophysistoxin-1, and dinophysistoxin-2 based on protein phosphatase 2A inhibition and cytotoxicity assays using neuro 2a cell line.

*Journal of Marine Science and Engineering*. 2021;9:1140. doi: 10.3390/jmse9101140

Diarrhetic shellfish poisoning (DSP) is a globally occurring disease threatening public health and trade. The causative toxins, okadaic acid (OA), dinophysistoxin-1 (DTX1), and dinophysistoxin-2 (DTX2) are collectively called OAs, and are quantified using the LC-MS/MS method. The hazardous effect of total OAs is expressed as the sum of OA equivalents defined for respective OAs based on mouse lethality, produced by either intraperitoneal (OAip) or oral administration (OAor). OAs are potent inhibitors of protein phosphatase 2A (PP2A) and are cytotoxic, necessitating expansion of the concept of OA equivalents to all relevant bioactivities. In this study, we determined OA equivalents for respective OA members in PP2A inhibition and cytotoxicity assays. To secure result credibility, we used certified OAs, reference materials, and PP2A produced using genetic engineering. The relative ratio of the OA equivalents determined by PP2A inhibition assays for OA, DTX1, and DTX2 were 1.0:1.6:0.3, while the ratio determined using the cytotoxicity assays indicated 1.0:1.5:0.5. OA equivalents showed a similar tendency in the PP2A inhibition and cytotoxicity assays, and matched better with oral toxicity data than intraperitoneal toxicity in mice. The PP2A inhibition assay, which measures the core activity of the OAs, suggested a higher OA equivalent for DTX1 than that currently used.

Keywords: DSP toxins, PP2A inhibition assay, OA equivalent

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Nagae M*, Igarashi T*, Mizukoshi K*, Kuniyoshi K, Oshiro N, Yasumoto T*: Development and validation of an LC-MS/MS method for the ultra-trace analysis of Pacific ciguatoxins in fish.

*Journal of AOAC INTERNATIONAL*. 2021;104:1272-81. doi: 10.1093/jaoacint/qsab052

Ciguatera fish poisoning (CFP) poses a serious threat to both public health and the use of aquatic resources from the various warm-water regions of the world. Hence, a process for the efficient determination of the relevant toxins is required. We sought to develop and validate the first LC-MS/MS method to quantify the major toxins prevalent in fish from the Pacific Ocean. Toxins were extracted from fish flesh (2 g) using a methanol-water mixture (9:1, v/v). The extract was heated at 80℃ , and low-polarity lipids were eliminated using hexane, initially from the basic solution and later from the acidic solution. The cleanup was performed using solid-phase extraction, Florisil, silica, reversed-phase C18, and primary secondary amine columns. A validation study was conducted by spiking fish flesh with two representative toxins having different skeletal structures and polarities and was calibrated by NMR (qNMR) spectroscopy. The validation

parameters for the ciguatera toxins CTX1B and CTX3C at spiked levels of 0.1 mg/kg were as follows: repeatabilities of 2.3-3.5% and 3.2-5.3%; intermediate precisions of 6.3-9.8% and 6.0-7.4%; recoveries of 80-107% and 95-120%, respectively. The lowest detection levels were 0.004 mg/kg for CTX1B, 0.005 mg/kg for 51-hydroxyCTX3C, and 0.009 mg/kg for CTX3C. The described method practically clears the international action level of 0.01 mg/kg CTX1B equivalents set by the U.S. Food and Drug Administration and the European Food Safety Authority and satisfies the global standards set by Codex and AOAC INTERNATIONAL.

Keywords: ciguatoxin, LC-MS/MS analysis, validation

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Oshiro N, Kuniyoshi K, Yamamoto S, Hotta A, Yamada T, Suzuki T, Sugita N, Matsuura K[*1], Nakashima A[*2], Anzai Y[*3], Asakura H: High levels of tetrodotoxin in the flesh, usually an edible part of the pufferfish *Takifugu flavipterus*, caused by migration from the skin and the regional characteristics of toxin accumulation.

*Journal of Marine Science and Engineering*. 2021;9:1312. doi: 10.3390/jmse9111312

The consumption of a pufferfish, *Takifugu flavipterus* or komonfugu in Japanese, formerly known as *Takifugu poecilonotus*, is popular in Japan. However, *T. flavipterus* is frequently involved in cases of tetrodotoxin (TTX) poisoning in Japan. Although victims have usually consumed inedible parts, some cases are related to consumption of flesh. To improve the risk management of pufferfish poisoning, we studied TTX level in the flesh and skin of *T. flavipterus*. Ninety-seven specimens obtained from the Seto Inland Sea and landed in Fukuoka Prefecture were analyzed by liquid chromatography-tandem mass spectrometry. The flesh from six specimens was toxic (>10 MU/g = 2.2 mg/kg): one was in poor condition (not freeze-thawed); three were freeze-thawed before sample preparation; and two freshly prepared and in good condition (not freeze-thawed). The fillets were divided into outer and inner portions; the TTX levels in the outer portions were notably higher. The skin of the six specimens was moderately to extremely toxic: 165 MU/g (36.3 mg/kg) in the fresh specimen not in good condition, 600-950 MU/g (132-200 mg/kg) in freeze-thawed specimens, and 4500 and 6000 MU/g (990 and 1320 mg/kg) in the two fresh specimens. We concluded that TTX in the flesh migrated from the highly toxic skin. In addition, TTX levels in the skin appeared to be regionally specific. We recommend that toxic portions of *T. flavipterus* are removed as soon as possible after individuals are caught, and that fish from known highly toxic areas are not consumed.

Keywords: *Takifugu flavipterus*, *Takifugu poecilonotus*, tetrodotoxin

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Campàs M[*1], Leonardo S[*1], Oshiro N, Kuniyoshi K, Tsumuraya T[*2], Hirama M[*2], Diogène J[*1]: A smartphone-controlled amperometric immunosensor for the detection of Pacific ciguatoxins in fish.

*Food Chemistry*. 2021;374:131687. doi: 10.1016/j.foodchem.2021.131687

Ciguatoxins (CTXs) are marine neurotoxins produced by microalgae of the genera *Gambierdiscus* and *Fukuyoa*. CTXs may reach humans through food webs and cause ciguatera fish poisoning (CFP). An immunosensor for the detection of Pacific CTXs in fish was developed using multiwalled carbon nanotube (MWCNT)-modified carbon electrodes and a smartphone-controlled potentiostat. The biosensor attained a limit of detection (LOD) and a limit of quantification (LOQ) of 6 and 27 pg/mL of CTX1B, respectively, which were 0.001 and 0.005 μg/kg in fish flesh. In the analysis of fish samples from Japan and Fiji, excellent correlations were found with sandwich enzyme-linked immunosorbent assays (ELISAs), a cell-based assay (CBA) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Stability of at least 3 months at −20℃ was predicted. In just over 2 h, the biosensor provides reliable, accurate and precise Pacific CTX contents in fish extracts, being suitable for monitoring and research programs.

Keywords: Pacific ciguatoxins (P-CTXs), fish, biosensor

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Oshiro N, Nagasawa H, Watanabe M, Nishimura M*1, Kuniyoshi K, Kobayashi N*2, Sugita-Konishi Y*2, Asakura H, Tachihara K*1, Yasumoto T*3: An extensive survey of ciguatoxins on grouper *Variola louti* from the Ryukyu Islands, Japan, using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

*Journal of Marine Science and Engineering*. 2022;10:423. doi:10.3390/jmse10030423

Ingesting fish contaminated with ciguatoxins (CTXs) originating from epibenthic dinoflagellates causes ciguatera fish poisoning (CFP). CFP occurs mainly in the tropical and subtropical Indo-Pacific region and the Caribbean Sea. Furthermore, it occurs sporadically in Japan, especially in the Ryukyu Islands between Taiwan and Kyushu, Japan. *Variola louti* is the most frequently implicated fish with a suggested toxin profile, consisting of ciguatoxin-1B and two deoxy congeners. Therefore, using the liquid chromatography-tandem mass spectrometry (LC-MS/MS), we analyzed CTXs in the flesh of 154 individuals from various locations and detected CTXs in 99 specimens (64%). In 65 fish (43%), CTX levels exceeded the Food and Drug Administration (FDA) guidance level (0.01 μg/kg). Furthermore, in four specimens (3%), the guideline level in Japan (>0.18 μg/kg) was met. Additionally, although the highest total CTX level was 0.376 μg/kg, the consumption of 180 g of this specimen was assumed to cause CFP. Moreover, only CTX1B, 52-*epi*-54-deoxyCTX1B, and 54-deoxyCTX1B were detected, with the relative contribution of the three CTX1B analogs to the total toxin content (35 ± 7.7 (SD)%, 27 ± 8.1%, and 38 ± 5.6%, respectively) being similar to those reported in this region in a decade ago. Subsequently, the consistency of the toxin profile in *V. louti* was confirmed using many specimens from a wide area. As observed, total CTX levels were correlated with fish sizes, including standard length ($r = 0.503$, $p = 3.08 \times 10-11$), body weight ($r = 0.503$, $p = 3.01 \times 10-11$), and estimated age ($r = 0.439$, $p = 3.81 \times 10-7$) of the specimens. Besides, although no correlation was observed between condition factor (CF) and total CTX levels, a significance difference was observed ($p = 0.039$) between the groups of skinnier and fattier fish, separated by the median CF (3.04). Results also showed that the CF of four specimens with the highest CTX level (>0.18 μg/kg) ranged between 2.49 and 2.87, and they were skinnier than the average (3.03) and median of all specimens.

Keywords: ciguatoxin, *Variola louti*, CTX1B

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長沢寛弥，國吉杏子，谷川敏明*1，小林直樹*2，小西良子*2，朝倉宏，大城直雅：小笠原群島産バラハタ*Variola louti*のシガトキシン類分析.

*食品衛生学雑誌* 2021;62:157-61. doi: 10.3358/shokueishi.62.157

小笠原群島（聟島列島，父島列島および母島列島）におけるシガテラの実態を調査するために，周辺海域で漁獲されたバラハタ*Variola louti* 65個体の筋肉を試料としてLC-MS/MSによるシガトキシン類（CTXs）分析を実施した．すべての試料からCTX 1 Bに近接するピークが検出されたが，CTX 1 Bの前駆体である52-*epi*-54-deoxyCTX 1 B，54-deoxyCTX 1 Bや，他のCTX類縁体は検出されなかった．バラハタ試料では通常，この3物質が同時に検出されることから夾雑物による影響を考え分析カラムを変更して分析した結果，全試料においてCTX 1 Bとは保持時間が異なったため夾雑物由来であると判断した．本研究に供したバラハタは体重2,170～7,000 gと大型の個体であったにも関わらず，65個体のいずれからもCTXsは検出されなかった．そのため，小笠原群島周辺海域のバラハタによるシガテラのリスクは低く，CTXs産生性渦鞭毛藻の分布密度は沖縄・奄美海域に比較して極めて低いことが示唆された．

Keywords：シガトキシン，バラハタ，小笠原群島

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Otake S*1, Okada Y, Forsythe SJ*2, Kasai M*1: Meningitis and brain abscess formation caused by *Cronobacter malonaticus* sequence type 440 in a full-term neonate.

*Journal of Infection and Chemotherapy*. 2021;27:1648-52. doi: 10.1016/j.jiac.2021.06.002

*Cronobacter* spp. cause serious diseases, such as necrotizing enterocolitis, bacteremia, and meningitis

in neonates and infants. Most *Cronobacter*-associated meningitis is reportedly due to *C. sakazakii* and the majority of infections caused by *C. malonaticus* occur in adults and are less severe. We report the case of meningitis and brain abscess caused by *C. malonaticus* Sequence Type (ST) 440 in a healthy full-term neonate. We should consider the possibility that full-term neonates may develop meningitis due to *C. malonaticus* and treat appropriately because its mortality rate is very high, and survivors are usually left with severe neurologic impairment. In addition, *C. malonaticus* ST440 may have virulence factors that cause neonatal meningitis akin to the previous report of meningitic ST307 strain.

Keywords: *Cronobacter malonaticus*, meningitis, brain abscess

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Fukunaga Y*1, Ogawa T*1, Suzuki H*2, Okada Y, Nakazawa T*1, Yamaguchi Y*1: Anterior segment dyxmorphogenesis of the eye and glaucoma in MG-W gerbils.

*Journal of Toxicologic Pathology*. 2021;34:245-9. doi: 10.1293/tox.2020-0090

Unilaterally swollen eyes were histopathologically characterized in four MG-W gerbils. The primary lesions resided in the anterior segment of the eye where neural crest cells play a critical role in embryonic development. They included indistinct filtration angle, unformed canal of Schlemm, hypoplastic iris, and ciliary body. The findings noted in the retina, optic nerve, optic tract, and lateral geniculate nucleus were consistent with the lesions induced following the persistent elevation of intraocular pressure as a result of insufficient drainage of aqueous humor. Thus, the present cases observed in the eyes of MG-W gerbils exemplified the anterior segment dysmorphogenesis associated with inadequate neural crest migration or differentiation, leading to subsequent glaucoma.

Keywords: MG-W gerbils, eye, anterior segment dysmorphogenesis

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Uema M, Yonemitsu K, Momose Y, Ishii Y*, Tateda K*, Inoue T, Asakura H: Effect of the photocatalyst under visible light irradiation in SARS-CoV-2 stability on an abiotic surface.

*Biocontrol Science*. 2021;26:119-25. doi: 10.4265/bio.26.119

There is a worldwide attempt to develop prevention strategies against SARS-CoV-2 transmission. Here we examined the effectiveness of tungsten trioxide ($WO_3$)-based visible light-responsive photocatalyst on the inactivation of SARS-CoV-2 under different temperatures and exposure durations. The viral titer on the photocatalyst-coated glass slides decreased from 5.93 ± 0.38 log$TCID_{50}$ /mL to 3.05 ± 0.25 log$TCID_{50}$/mL after exposure to 3,000 lux of the visible light irradiation for 6h at 20℃ . On the other hand, lighting without the photocatalyst, or the photocatalyst-coat without lighting retained viral stability. Immunoblotting and electron microscopic analyses showed the reduced amounts of spike protein on the viral surface after the photocatalyst treatment. Our data suggest a possible implication of the photocatalyst on the decontamination of SARS-CoV-2 in indoor environments, thereby preventing indirect viral spread.

Keywords: COVID-19, SARS-CoV-2, visible light responsive photocatalyst

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Arai S, Ohtsuka K*1, Konishi N*2, Ohya K, Konno T*3, Tokoi Y*4, Nagaoka H*5, Asano Y*6, Maruyama H*7, Uchiyama H*8, Takara T*9, Hara-Kudo Y: Evaluating methods for detecting *Escherichia albertii* in chicken meat.

*J Food Prot*. 2021;84(4):553-562. doi: 10.4315/JFP-20-206.

*Escherichia albertii* is an emerging foodborne pathogen. The source of the *E. albertii* infection in most foodborne outbreaks is unknown because *E. albertii* is difficult to isolate from suspected food or water. *E. albertii* has a broad host range among birds and can be isolated from chicken meat. In this study, PCR assay, enrichment, and isolation conditions for detecting *E. albertii* in chicken meat were evaluated. The growth of 47 *E. albertii* strains isolated in Japan between 1994 and 2018 and a type strain was evaluated

in modified EC broth (mEC) and mEC supplemented with novobiocin (NmEC) and on media containing carbohydrates. The enzyme used for the nested PCR, the enrichment conditions, the most-probable-number (MPN) method, and agar media were also evaluated with chicken meat. To distinguish *E. albertii* from presumptive non-*E. albertii* bacteria, desoxycholate hydrogen sulfide lactose agar (DHL), MacConkey agar (MAC), and these agars supplemented with rhamnose and xylose (RX-DHL and RX-MAC, respectively) were used. All *E. albertii* strains grew in mEC and NmEC at both 36 and 42℃ and did not utilize rhamnose, sucrose, or xylose. Both the first and nested PCRs with TaKaRa Ex Taq, which was 10 to 100 times more active than the other enzymes, produced positive results in enrichment culture of 25 g of chicken meat inoculated with >20 CFU of *E. albertii* and incubated in mEC and NmEC at 42℃ for 22 ± 2 h. Thus, the first PCR was sensitive enough to detect *E. albertii* in chicken meat. The MPN values in mEC and NmEC were 0.5- and 2.3-fold higher than the original inoculated bacterial levels, respectively. *E. albertii* in chicken meat was more efficiently isolated with enrichment in NmEC (70.1 to 100%) and plating onto RX-DHL (85.4%) and RX-MAC (100%) compared with enrichment in mEC (53.5 to 83.3%) and plating onto DHL (70.1%) and MAC (92.4%). Thus, optimized conditions for the surveillance of *E. albertii* contamination in food and investigations of *E. albertii* outbreaks, including the infectious dose, were clarified.

Keywords: Chicken meat, *Escherichia albertii*, Nested PCR

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[*7] Fukuoka City Institute of Health and Environment
[*8] Miyazaki Prefectural Institute for Public Health and Environment
[*9] Okinawa Prefectural Institute of Health and Environment

Arai S, Yamaya S[*1], Ohtsuka S[*2], Konishi N[*3], Obata H[*3], Ooka T[*4], Hirose S, Kai A[*5], Hara-Kudo Y: Detection of *Escherichia albertii* in retail oysters.
*J Food Prot.* 2022;85(1):173-179. doi: 10.4315/JFP-21-222

*Escherichia albertii* is an emerging foodborne pathogen. Owing to its distribution in river water, it is important to determine the presence of *E. albertii* in aquaculture-related foods. In this study, we investigated the distribution of *E. albertii* in retail oyster samples. A total of 427 raw oyster samples (385 Pacific oysters and 42 Japanese rock oysters) were enriched in modified *Escherichia coli* broth (mEC) or mEC supplemented with novobiocin (NmEC) at 42℃ . The cultures were used for *E. albertii*-specific nested PCR assay, as well as for *E. albertii* isolation using deoxycholate hydrogen sulfide lactose agar (DHL), DHL supplemented with rhamnose and xylose, and MacConkey agar supplemented with rhamnose and xylose. The population of *E. albertii* in nested PCR-positive samples was determined using the most-probable-number (MPN) method. *E. albertii* isolates were subjected to biochemical and genetic characterization. *E. albertii* was detected in 5 (1.6%) of 315 Pacific oyster samples (one piece each), 2 (2.9%) of 70 Pacific oyster samples (25 g each), and 2 (4.8%) of 42 Japanese rock oyster samples procured from four geographically distinct regions. A total of 64 *E. albertii* strains were isolated from eight of the nine nested PCR assay-positive oyster samples, and the MPN value was under the detection limit (<3 MPN/10 g). A specific season or month for detecting *E. albertii* was not observed in this study, suggesting that the pathogen is present in seawater. All the *E. albertii* isolates, except one, were positive for the virulence factor *eae*, indicating that these isolates have the potential to infect humans.

Keywords: *Escherichia albertii*, Nested PCR, Oyster

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Hashimoto K[*1], Kawakami Y[*1], Hashimoto R[*2], Kitaoka Y[*3], Onji Y[*3], Oda H[*1], Watanabe M, Takahashi H, Yokoyama K[*4]: Distribution of *Aspergillus* section Nigri at shochu fermenting places in Japan.

*J Air Waste Manag Assoc*. 2022;72(1):61-68. doi: 10.1080/10962247.2021.1880497

Koji mold, which belongs to the *Aspergillus* section *Nigri*, is used in the production of shochu. The section Nigri is composed of very morphologically similar members that in some cases produce mycotoxins, which rises concerns as to whether the presence of mycotoxin-producing fungi in shochu producing sites can compromise consumer safety. Thus, we examined the presence of mycotoxin-producing sec. *Nigri* fungi in six shochu factories (named A-F) in Japan. Isolates of sec. *Nigri* fungi were identified morphologically and confirmed via cytochrome b gene analysis. In factory A (Nago city), airborne fungal levels of sec. *Nigri* were 4,000 and 100 cfu/$m^3$ in the koji-making and fermentation rooms, respectively. In factories B, C, and D, the levels were 40, >104 cfu/$m^3$, and 100 cfu/$m^3$, respectively. The most dominant fungal species of sec. *Nigri* was isolated and identified as *Asp. luchuensis* via genetic analysis. This is likely to have originated from the commercial fermentation culture used. Mycotoxin production (ochratoxin and fumonisin B2) by *Asp. luchuensis* (eight strains) and *Asp. niger* (three strains) was virtually inexistent; only one strain of *Asp. niger* was positive for fumonisin B2. This study clearly shows that mycotoxin-producing fungi are not dominant in the fungal flora present in the shochu factories examined and therefore, that the liquor can be safely fermented.

Keywords: Koji mold, *Asp. luchuensis*, fumonisin B

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Hayashi K, Misawa T, Goto C[*1], Demizu Y, Hara-Kudo Y, Kikuchi Y[*2]: The effects of magainin 2-derived and rationally designed antimicrobial peptides on *Mycoplasma pneumoniae*.

*PLoS ONE*, 2022;17(1):e0261893, doi: 10.1371/journal.pone.0261893

We evaluated the anti-microbial effects of antimicrobial peptides (AMPs) to *Mycoplasma pneumoniae*: four magainin 2 derivatives, three rationally designed AMPs, and NK2A which has the antimicrobial effects on *Mycoplasma bovi*s. We found that three synthesised AMPs, namely 17base-Ac6c, 17base-Hybrid, and Block, had anti-*M. pneumoniae* (anti-Mp) effect at 8-30 μM, whereas others, including NK2A, did not have any such effect. For the further analysis, the membrane disruption activities of AMPs were measured by propidium iodide uptake assays, which showed the membrane-peptide interaction in order of the anti-Mp effect, however, also showed the NK2A strong interaction to cell membrane. These results indicated that anti-Mp effect was not simply determined by the membrane disruption activities of AMPs, but also that the sequence of AMPs were important for killing of *M. pneumoniae*.

Keywords: *Mycoplasma pneumoniae*, antimicrobial peptide

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Ikeuchi S[*1], Hien BT[*1], Nguyen TK [*1,2], Ly LKT [*2], Hara-Kudo Y, Taniguchi T[*1], Hayashidani H[*1]: Contamination of pathogenic *Escherichia coli* in retail fresh vegetables in the Mekong Delta, Vietnam.

*Shokuhin Eiseigaku Zasshi*. 2021;62(3):94-99. doi: 10.3358/shokueishi.62.94

From July 2017 to January 2019, total of 645 retail fresh vegetables collected from 19 retail shops and markets was investigated to know the contamination of enterohemorrhagic *Escherichia coli* (EHEC) and enterotoxigenic *E. coli* (ETEC). Of 645 samples, 2 samples (0.3%) were positive for pathogenic *E. coli*. Of 2 pathogenic *E. coli* positive samples, 1 was EHEC (*stx*2 positive) and the other was ETEC (*sta* positive). Two pathogenic *E. coli* strains were isolated from crisphead lettuce. EHEC strain was not serotyped by commercial antisera and ETEC was serotyped as O20. EHEC and ETEC strains showed multi-drug resistance against 4 and 7 antibiotics, respectively. These results indicate that retail fresh vegetables seem to be not an important source of human EHEC and ETEC infection in the Mekong Delta, Vietnam.

Keywords: EHEC, ETEC, vegetable

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Ksieniewicz-Woźniak E*1, Bryła M*1, Michałowska D*1, Waśkiewicz A*2, Yoshinari T: Transformation of Selected *Fusarium* Toxins and Their Masked Forms during Malting of Various Cultivars of Wheat.
*Toxins (Basel)*. 2021;13(12):866. doi: 10.3390/toxins13120866

This study investigated the impact of malting of six wheat cultivars inoculated with *Fusarium culmorum* on the dynamics of content changes of selected *Fusarium* toxins. The grains of all the tested cultivars showed a high content of deoxynivalenol (DON), zearalenone (ZEN), and their derivatives, whereas nivalenol (NIV) and its glucoside were found only in the Legenda cultivar. Our experiments confirmed that the malting process of wheat grain enables the secondary growth of *Fusarium*, and mycotoxin biosynthesis. The levels of toxins in malt were few-fold higher than those in grain; an especially high increase was noted in the case of ZEN and its sulfate as the optimal temperature and pH conditions for the biosynthesis of these toxins by the pathogen are similar to those used in the grain malting process. This is the first paper reporting that during the malting process, biosynthesis of ZEN sulfate occurs, instead of glycosylation, which is a typical modification of mycotoxins by plant detoxication enzymes.
Keywords: *Fusarium* toxins, biotransformation, modified mycotoxins, wheat

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Matsui K*1, Takeda H*2, Shinkai K*2, Kakinuma T*2, Koizumi Y*2, Kase M*2, Yoshinari T, Minegishi H*2, Nakajima Y*1, Aikawa S*2, Takahashi-Ando N*2, Kimura M*1: 4-*O*-Glucosylation of Trichothecenes by *Fusarium* Species: A Phase II Xenobiotic Metabolism for t-Type Trichothecene Producers.
*Int J Mol Sci*. 2021;22(24):13542. doi: 10.3390/ijms222413542

The t-type trichothecene producers *Fusarium sporotrichioides* and *Fusarium graminearum* protect themselves against their own mycotoxins by acetylating the C-3 hydroxy group with Tri101p acetylase. To understand the mechanism by which they deal with exogenously added d-type trichothecenes, the Δ*tri*5 mutants expressing all but the first trichothecene pathway enzymes were fed with trichodermol (TDmol), trichothecolone (TCC), 8-deoxytrichothecin, and trichothecin. LC-MS/MS and NMR analyses showed that these C-3 unoxygenated trichothecenes were conjugated with glucose at C-4 by α-glucosidic linkage. The toxicities of 4-*O*-glucosides of TDmol, TCC, and HT-2 were much weaker than their corresponding aglycons, suggesting that 4-*O*-glucosylation serves as a phase II xenobiotic metabolism for t-type trichothecene producers.
Keywords: *Fusarium graminearum*, d-type trichothecene, glucosylation, phase II xenobiotic metabolism

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Nakae K*1, Ooka T*2, Murakami K*3, Hara-Kudo Y, Imuta N*2, Gotoh Y*4, Ogura Y*4,5, Hayashi T*4, Okamoto Y*1, Nishi J*2: Diversification of *Escherichia albertii* H-Antigens and Development of H-Genotyping PCR.
*Front Microbiol*. 2021;12:737979. doi: 10.3389/fmicb.2021.737979

*Escherichia albertii* is a recently recognized human enteropathogen that is closely related to *Escherichia coli*. As *E. albertii* sometimes causes outbreaks of gastroenteritis, rapid strain typing systems, such as the O- and H-serotyping systems widely used for *E. coli*, will be useful for outbreak investigation and surveillance. Although an O-genotyping system has recently been developed, the diversity of *E. albertii* H-antigens (flagellins) encoded by *fliC* genes remains to be systematically investigated, and no H-serotyping or genotyping system is currently available. Here, we analyzed the *fliC* genes of 243 genome-sequenced *E. albertii* strains and identified 73 sequence types, which were grouped into four clearly distinguishable types designated *E. albertii* H-genotypes 1-4 (EAHg1-EAHg4). Although there was a clear sign of intraspecies transfer of *fliC* genes in *E. albertii*, none of the four *E. albertii* H-genotypes (EAHgs) were closely related to any of the 53 known *E. coli*

H-antigens, indicating the absence or rare occurrence of interspecies transfer of *fliC* genes between the two species. Although the analysis of more *E. albertii* strains will be required to confirm the low level of variation in their *fliC* genes, this finding suggests that *E. albertii* may exist in limited natural hosts or environments and/or that the flagella of E. albertii may function in a limited stage(s) in their life cycle. Based on the *fliC* sequences of the four EAHgs, we developed a multiplex PCR-based H-genotyping system for *E. albertii* (EAH-genotyping PCR), which will be useful for epidemiological studies of *E. albertii* infections.

Keywords: *Escherichia albertii*, H-antigen, genotyping

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Nguyen TK*1,2, Bui HT*1, Truong TA*2, Lam DN*2, Ikeuchi S*1, Ly LKT*2, Hara-Kudo Y, Taniguchi T*1, Hayashidani H*1: Retail fresh vegetables as a potential source of *Salmonella* infection in the Mekong Delta, Vietnam.

*Int J Food Microbiol*. 2021;341:109049. doi: 10.1016/j.ijfoodmicro.2021.109049

From July 2017 to Jan 2019, a total of 572 retail fresh vegetables were collected to clarify the contamination of *Salmonella* in the Mekong Delta, Vietnam. *Salmonella* was isolated from 74 (12.9%) of 572 samples. The isolation rate of *Salmonella* from retail fresh vegetables in the rainy season (15.3%) was significantly higher than that in the dry season (7.6%) ($P < 0.05$). Of 74 *Salmonella* isolates, *Salmonella* Weltevreden was the most predominant serovar (35.1%) identified from retail fresh vegetables in all of the wet markets. All *S.* Weltevreden isolates (100%) were susceptible to nine antibiotics examined. Thus, retail fresh vegetables were considered as an important potential vehicle of *Salmonella* transmission to humans in the Mekong Delta. These results provide important data for preventing and controlling human salmonellosis in this area.

Keywords: vegetable, *Salmonella*, wet market

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Ohnishi T, Hara-Kudo Y: Presence and quantification of pathogenic *Arcobacter* and *Campylobacter* species in retail meats available in Japan.

*Lett Appl Microbiol*. 2021;73(1):81-87. doi: 10.1111/lam.13478

We present estimations for the amounts of *Arcobacter* (*A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*) and *Campylobacter* (*C. jejuni*, *C. coli*, and *C. fetus*) species in retail chicken, pork, and beef meat using PCR-MPN. *A. butzleri*, *A. cryaerophilus*, and *C. jejuni* were found in 100%, 60%, and 55% of chicken samples, respectively. No other *Arcobacter* or *Campylobacter* species were found in chicken. The MPNs of *A. butzleri*, *A. cryaerophilus*, and *C. jejuni* were greater than $10^3$/100 g in 50%, 0%, and 5% of samples, respectively. The MPN of *A. butzleri* was higher than that of *C. jejuni* in 95% of samples. In pork, *A. butzleri* and *A. cryaerophilus* were detected in 10 and 11 (50 and 55%) of 20 samples, respectively. No other *Arcobacter* or *Campylobacter* species were found in pork. Only one pork sample had more than $10^3$ MPN/100 g of *A. cryaerophilus*. For beef, only two samples tested positive for *A. cryaerophilus*, at 4,600 and 92 MPN/100 g. Overall, we found that the presence and MPNs of *Arcobacter* species is very high in chicken. In contrast, the positive ratios of *Arcobacter* in pork was high as chicken samples, but MPNs were lower than in chicken.

Keywords: Arcobacter, *Campylobacter*, food-borne disease

Oshikata C*1,2, Watanabe M, Ishida M*3, Kobayashi S*3, Hashimoto K*4, Kobayashi N*5, Yamazaki A*6, Konuma R*7, Kaneko T*2, Kamata Y*8, Kuriyama S*9, Yanai M*3, Tsurikisawa N*1,2: Association between temporary housing habitation after the 2011 Japan earthquake and mite allergen sensitization and asthma development.

*Int Arch Allergy Immunol*. 2021;182(10):949-961. doi: 10.1159/000515870

We previously reported an increased prevalence of asthma in adults who lived in temporary housing after the 2011 Great East Japan Earthquake. By using the Global Initiative for Asthma guidelines, we diagnosed asthma in Ishinomaki city temporary housing residents

aged 15 years or older. We then analyzed serum antigen-specific IgE levels to *Dermatophagoides farinae* (Der f), *Dermatophagoides pteronyssinus* (Der p), and *Aspergillus fumigatus*. The prevalence of asthma exceeded 20% across all age-groups throughout the study period. The proportion of study participants with a "positive" antigen-specific IgE titer (i.e., ≥0.35 IUA/mL) was higher in asthmatics than in nonasthmatics for Der f and Der p but not for *A. fumigatus*. The Der p-specific IgE level was positively correlated with the duration of temporary housing ($p < 0.05$, $r = 0.41$) and inversely correlated with the time elapsed since moving out of temporary housing ($p < 0.05$, $r = -0.35$). Mite allergen sensitization was found in both asthmatic and nonasthmatic temporary housing residents after the 2011 Japan earthquake and tsunami; asthma developed even after subjects moved out of temporary housing.

Keywords: adult asthma, *Aspergillus fumigatus*, *Dermatophagoides*, Great East Japan Earthquake

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Oshikata C*1,2, Watanabe M, Ishida M*3, Kobayashi S*3, Hashimoto K*4, Kobayashi N*5, Yamazaki A*6, Konuma R*7, Shimada T*8, Kaneko T*2, Kamata Y*9, Kuriyama S*10, Yanai M*3, Tsurikisawa N*1,2: Mite avoidance decreased mite-specific IgE levels and ameliorated asthma symptoms in subjects who lived in temporary housing after natural disasters.

*Allergol Immunopathol (Madr)*. 2021;49(4):171-179. doi: 10.15586/aei.v49i4.240

We previously reported an increased prevalence of asthma among patients who had lived in temporary housing after the 2011 Great East Japan Earthquake. We investigated the prognosis of asthma in former residents of temporary housing after allergen avoidance. Asthma was diagnosed in adults ≥ 15 years from 2014 to 2019 who had lived in temporary housing in Ishinomaki City for at least 1 year. The disease prognosis after the intervention of allergen avoidance in cases that were followed for more than 3 years during the 6-year study period was analyzed. We measured the *Dermatophagoides farinae* -specific immunoglobulin E (IgE) levels in serum, and the amount of *Dermatophagoides* group 1 (Der 1) antigen on their futons or mattresses. We instructed residents in an allergen avoidance strategy that included 32 tasks, including using microfiber bedding covers. Of the 202 examinees who were followed for at least 3 years during the 6-year study period, 72(35.6%) were asthmatic during at least one examination. Of these 72 asthmatics, 55(76.4%) developed the disease after the earthquake, and more than half of the cases that we diagnosed at the examination were mild intermittent asthma. After the allergen-avoidance intervention, both the Der 1 level on the futons or mattresses of residents who were diagnosed with asthma but who were nonasthmatic at the final screening and their serum Der f-specific IgE levels significantly decreased ($P < 0.01$) at the final examination. Antigen avoidance ameliorated mild asthma that was prevalent among residents of temporary housing after the earthquake.

Keywords: adult asthma, *Dermatophagoides*, allergen avoidance, temporary housing

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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*11, Yanai M*7, Tsurikisawa N*1,2: Mite allergen avoidance decreases

allergic symptoms in children in Ishinomaki city of Japan after natural disasters.

*Allergol Immunopathol (Madr)*. 2022;50(2):23-32. doi: 10.15586/aei.v50i2.483

We investigated the prevalence of asthma, rhinitis, and atopic dermatitis in children, evaluated the mite allergen levels in their bedding after the Great East Japan Earthquake, and assessed changes in allergic symptoms in children and their families after allergen avoidance practices. We performed a survey for the International Study of Asthma and Allergies in Childhood (ISAAC) comprising 1109 children, aged 7-8 years, living in Ishinomaki, Japan. We collected responses from 464 children, and in 2016, measured the level of *Dermatophagoides* group 1 (Der 1) in the bedding of 202 of these children. The levels of Der 1 in their bedding were measured, along with changes in allergic symptoms, in 17 children in 2017 and 14 children in 2018. The levels of Der 1 in the intervention group-but not in the nonintervention group-significantly decreased in 2017 and 2018. The symptoms of asthma, allergic rhinitis, and atopic dermatitis in the children of intervention group and their families decreased after allergen avoidance practices.

Keywords: *Dermatophagoides farina*, Great East Japan Earthquake, allergic rhinitis, asthma

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Sasaki Y, Kakizawa H*1, Baba Y*1, Ito T*1, Haremaki Y*2, Yonemichi M*2, Ikeda T*3, Kuroda M*4, Ohya K, Hara-Kudo Y, Asai T*5, Asakura H: Antimicrobial resistance in *Salmonella* isolated from food workers and chicken products in Japan.

*Antibiotics*. 2021;10(12):1541. doi: 10.3390/antibiotics10121541

*Salmonella* is an enteric bacterial pathogen that causes foodborne illness in humans. Third-generation cephalosporin (TGC) resistance in *Salmonella* remains a global concern. Food workers may represent a reservoir of *Salmonella*, thus potentially contaminating food products. Therefore, we aimed to investigate the prevalence of *Salmonella* in food workers and characterize the isolates by serotyping and antimicrobial susceptibility testing. *Salmonella* was isolated from 583 (0.079%) of 740,635 stool samples collected from food workers between January and December 2018, and then serotyped into 76 *Salmonella enterica* serovars and 22 untypeable *Salmonella* strains. High rates of antimicrobial resistance were observed for streptomycin (51.1%), tetracycline (33.1%), and kanamycin (18.4%). Although isolates were susceptible to ciprofloxacin, 12 (2.1%) strains (one S. Infantis, one *S.* Manhattan, two *S.* Bareilly, two *S.* Blockley, two *S.* Heidelberg, two *S.* Minnesota, one *S.* Goldcoast, and one untypeable *Salmonella* strain) were resistant to the TGC cefotaxime, all of which harbored β-lactamase genes (blaCMY-2, blaCTX-M-15, blaCTX-M-55, and blaTEM-52B). Moreover, 1.3% (4/309) of *Salmonella* strains (three *S.* Infantis and one *S.* Manhattan strains) isolated from chicken products were resistant to cefotaxime and harbored blaCMY-2 or blaTEM-52B. Thus, food workers may acquire TGC-resistant *Salmonella* after the ingestion of contaminated chicken products and further contaminate food products.

Keywords: antimicrobial resistance, chicken product, food worker, *Salmonella*

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Takashima K*1, Nakajima K*1, Shimizu S*1, Ojiro R*1, Tang Q*1, Okano H*1, Takahashi Y*1, Ozawa S*1, Jin M*2, Yoshinari T, Yoshida T*1, Sugita-Konishi Y*3, Shibutani M*1: Disruption of postnatal neurogenesis and adult-stage suppression of synaptic plasticity in the hippocampal dentate gyrus after developmental

exposure to sterigmatocystin in rats.
*Toxicol Lett*. 2021;349:69-83. doi: 10.1016/j.toxlet.2021.06.006

The present study investigated the effects of maternal oral STC exposure on postnatal hippocampal neurogenesis of offspring in rats. Dams were exposed to STC (1.7, 5.0, and 15.0 ppm in diet) from gestational day 6 until day 21 post-delivery (weaning), and offspring were maintained without STC exposure until adulthood on postnatal day (PND) 77, in accordance with OECD chemical testing guideline Test No. 426. On PND 21, 15.0-ppm STC decreased type-3 neural progenitor cell numbers in the subgranular zone (SGZ) due to suppressed proliferation. Increased γ-H2AX-immunoreactive ($^+$) cell numbers in the SGZ and Ercc1 upregulation and Brip1 downregulation in the dentate gyrus suggested induction of DNA double-strand breaks in SGZ cells. The no-observed-adverse-effect level of maternal oral STC exposure for offspring neurogenesis was determined to be 5.0 ppm, translating to 0.34-0.85 mg/kg body weight/day.
Keywords: sterigmatocystin, genotoxicity, oxidative stress, synaptic plasticity

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Yoshinari T, Hayashi K, Hirose S, Ohya K, Ohnishi T, Watanabe M, Taharaguchi S*1, Mekata H*2, Taniguchi T*3, Maeda T*4, Orihara Y*4, Kawamura R*4, Arai S, Saito Y, Goda Y, Hara-Kudo Y: Matrix-Assisted Laser Desorption and Ionization Time-of-Flight Mass Spectrometry Analysis for the Direct Detection of SARS-CoV-2 in Nasopharyngeal Swabs.
*Anal Chem*. 2022;94(10):4218-4226. doi: 10.1021/acs.analchem.1c04328

We developed a method for the direct detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in nasopharyngeal swabs, which uses matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-ToF MS) to identify specific peptides from the SARS-CoV-2 nucleocapsid phosphoprotein (NP). Seven NP-derived peptides were selected as the target molecules for the detection of SARS-CoV-2 in clinical specimens. The method detected between two and seven NP-derived peptides in 19 nasopharyngeal swab specimens from contagious COVID-19 patients. Our results provide evidence that the developed MALDI-ToF MS-based method in a combination of straightforward purification steps and a rapid detection step directly detect SARS-CoV-2-specific peptides in nasopharyngeal swabs and can be a reliable high-throughput diagnostic method for COVID-19.
Keywords: COVID-19, SARS-CoV-2, MALDI-ToF MS

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古賀舞香*，野上有美*，中野朝美*，松永典久*，大屋賢司，工藤由起子，日高千恵*：腸管凝集性大腸菌O15:H 1を原因とする食中毒事例.
*日本食品微生物学会誌* 2021;38(4):153-159. doi: 10.5803/jsfm.38.153

大腸菌は通常，病原性を有しないが，特定の感染症を引き起こす大腸菌を病原性大腸菌と呼ぶ．病原性大腸菌は下痢原性大腸菌及び腸管外病原性大腸菌に大別される．さらに，下痢原性大腸菌は病原機構に基づいて腸管病原性大腸菌（enteropathogenic *Escherichia coli*, EPEC），腸管侵入性大腸菌（enteroinvasive *E. coli*, EIEC），腸管毒素原性大腸菌（enterotoxigenic *E. coli*, ETEC），腸管出血性大腸菌（enterohemorrhagic *E. coli*, EHEC）及び腸管凝集性大腸菌（enteroaggregative *E. coli*, EAggEC）に大別される．調査等に報告された集団事例は21事例であり，そのうち，O126:H27が７事例で分離されており最も多かった．その他の血清型としては，O111，O125，O44，O55，O86aが検出されており，５事例はOUTであった．今回，福岡市内で発生した食中毒について原因究明を行ったところ，病因物質は，日本では報告の少ないEAggEC O15:H 1であることが判明したので報告する．
Keywords：腸管凝集性大腸菌，食中毒，血清型

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Naganuma M, Ohoka N, Tsuji G, Tsujimura H, Matsuno K*, Inoue T, Naito N, 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: ubiqutin-proteasome system, protein knockdown, decoy, transcription factors, estrogen receptor

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Kurohara T, Ito T, Tsuji G, Misawa T, Yokoo H, Yanase Y, Shoda T, Sakai T, Hosoe J, Uchiyama N, Akiyama H*, Demizu Y: Synthesis of Norgestomet and its 17β-isomer and evaluation of their agonistic activities against progesterone receptor.

*Bioorganic & Medicinal Chemistry*, 2021;49:116425. doi: 10.1016/j.bmc.2021.116425

Norgestomet is a synthetic progesterone derivative applied in veterinary medicine to control estrus and ovulation in cattle. Norgestomet has been widely used in the livestock industry to promote the synchronization of estrus in cattle and increase pregnancy rates. However, highly reproducible synthetic methods for Norgestomet have been rarely reported. Here, we described a method for the synthesis of Norgestomet and performed quantitative NMR analysis to determine the purity of the products. Moreover, the agonistic activity of the synthesized compounds against progesterone receptors (PRs) was evaluated using an alkaline phosphatase assay. We synthesized Norgestomet with 97.9% purity that exhibited agonistic activity against PR with EC50 values of 4.5 nM. We also synthesized the 17β-isomer of Norgestomet with 92.7% purity that did not exhibit any PR agonistic activity. The proposed synthetic route of Norgestomet can facilitate the assessment of residual Norgestomet in foods.

Keywords: Norgestomet, Progesterone receptor, Agonist, Hormone-like activity, Residual drugs in food

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Yokoo H, Shibata N, Endo A*1, Ito T, Yanase Y, Murakami Y, Fujii K*2, Hamamura K*2, Saeki Y*1, Naito M*3, Aritake K*2, Demizu Y: Discovery of a highly potent and selective PROTAC targeting hematopoietic prostaglandin D synthase via *in silico* design.

*Journal of Medicinal Chemistry*. 2021;64:15868-82. doi: 10.1021/acs.jmedchem.1c01206

Targeted protein degradation by proteolysis-targeting chimera (PROTAC) is one of the exciting modalities for drug discovery and biological discovery. It is important to select an appropriate linker, an E3 ligase ligand, and a target protein ligand in the development; however, it is necessary to synthesize a large number of PROTACs through trial and error. Herein, using a docking simulation of the ternary complex of a hematopoietic prostaglandin D synthase (H-PGDS) degrader, H-PGDS, and cereblon, we have succeeded in developing PROTAC(H-PGDS)-7 (6), which showed potent and selective degradation activity ($DC_{50}$ = 17.3 pM) and potent suppression of prostaglandin D2 production in KU812 cells. Additionally, in a Duchenne muscular dystrophy model using mdx mice with cardiac hypertrophy, compound 6 showed better inhibition of inflammatory cytokines than a potent H-PGDS inhibitor TFC-007. Thus, our results demonstrated that *in silico* simulation would be useful for the rational development of PROTACs.

Keywords: H-PGDS, prostaglandin D2, PROTAC

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Yokoo, H Ohoka N, Takyo M, Ito T, Tsuchiya K, Kurohara T, Fukuhara K[*1], Inoue T, Naito M[*2], Demizu Y: Peptide stapling improves the sustainability of a peptide-based chimeric molecule that induces targeted protein degradation.

*International Journal of Molecular Sciences*, 2021;22:877210. doi: 3390/ijms22168772

Peptide-based target protein degradation inducers called PROTACs/SNIPERs have low cell penetrability and poor intracellular stability as drawbacks. These shortcomings can be overcome by easily modifying these peptides by conjugation with cell penetrating peptides and side-chain stapling. In this study, we succeeded in developing the stapled peptide stPERML-R7, which is based on the estrogen receptor alpha (ER $\alpha$)-binding peptide PERML and composed of natural amino acids. stPERML-R7, which includes a hepta-arginine motif and a hydrocarbon stapling moiety, showed increased $\alpha$-helicity and similar binding affinity toward ER $\alpha$ when compared with those of the parent peptide PERML. Furthermore, we used stPERML-R7 to develop a peptide-based degrader LCL-stPERML-R7 targeting ER $\alpha$ by conjugating stPERML-R7 with a small molecule LCL161 (LCL) that recruits the E3 ligase IAPs to induce proteasomal degradation via ubiquitylation. The chimeric peptide LCL-stPERML-R7 induced sustained degradation of ER $\alpha$ and potently inhibited ER $\alpha$-mediated transcription more effectively than the unstapled chimera LCL-PERML-R7. These results suggest that a stapled structure is effective in maintaining the intracellular activity of peptide- based degraders.

Keywords: estrogen receptors, helical peptide, protein-protein interaction, protein knockdown

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Ikeda K, Shoda T, Demizu Y, Tsuji G: Discovery of non-proteinogenic amino acids inhibiting biofilm formation of *S. aureus* and methicillin-resistant *S. aureus*.

*Bioorg. Med. Chem. Lett.* 2021;48:128259. doi: 10.1016/j.bmcl.2021.128259

Bacterial biofilms often cause medical complications and there has been a great deal of interest in the discovery of small-molecule agents that can inhibit the formation of biofilms. Among these agents, it has been reported that several d-amino acids, such as d-Leu, d-Trp, d-Tyr, and d-Met, exhibit weak inhibitory activity toward bacterial biofilm formation. In this study, we have screened a library of 332 non-proteinogenic amino acids for new biofilm inhibitory agents and discovered several compounds exhibiting biofilm-inhibitory activity against Gram-positive bacteria. In particular, H-DL-β-(3,4-dihydroxyphenyl)-dl-Ser-OH (**253**) showed potent activity against *S. aureus*, including methicillin-resistant *S. aureus*.

Keywords: Biofilm formation inhibitor, Gram-positive bacteria, MRSA, Non-proteinogenic amino acid

Matsuo K*, Kuriyama M*, Yamamoto K*, Demizu Y, Nishida K*, Onomura O*: Nickel-catalyzed hydrodeoxygenation of aryl sulfamates with alcohols as mild reducing agents.

*Synthesis*. 2021;53:4449-60. doi: 10.1055/a-1548-8362

The nickel-catalyzed hydrodeoxygenation of aryl sulfamates has been developed with alcohols as mild reductants. A variety of functional groups and heterocycles were tolerated in this reaction system to give the desired products in high yields. In addition, the gram-scale process and stepwise cine-substitution were also achieved with high efficiency.

Keywords: hydrodeoxygenation, nickel, reduction

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Yokoo H*, Hirano M, Ohoka N, Misawa T, Demizu Y: Structure-activity relationship study of amphipathic antimicrobial peptides using helix-destabilizing sarcosine.

*J. Pept. Sci*. 2021;e3360:1-6. https://doi.org/10.1002/psc.3360

Antimicrobial peptides (AMPs) are potential therapeutic agents against bacteria. We recently showed that a rationally designed AMP, termed **Stripe**, with an amphipathic distribution of native cationic and hydrophobic amino acids on its helical structure exhibited potent antimicrobial activity against Gram-positive and Gram-negative bacteria with negligible hemolytic activity and cytotoxicity. In this study, the structure-activity relationship of **Stripe**

was elucidated by designing a series of antimicrobial peptides whereby amino acid residues of **Stripe** were exchanged with helix-destabilizing sarcosine residues. **Stripe 1-5** peptides with hydrophobic amino acids substituted with sarcosine were predominantly unstructured and showed no antimicrobial activity, except against *Escherichia coli (E. coli)* (DH5α) cells. The activity against *E. coli* (DH5α) cells and the helicity of **Stripe 1-5** peptides decreased concomitantly as the number of sarcosine residue substitutions increased. **Stripe 1-5** peptides showed no hemolytic activity or cytotoxicity. The results indicate that sarcosine substitutions provide an approach to study the structure–activity relationship of helical AMPs, and the helicity of **Stripe** is an important feature defining its activity.

Keywords: Antimicrobialpeptides, Helical structures, Sarcosine, Amphipathicity

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Yokoo H, Yamamoto E, Masada S, Uchiyama N, Tsuji G, Hakamatsuka T, Demizu Y, Izutsu K, Goda Y: *N*-Nitrosodimethylamine (NDMA) formation from ranitidine impurities: Possible root causes of the presence of NDMA in ranitidine hydrochloride.
*Chem. Pharm. Bull.* 2021;69;872-6. doi: 10.1248/cpb.c21-00289

*N*-Nitrosodimethylamine (NDMA) is a probable human carcinogen. This study investigated the root cause of the presence of NDMA in ranitidine hydrochloride. Forced thermal degradation studies of ranitidine hydrochloride and its inherent impurities (**Imps. A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, **I**, **J**, and **K**) listed in the European and United States Pharmacopeias revealed that in addition to ranitidine, **Imps. A**, **C**, **D**, **E**, **H**, and **I** produce NDMA at different rates in a solid or an oily liquid state. The rate of NDMA formation from amorphous **Imps. A**, **C**, and **E** was 100 times higher than that from crystalline ranitidine hydrochloride under forced degradation at 110℃ for 1 h. Surprisingly, crystalline **Imp. H**, bearing neither the *N*,*N*-dialkyl-2-nitroethene-1,1-diamine moiety nor a dimethylamino group, also generated NDMA in the solid state, while **Imp. I**, as an oily liquid, favorably produced NDMA at moderate temperatures (*e.g.*, 50℃). Therefore, strict control of the aforementioned specific impurities in ranitidine hydrochloride during manufacturing and storage allows appropriate control of NDMA in ranitidine and its pharmaceutical products. Understanding the pathways of the stability related NDMA formation enables improved control of the pharmaceuticals to mitigate this risk.

馬庭愛加，辻厳一郎，伊藤貴仁，内山奈穂子，細江潤子，大槻崇*，松藤寛*，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２報）：ロラゼパムのHPLCによる定量法設定に向けた検討.
*Yakugaku Zasshi*, 2021;141;961-70. doi: 10.1248/yakushi.21-00010

The Japanese Pharmacopoeia (JP) is an official normative publication that is referred to, for establishing the authenticity and properties and maintaining the quality of pharmaceutics in Japan. Partial amendments are periodically made to these guidelines to keep up with the progress of science and technology, and the international harmonization is revised every 5 years. Thus, "Internationalization of the JP" is one of the more important issues to address for the revision of the JP. For example, the incorporation of the test methods that have been used in other pharmacopeias, such as the United States Pharmacopeia (USP) and the European Pharmacopoeia (EP), into the JP is a useful approach. In light of this, we have recently reported changes in test methods in the 17th JP, "Establishment of a quantitative test method for clonidine hydrochloride from using a potentiometric titration method to using HPLC". As a part of our ongoing research to change test methods for internationalization, we selected lorazepam. Lorazepam is analyzed using a potentiometric titration method as listed in the 17th JP; however, both the USP and EP use HPLC for quantitative analysis of this drug. In this study, we synthesized the related impurities of lorazepam listed in the USP and the EP and determined their purities using quantitative NMR. The separation conditions of these compounds, including lorazepam, were examined using HPLC and simultaneous analyses were performed. In addition, lorazepam extracted from the tablets was analyzed using conditions similar to those used for the analysis of the related impurities.

Keywords: international harmonization, the Japanese

Pharmacopoeia, HPLC, quantitative NMR, lorazepam, impurity

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Xu H, Ohoka N, Yokoo H, Nemoto K, Ohtsuki T*, Matsufuji H*, Naito M, Inoue T, Tsuji G, Demizu Y: Development of agonist-based PROTACs targeting liver X receptor.

*Front. Chem*. 2021;9;674967. doi: 10.3389/fchem.2021.674967

Liver X receptors (LXRs) belong to the nuclear hormone receptor superfamily and function as ligand-dependent transcription factors that regulate cholesterol homeostasis, lipid homeostasis, and immune responses. LXR antagonists are promising treatments for hypercholesterolemia and diabetes. However, effective LXR antagonists and inhibitors are yet to be developed. Thus, we aimed to develop LXR degraders (proteolysis targeting chimeras PROTACs against LXR) as a complementary strategy to provide a similar effect to LXR inhibition. In this study, we report the development of GW3965-PEG5-VH032 (**3**), a PROTAC capable of effectively degrading LXRβ protein. Compound **3** induced the ubiquitin-proteasome system-dependent degradation of the LXRβ protein, which requires VHL E3 ligase. We hope that PROTACs targeting LXR proteins will become novel therapeutic agents for LXR-related diseases.

Keywords: liver X receptor, PROTAC, ubiquitin-proteasome system, von Hippel-Lindau, protein degradation

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Tsuji G, Yonemitsu K, Ito T, Yanase Y, Uema M, Ohoka N, Inoue T, Asakura H, Demizu Y: Development of ciclesonide analogues that block SARS-CoV-2 RNA replication.

*Bioorganic and Medicinal Chemistry Letters*. 2021;43;128052. doi: 10.1016/j.bmcl.2021.128052

Ciclesonide is an inhaled corticosteroid used to treat asthma and is currently undergoing clinical trials for treatment of coronavirus disease 2019 (COVID-19). An active metabolite of ciclesonide, **Cic2**, was recently reported to repress severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) genomic RNA replication. Herein, we designed and synthesized a few types of ciclesonide analogues. **Cic4** (bearing an azide group) and **Cic6** (bearing a chloro group) potently decreased SARS-CoV-2 viral replication and had low cytotoxicity compared with **Cic2** (bearing a hydroxy group). These compounds are promising as novel therapeutic agents for COVID-19 that show significant antiviral activity.

Keywords: SARS-CoV-2, COVID-19, Endonuclease, Ciclesonide, Viral growth inhibition

Moriya S*1, Shibasaki H*1, Kohara M*1, Kuwata K*2, Imamura Y*3, Demizu Y, Kurihara M*4, Kittaka A*1, Sugiyama T*1: Synthesis and characterization of PNA oligomers containing preQ1 as a positively charged guanine analogue.

*Bioorganic and Medicinal Chemistry Letters*. 2021;39:127850. doi: 10.1016/j.bmcl.2021.127850

We report the synthesis of a peptide nucleic acid (PNA) monomer containing preQ1, a positively charged guanine analogue. The new monomer was incorporated into PNA oligomers using standard Fmoc-chemistry-based solid-phase synthesis. The preQ1 unit-containing PNA oligomers exhibited improved affinity for their complementary DNA through electrostatic attraction, and their sequence specificity was not compromised. It could be beneficial to incorporate preQ1 into PNA oligomers instead of guanine when creating antisense/antigene agents or research tools.

Keywords: nucleic acid, preQ1, Strand invasion

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Soma-Kaiho A*1, Akizuki Y*1, Igarashi K*1 Endo A*2, Kawase Y*2, Shoda T, Demizu Y, Naito M, Saeki Y*2, Tanaka K*2, Ohtake F*1: TRIP12 enhances small molecule-induced degradation of BRD4 through K29/K48 branched ubiquitin chains

*Mol. Cell*, 2021;81:1411-248. doi: 10.1016/j.molcel.2021.01.023

Targeted protein degradation is an emerging therapeutic paradigm. Small-molecule degraders

such as proteolysis-targeting chimeras (PROTACs) induce the degradation of neo-substrates by hijacking E3 ubiquitin ligases. Although ubiquitylation of endogenous substrates has been extensively studied, the mechanism underlying forced degradation of neo-substrates is less well understood. We found that the ubiquitin ligase TRIP12 promotes PROTAC-induced and CRL2VHL-mediated degradation of BRD4 but is dispensable for the degradation of the endogenous CRL2VHL substrate HIF-1$\alpha$. TRIP12 associates with BRD4 via CRL2VHL and specifically assembles K29-linked ubiquitin chains, facilitating the formation of K29/K48-branched ubiquitin chains and accelerating the assembly of K48 linkage by CRL2VHL. Consequently, TRIP12 promotes the PROTAC-induced apoptotic response. TRIP12 also supports the efficiency of other degraders that target CRABP2 or TRIM24 or recruit CRBN. These observations define TRIP12 and K29/K48-branched ubiquitin chains as accelerators of PROTAC-directed targeted protein degradation, revealing a cooperative mechanism of branched ubiquitin chain assembly unique to the degradation of neo-substrates.

Keywords: apoptosis, cancer, cullin-RING ligase, epigenetics, targeted protein degradation, ubROTAC, ubiquitin

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馬庭愛加，辻厳一郎，伊藤貴仁，内山奈穂子，細江潤子，大槻崇*，松藤寛*，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１報）：クロニジン塩酸塩のHPLCによる定量法設定に向けた検討

*Yakugaku Zasshi*, 2021;141;591-8. doi: 10.1248/yakushi.20-00237

The Japanese Pharmacopoeia (JP) is an official normative guide for maintaining the authenticity of properties and qualities of medicine in Japan. The JP is revised every 5 years, and partial amendments are made from time to time to keep abreast with progress in science and technology and international harmonization. We are conducting a related study on the elimination of toxic reagents from the JP. The elimination of toxic reagents is an important study in relation to the five pillars of the revision of the 18th JP, "Improvement in quality by proactively introducing the latest knowledge and technological advances". In addition, "Internationalization of the JP" is an important issue to be addressed during revision of the JP. Considering international harmonization of the JP, it is important to incorporate the test methods that have been used in other pharmacopoeia, such as the United States Pharmacopeia (USP) and the European Pharmacopoeia (EP) in the JP. To achieve the above, herein, we selected clonidine hydrochloride, which is listed in the 17th JP. A potentiometric titration method is used as a quantitative method for clonidine hydrochloride in the 17th JP; in contrast, a HPLC method is utilized in the USP and the EP. In this study, we synthesized impurities of clonidine hydrochloride and determined their purities using quantitative NMR. In addition, the complete separation conditions of these compounds by HPLC were examined, and simultaneous analysis was performed.

Keywords: international harmonization, the Japanese Pharmacopoeia, HPLC, quantitative NMR, lorazepam, impurity

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Shibata N, Cho N*1, Koyama H*1, Naito M*2: Development of a degrader against oncogenic fusion protein FGFR3-TACC3

*Bioorg. Med. Chem. Lett.* 2022;60:128584. doi: https://doi.org/10.1016/j.bmcl.2022.128584

Fibroblast growth factor receptor 3-transforming acidic coiled-coil containing protein 3 (FGFR3-TACC3), which has been identified in many cancers such as glioblastoma and bladder cancer, is a potent oncogenic fusion protein that induces constitutive activation of FGFR signaling, resulting in uncontrolled cell proliferation. Although several tyrosine kinase inhibitors against FGFR are currently under development, resistance to such types of inhibitors in patients has become a concern. In this study, a chimeric molecule SNIPER(TACC3)-11 (5a) was developed and found to reduce FGFR3-TACC3 levels effectively. Compound 5a conjugated KHS108 (a TACC3 ligand) to an LCL161 derivative (11) (an inhibitor of apoptosis protein [IAP] ligand) with a PEG linker (n = 2). Mechanistical analysis showed that cellular IAP1 was required for the reduction of FGFR3-

TACC3 levels. Consistent with the decrease in FGFR3-TACC3 levels, compound 5a suppressed the growth of FGFR3-TACC3 positive cells. Thus, compound 5a is a candidate therapeutic with a novel drug modality against cancers that exhibit FGFR3-TACC3-dependent proliferation and exerts pharmacological effects distinct from FGFR3 kinase inhibitors because it lacks substructures crucial for kinase inhibition.

Keywords: FGFR3-TACC3, oncogenic fusion protein, SNIPER

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Cui H, Soga K, Tamehiro N, Adachi R, Hachisuka A, Hirose A, Kondo K, Nishimaki-Mogami T: Statins repress needle-like carbon nanotube- or cholesterol crystal-stimulated IL-1β production by inhibiting the uptake of crystals by macrophages.
*Biochem Pharmacol*. 2021;188:114580. doi: https://doi.org/10.1016/j.bcp.2021.114580

Statins are 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors that lower atherogenic LDL-cholesterol levels. Statins exert clinically relevant anti-inflammatory effects; however, the underlying molecular mechanism remains unclear. Studies have shown that endogenous and exogenous pathogenic crystals, such as cholesterol and monosodium urate (MSU), and needle-like nanomaterials, such as multi-wall carbon nanotubes (MWCNT), induce the production of IL-1β and play a critical role in the development of crystal-associated sterile inflammatory pathologies. In this study, we evaluated the effect of statins on crystal-induced IL-1β production in macrophages. We found that various statins, including pitavastatin, atorvastatin, fluvastatin, and lovastatin, but not squalene synthase inhibitor, repressed IL-1β release upon MWCNT stimulation. In addition, IL-1β production induced by cholesterol crystals and MSU crystals, but not by ATP or nigericin, was diminished. MWCNT-stimulated IL-1β release was dependent on the expression of NLRP3, but not AIM2, NLRC4, or MEFV. Statin-induced repression was accompanied by reduced levels of mature caspase-1 and decreased uptake of MWCNT into cells. Supplementation of mevalonate, geranylgeranyl pyrophosphate, or farnesyl pyrophosphate prevented the reduction in IL-1β release, suggesting a crucial role of protein prenylation, but not cholesterol synthesis. The statin-induced repression of MWCNT-elicited IL-1β release was observed in THP-1-derived and mouse peritoneal macrophages, but not in bone marrow-derived macrophages where statins act in synergy with lipopolysaccharide to enhance the expression of IL-1β precursor protein. In summary, we describe a novel anti-inflammatory mechanism through which statins repress mature IL-1β release induced by pathogenic crystals and nanoneedles by inhibiting the internalization of crystals by macrophages.

Keywords: carbon nanotube, inflammasome, statin

Tanaka H*1, Ito S*2, Ojika M*3, Nishimaki-Mogami T, Kondo K, Wakamatsu K*2: The Oxidation of Equol by Tyrosinase Produces a Unique Di- *ortho*-Quinone: Possible Implications for Melanocyte Toxicity

*Int. J. Mol. Sci*. 2021;22:9145. doi: https://doi.org/10.3390/ijms22179145

Equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman, EQ), one of the major intestinally derived metabolites of daidzein, the principal isoflavane found in soybeans and most soy foods, has recently attracted increased interest as a health-beneficial compound for estrogen-dependent diseases. However, based on its structure with two *p*-substituted phenols, this study aimed to examine whether EQ is a substrate for tyrosinase and whether it produces *o*-quinone metabolites that are highly cytotoxic to melanocyte. First, the tyrosinase-catalyzed oxidation of EQ was performed, which yielded three EQ-quinones. They were identified after being reduced to their corresponding catechols with $NaBH_4$ or L-ascorbic acid. The binding of the EQ-quinones to *N*-acetyl-L-cysteine (NAC), glutathione (GSH), and bovine serum albumin via their cysteine residues was then examined. NAC and GSH afforded two mono-adducts and one di-adduct, which were identified by NMR and MS analysis. It was also found that EQ was oxidized to EQ-di-quinone in cells expressing human tyrosinase. Finally, it was confirmed that the EQ-oligomer, the EQ oxidation product, exerted potent pro-oxidant activity by oxidizing GSH to the oxidized GSSG and concomitantly producing $H_2O_2$. These results suggest that EQ-quinones could be cytotoxic to melanocytes due to their binding to cellular proteins.

Keywords: equol, melanocyte toxicity, ortho-quinone

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Miyagi E*1, Arakawa N, Sakamaki K*1, Yokota NR*1, Yamanaka T*1, Yamada Y*2, Yamaguchi S*3, Nagao S*3, Hirashima Y*4, Kasamatsu Y*4, Kato H*5, Mogami T*1, Miyagi Y*5, Kobayashi H*2. Validation of tissue factor pathway inhibitor 2 as a specific biomarker for preoperative prediction of clear cell carcinoma of the ovary.

*Int J Clin Oncol*. 2021;26:1336-44. doi:10.1007/s10147-021-01914-y

Methods: Serum samples were obtained preoperatively from patients with ovarian masses, who needed surgical treatment at five hospitals in Japan. The diagnostic powers of TFPI2 and cancer antigen 125 (CA125) serum levels to discriminate CCC from BOTs, other EOCs, and benign lesions were compared.

Results: A total of 351 patients including 69 CCCs were analyzed. Serum TFPI2 levels were significantly higher in CCC patients (mean ± SD, 508.2 ± 812.0 pg/mL) than in patients with benign lesions (154.7 ± 46.5), BOTs (181 ± 95.5) and other EOCs (265.4 ± 289.1). TFPI2 had a high diagnostic specificity for CCC (79.5%). In patients with benign ovarian endometriosis, no patient was positive for TFPI2, but 71.4% (15/21) were CA125 positive. TFPI2 showed good performance in discriminating stage II-IV CCC from BOTs and other EOCs (AUC 0.815 for TFPI2 versus 0.505 for CA125) or endometriosis (AUC 0.957 for TFPI2 versus 0.748 for CA125). The diagnostic sensitivity of TFPI2 to discriminate CCC from BOTs and other EOCs was improved from 43.5 to 71.0% when combined with CA125.

Conclusions: High specificity of TFPI2 for preoperative detection of CCC was verified with the defined cutoff level of TFPI2 in clinical practice. TFPI2 and CA125 may contribute substantially to precise prediction of intractable CCC.

Keywords: Ovarian cancer, TFPI2, Serum tumor marker

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Imai K*1,2, Matsuoka M*3, Tabata S*2, Kitagawa Y*3, Nagura-Ikeda M*2, Kubota K*3, Fukada A*3, Takada T*3, Sato M*3, Noguchi S*3, Takeuchi S*3, Arakawa N, Miyoshi K*2, Saito Y, Maeda T*3. Cross-reactive humoral immune responses against seasonal human coronaviruses in COVID-19 patients with different disease severities.

*Int J Infect Dis*. 2021;111:68-75. doi: 10.1016/j.ijid.2021.08.026.

Background: The cross-reactive antibody response against seasonal human coronaviruses (HCoVs) was evaluated according to disease severity in patients with COVID-19 in Japan. Methods: In total, 194 paired serum samples collected from 97 patients with COVID-19 (mild, 35; severe, 62) were analyzed on admission and during convalescence. IgG antibodies against the nucleocapsid (N) and spike (S) proteins of SARS-CoV-2 and four seasonal HCoVs (HCoV-NL63, -229E, -OC43, and -HKU1) were detected by enzyme-linked immunosorbent assays. Results: There was no difference in optical density (OD) values for seasonal HCoVs on admission between the severe and mild cases. In addition, a specific pattern of disease severity-associated OD values for HCoVs was not identified. Significant increases in OD values from admission to convalescence for HCoV-HKU1and -OC43 IgG-S, and for HCoV-NL63 and -229E IgG-N were observed in the severe cases. Significant differences were observed between the mild and severe cases for HCoV-HKU1 and -OC43 IgG-S OD values during convalescence. Correlations were found between the fold changes for HCoV-OC43 IgG-S OD values, and for SARS-CoV-2 IgG-S OD values, and C-reactive protein, lactate dehydrogenase, and lymphocyte levels. Conclusion: There was no association between the antibody titer for seasonal HCoVs in the early phase of COVID-19 and disease severity.

Keywords: COVID-19, SARS-CoV-2, seasonal human coronavirus

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Kimura A*1,2, Arakawa N, Kagawa H*1, Kimura Y*1, Hirano H*1,2. Phosphorylation of Ser1452 on BRG1 inhibits the function of the SWI/SNF complex in chromatin activation.
*J Proteomics*. 2021;15;247:104319. doi: 10.1016/j.jprot.2021.104319-29.

BRG1, one of core subunits of the SWI/SNF chromatin remodeling complex, is frequently mutated in cancers. Previously, we reported significant downregulation of the phosphorylation level of BRG1 on Ser1452 (<10%) in cell lines derived from ovarian clear cell carcinoma with frequent recurrence and acquired drug resistance. In this study, we tried to elucidate the roles of BRG1 phosphorylation, using cell lines expressing wild-type, phosphorylation-mimic (brg1-S1452D), or non-phosphorylatable (brg1-S1452A) BRG1. Quantitative proteomic analyses revealed upregulation of proteins and phosphoproteins related to linker histone H1s, histone methylation, and protein ubiquitylation in brg1-S1452D cells, which may coordinately promote the chromatin inactivation and ubiquitin-dependent degradation of target proteins. Consistent with these results, brg1-S1452D cells exhibited an increase in condensed chromatin and polyubiquitylated proteins. In brg1-S1452D cells, we also detected downregulation of various cancer-related proteins (e.g., EGFR and MET) as well as decreased migration, proliferation, and sensitivity to taxanes and oxaliplatin. Together, our results reveal that BRG1 phosphorylation drives tumor malignancy by inhibiting the functions of SWI/SNF complex in chromatin activation, thereby promoting expression of various cancer-related proteins. SIGNIFICANCE: For the first time we demonstrated that the mutation on Ser1452 phosphorylation site of BRG1, a component of SWI/SNF chromatin remodeling complex, changed protein and phosphoprotein levels of linker histone H1s, binding competitor of histone H1s, and histone methylase/demethylase involved in the heterochromatic histone modifications to promote the chromatin inactivation. In phosphorylation-mimic mutant, significant decrease of various cancer-related proteins as well as migration, proliferation, and sensitivity to specific antitumor agents were detected. Our results reveal that BRG1 phosphorylation drives tumor malignancy by inhibiting the functions of SWI/SNF complex in chromatin activation, thereby promoting expression of various cancer-related proteins.
Keywords: BRG1, Linker histone, Ovarian cancer

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Ishikawa R, Saito K, Matsumura T*1, Arai K*2, Yamauchi S*3, Goda R*4, Tachiki H*5, Kawabata M*1, Nitta SI*2, Nagao A*3, Suga T*4, Uchiyama H*5, Nakai K*2, Asahina K*3, Yamaoka M*5, Saito Y. A multilaboratory validation study of LC/MS biomarker assays for three lysophosphatidylcholines. *Bioanalysis*. 2021;13:1533-1546. doi:10.4155/bio-2021-0150.

Aim: Although the fit-for-purpose approach has been proposed for validation procedures and acceptance criteria for biomarker assays, practical biomarker assays to facilitate clinical application and regulatory documents on biomarker assays remain limited. Materials & methods: We assigned six independent laboratories and selected three lysophosphatidylcholines (LPCs): LPC(16:0), LPC(18:0) and LPC(18:1) as model biomarkers. Using LC-MS, the following key validation parameters were evaluated: calibration curve, carryover, parallelism, precision and relative accuracy and these values were similar among all laboratories. Further, we determined LPC levels in six lots of rat plasma at unknown concentrations and compared them among the laboratories. Conclusion: Our multilaboratory validation and reproducibility data are useful for the development of future biomarker assay validation procedures, as well as regulatory documents.
Keywords: LC/MS, biomarker assay, interlaboratory reproducibility

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Yang MS[*3], Fujita M[*1], Kumagai Y[*4], Tohkin M[*5], Saito Y, Sai K. Real-world evidence of population differences in allopurinol-related severe cutaneous adverse reactions in East Asians: A population-based cohort study.
*Clin Transl Sci*. 2021;14:1002-1014. doi: 10.1111/cts.12964.

Allopurinol-related severe cutaneous adverse reactions (SCARs) are strongly associated with *HLA-B*58:01*, the allele frequency (AF) of which is largely different among East Asians. This study aimed to evaluate population differences in allopurinol-related SCAR incidence related to genetic and/or other risk factors among East Asians in the real-world. A population-based cohort study was conducted using claims databases from Taiwan, Korea, and Japan. New users of allopurinol were followed up to 1 year. As control drugs, phenytoin and carbamazepine were used. The crude incidence rate ratios (IRRs) of SCARs for allopurinol against phenytoin or carbamazepine were the highest in Taiwan (IRR, 0.62 and 1.22; 95% confidence interval [CI], 0.54-0.72 and 1.01-1.47, respectively), followed by Korea (IRR, 0.34 and 0.82; 95% CI, 0.29-0.40 and 0.77-0.87), and the lowest in Japan (IRR, 0.04 and 0.16; 95% CI, 0.02-0.08 and 0.09-0.29). This order was accordant with that of AF ratios (AFRs) reported of *HLA-B*58:01* against alleles responsible for phenytoin- or carbamazepine-related SCARs. The IRRs were higher in patients with chronic kidney disease, females, and elderly. This study demonstrated population differences in the risk of allopurinol-related SCAR development among East Asians based on genetic and other common risk factors. This finding will help to promote appropriate risk management for allopurinol-related SCARs based on ethnic origins.
Keywords: severe cutaneous adverse reactions, allopurinol, population difference

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Saito K, Hattori K[*1], Hidese S[*1], Sasayama D[*1], Miyakawa T[*1], Matsumura R[*1], Tatsumi M[*1], Yokota Y[*1], Ota M[*1], Hori H[*1], Kunugi H[*1, 2]. Profiling of Cerebrospinal Fluid Lipids and Their Relationship with Plasma Lipids in Healthy Humans.
*Metabolites*. 2021;11:268. doi:10.3390/metabo11050268.

Lipidomics provides an overview of lipid profiles in biological systems. Although blood is commonly used for lipid profiling, cerebrospinal fluid (CSF) is more suitable for exploring lipid homeostasis in brain diseases. However, whether an individual's background affects the CSF lipid profile remains unclear, and the association between CSF and plasma lipid profiles in heathy individuals has not yet been defined. Herein, lipidomics approaches were employed to analyze CSF and plasma samples obtained from 114 healthy Japanese subjects. Results showed that the global lipid profiles differed significantly between CSF and plasma, with only 13 of 114 lipids found to be significantly correlated between the two matrices. Additionally, the CSF total protein content was the primary factor associated with CSF lipids. In the CSF, the levels of major lipids, namely, phosphatidylcholines, sphingomyelins, and cholesterolesters, correlated with CSF total protein levels. These findings indicate that CSF lipidomics can be applied to explore changes in lipid homeostasis in patients with brain diseases.
Keywords: cerebrospinal fluid, lipid profiling, lipidomics

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Sai K, Nakatani E[*1], Iwama Y[*2], Hiraoka S[*2], Tohkin M[*3], Uyama Y[*4], Saito Y. Efficacy comparison for a schizophrenia and a dysuria drug among East Asian populations: A retrospective analysis using multi-regional clinical trial data.
*Ther Innov Regul Sci*. 2021:55:523-538. doi: 10.1007/s43441-020-00246-9.

In planning multi-regional clinical trials (MRCTs) according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use E17 guideline, it is expected that East Asian populations with relatively similar ethnicity can be pooled as one population. However, evidence supporting this assumption is limited. This study aimed to investigate population/regional differences considering influencing factors among East Asian regions using MRCT data as

a research model. A retrospective analysis was conducted to determine the efficacy of two drugs, asenapine, a schizophrenia drug, and tadalafil, a dysuria drug for benign prostatic hyperplasia, using MRCT data from Japan, Korea, and Taiwan.. Among the 4 outcomes for the two drugs, no significant population/regional differences were detected ($P > 0.05$) by the adjusted regression models. The effect modifiers, such as pretreatment drug status or concurrent diseases, were common among countries. This finding supported the possible applicability of the region pooling strategy for MRCTs in East Asia, emphasizing the benefits of exploring ethnic difference/influencing factors at an early stage to design further confirmatory studies.

Keywords: Ethnic difference, ICH E17, Multi-regional clinical trial

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*Int J Mol Sci*. 2022;23:575. doi: 10.3390/ijms23010575.

Conventional anti-cancer therapies based on chemo- and/or radiotherapy represent highly effective means to kill cancer cells but lack tumor specificity and, therefore, result in a wide range of iatrogenic effects. A promising approach to overcome this obstacle is spliceosome-mediated RNA trans-splicing (SMaRT), which can be leveraged to target tumor cells while leaving normal cells unharmed. Notably, a previously established RNA trans-splicing molecule (RTM44) showed efficacy and specificity in exchanging the coding sequence of a cancer target gene (Ct-SLCO1B3) with the suicide gene HSV1-thymidine kinase in a colorectal cancer model, thereby rendering tumor cells sensitive to the prodrug ganciclovir (GCV). In the present work, we expand the application of this approach, using the same RTM44 in aggressive skin cancer arising in the rare genetic skin disease recessive dystrophic epidermolysis bullosa (RDEB). Stable expression of RTM44, but not a splicing-deficient control (NC), in RDEB-SCC cells resulted in expression of the expected fusion product at the mRNA and protein level. Importantly, systemic GCV treatment of mice bearing RTM44-expressing cancer cells resulted in a significant reduction in tumor volume and weight compared with controls. Thus, our results demonstrate the applicability of RTM44-mediated targeting of the cancer gene Ct-SLCO1B3 in a different malignancy.

Keywords: cancer gene therapy, epidermolysis bullosa, trans-splicing

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Tsuji D*1, Saito Y, Mushiroda T*2, Miura M*3, Hira D*4, Terada T.*5: Results of a national survey of Japanese pharmacists in relation to the application of pharmacogenomic testing for precision medicine.

*J Clin Pharm Ther*. 2021;46:649-657. doi: 10.1111/jcpt.13367.

Pharmacogenomics (PGx) testing can be effective for supporting precision medicine. The purpose of this study was to assess the knowledge, attitude and practice behaviours of pharmacists in relation to such testing through a survey. We also aimed to identify potential obstacles to implementation of PGx testing by pharmacists and the characteristics of hospital pharmacists involved. We performed a web-based survey regarding PGx in Japan. The survey contained a questionnaire related to PGx, which consisted of 30 items and was made accessible via the official Japanese Society of Pharmaceutical Health Care and Sciences (JSPHCS) website. The characteristics of hospital pharmacists associated with involvement in PGx testing were evaluated using univariate and multivariate analyses. One thousand three-hundred and thirteen pharmacists responded to the survey. The results revealed that the majority of respondents recognized the role that germline PGx testing can play in determining individual drug responses and

that pharmacists have embraced the potential of PGx testing to improve patient care. However, only 26% of pharmacists were involved in PGx testing. We also found that most respondents (81.0%) believed that the lack of insurance coverage for PGx testing was a major barrier to its clinical implementation. Hospital pharmacists involved in PGx testing included certified pharmacists in JSPHCS and pharmacists who had studied PGx in university; however, only 12.4% of pharmacists had received specific PGx-related education. The findings of this survey highlight the necessity to increase the number of PGx tests covered by insurance, and the importance of effective education to inform and facilitate clinical implementation of PGx testing.

Keywords: pharmacogenomics, precision medicine, questionnaire survey

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Maekawa K*1, Yamamura M*2, Matsuki A*3, Ishikawa T*3, Hirai T*4, Yamaguchi Y*2, Saito Y, Kanda T*3, Hatakeyama K*1. Impacts of SNPs on Adverse Events and Trough Concentration of Imatinib in Patients with Gastrointestinal Stromal Tumors.

*Drug Metab. Pharmacokinet.* 2021;43:100441. doi: 10.1016/j.dmpk.2021.100441.

Although imatinib has dramatically improved the outcomes of patients with gastrointestinal stromal tumor (GIST), marked inter-individual differences in its efficacy and toxicity have been observed. Extensive pharmacogenetic studies in Caucasian and Asian populations have demonstrated that several genetic polymorphisms are involved in these differences; however, no studies have focused on Japanese patients with GIST. This study aimed to evaluate the impacts of genetic polymorphisms of drug metabolizing enzymes and transporters on the incidence of adverse events and trough plasma concentrations ($C_{troughs}$) of imatinib in Japanese patients with GIST. Of 35 candidate SNPs genotyped from 65 patients, ABCG2 421C>A was significantly associated with increased incidence rates of grade 2 or higher rash. When relationships between the genotypes and $C_{troughs}$ were examined in a subgroup of 38 patients from whom plasma was available, 5 SNPs were associated with significant trends toward increased or decreased dose-adjusted $C_{troughs}$. Of them, SLCO1B3 334T>G and SLCO1A2 -1032G>A made significant contributions to the individual variability of $C_{trough}$ by multivariate regression analysis. Genetic variations in ABCG2, SLCO1B3, and SLCO1A2 may play important roles in the safety and pharmacokinetics of imatinib in Japanese patients with GIST, although a replication study is necessary for validation.

Keywords: adverse drug reaction, gastrointestinal stromal tumors, genetic polymorphism

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Yokota S, Sekine N*1, Wakayama T*2, Oshio S*1: Impact of chronic vitamin A excess on sperm morphogenesis in mice.

*Andrology*. 2021;9(5):1579-1592. doi: 10.1111/andr.13013

The increasing availability of fortified foods and supplements has caused an overconsumption of vitamin A (VA), above the recommended level. To date, the effects of chronic VA excess (VAE) on spermatogenesis remain unclear. This study aims to investigate the long-term excessive intake of VA effects on spermatogenesis in mice. Dams were initially fed a control diet (4 IU/g) or a VAE diet (250 IU/g), 4 weeks prior to mating and during pregnancy. Dams and their male pups continued this diet regimen until the offspring reached 12 weeks of age. At 12 weeks of age, epididymis caudal spermatozoa and testes were collected. For histological analysis, sections were stained with periodic acid-Schiff-hematoxylin, and quantitative PCR was used to detect changes in gene expression in the testes of the VAE mice. Sperm motility and morphology were evaluated to detect the endpoint of VAE toxicity. Body weights were not significantly different between the control and VAE groups. Testicular cross-sections from the control and VAE mice contained a normal array of germ cells, and

the daily sperm production was similar between the two groups. However, the percentage of seminiferous tubules in stages VII and VIII was significantly lower in the VAE mice than in the control. In addition, significant changes in the expression of genes involved in retinoid metabolism, spermatogenesis, and spermiogenesis were detected in the testes of the VAE mice. Consistently, sperm motility and head morphology were significantly impaired in the VAE mice. Our findings suggest that long-term dietary intake of VAE was able to influence both pre- and post-meiotic spermatogenesis. As a result of testicular toxicity, we demonstrated, to the best of our knowledge, for the first time that long-term VAE caused sperm-head abnormalities.

Keywords: spermatogenesis, toxicology, vitamin A

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Sasaki T[*], Saito H, Hiradate Y[*], Hara K[*], Tanemura K[*]: Behavioural effects in mice orally exposed to domoic acid or ibotenic acid are influenced by developmental stages and sex differences.
*Biochem Biophys Res Commun*. 2021;558:175-182. doi: 10.1016/j.bbrc.2021.04.080

The structure of the brain is dramatically altered during the critical period. Physiological substances (neurotransmitters, hormones, etc.) in the body fluctuate significantly before and after sexual maturation. Therefore, the effect of chemical exposure on the central nervous system often differs depending on the developmental stage and sex. We aimed to compare the behavioural effects that emerged from the administration of chemicals to mice of different life stages (immature or mature) and different sex (male or female). We administered mice with domoic acid (DA), a marine poison, and ibotenic acid (IA), found in poisonous mushrooms. These excitatory amino acids act as agonists for glutamate and are potent neurotoxins. Interestingly, the behavioural effects of these chemicals were completely different. Following DA administration, we observed memory deficits only in groups of male mice treated at maturity. Following IA administration, we observed deviations in emotional behaviour in groups of male mice treated at both immaturity and maturity. In contrast, few characteristic changes were detected in all groups of females. Our results support the theory that the behavioural effects of chemical administration vary considerably with developmental stages and sex. In conclusion, our findings promote better understanding of individual differences in excitatory chemical-induced neurotoxicity and provide evidence for future risk strategies and treatments.

Keywords: behavioural analysis, early exposure and delayed effect, sex differences

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Nagaishi T[*1,2], Watabe T[*3], Kotake K[*4,5], Kumazawa T[*1,4,5], Aida T[*6], Tanaka K[*6], Ono R, Ishino F[*7], Usami T[*8], Miura T[*9], Hirakata S[*9], Kawasaki H[*9], Tsugawa N[*3], Yamada D[*3], Hirayama K[*10], Yoshikawa S[*11,12], Karasuyama H[*11,13], Okamoto R[*4], Watanabe M[*3,13], Blumberg RS[*14], Adachi T[*15,16]: Immunoglobulin A-specific deficiency induces spontaneous inflammation specifically in the ileum.
*Gut*. 2021;71(3):487-496. doi: 10.1136/gutjnl-2020-322873

Objective: Although immunoglobulin A (IgA) is abundantly expressed in the gut and known to be an important component of mucosal barriers against luminal pathogens, its precise function remains unclear. Therefore, we tried to elucidate the effect of IgA on gut homeostasis maintenance and its mechanism.

Design: We generated various IgA mutant mouse lines using the CRISPR/Cas9 genome editing system. Then, we evaluated the effect on the small intestinal homeostasis, pathology, intestinal microbiota, cytokine production, and immune cell activation using intravital imaging.

Results: We obtained two lines, with one that contained a <50 base pair deletion in the cytoplasmic region of the IgA allele (IgA tail-mutant; IgAtm/tm) and the other that lacked the most constant region of the IgH $\alpha$ chain, which resulted in the deficiency of IgA production (IgA-/-). IgA-/- exhibited spontaneous inflammation in the ileum but not the other parts of the gastrointestinal tract. Associated with this,

there were significantly increased lamina propria CD4+ T cells, elevated productions of IFN-γ and IL-17, increased ileal segmented filamentous bacteria and skewed intestinal microflora composition. Intravital imaging using Ca2+ biosensor showed that IgA-/- had elevated Ca2+ signalling in Peyer's patch B cells. On the other hand, IgAtm/tm seemed to be normal, suggesting that the IgA cytoplasmic tail is dispensable for the prevention of the intestinal disorder.

Conclusion: IgA plays an important role in the mucosal homeostasis associated with the regulation of intestinal microbiota and protection against mucosal inflammation especially in the ileum.

Keywords: IgA, ileitis, inflammation

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Sekine N*, Yokota S, Oshio S*: Sperm morphology is different in two common mouse strains.
*BPB Reports*. 2021;4:162-165. doi: 10.1248/bpbreports.4.5_162

ICR and C57BL/6J mice have been widely used in several research fields. The reproductive toxicology parameters, such as fertilization rate, which may differ between the two strains, are well known. However, the details of the sperm quality parameters are not well known. To reveal these, we compared the sperm morphology of the two strains. Eosin-stained sperm smears from adult ICR and C57BL/6J mice were analyzed. We observed that 79.6 ± 1.2 and 49.5 ± 1.7% of ICR and C57BL/6J mice sperm, respectively, showed a normal form. Furthermore, abnormal sperm samples were classified into ten types based on their defective sites. The percentage of abnormal sperm with an amorphous head, bent head, no head, hairpin loop, short tail, and two tails in ICR mice was significantly lower than that in C57BL/6J mice. In contrast, the percentage of coil-tailed sperm in ICR mice was significantly higher than that in C57BL/6J mice. These results suggest that C57BL/6J mice have a limited ability to remove the cytoplasm during spermiation and ICR mice have fewer sperm abnormalities than C57BL/6J mice. The characteristics of male reproductive traits among mouse strains should be taken into consideration in sperm analysis, as the negligence of this could generate an increased potential for a misleading in toxicology evaluation.

Keywords: sperm morphology, mouse strain differences

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Shiura H*1,2, Ono R, Tachibana S*2, Kohda T*1,2, Kaneko-Ishino T*3, Ishino F*2: PEG10 viral aspartic protease domain is essential for the maintenance of fetal capillary structure in the mouse placenta.
*Development*. 2021;148(19):dev199564. doi: 10.1242/dev.199564

The therian-specific gene paternally expressed 10 (Peg10) plays an essential role in placenta formation: Peg10 knockout mice exhibit early embryonic lethality as a result of severe placental defects. The PEG10 protein exhibits homology with long terminal repeat (LTR) retrotransposon GAG and POL proteins; therefore, we generated mice harboring a mutation in the highly conserved viral aspartic protease motif in the POL-like region of PEG10 because this motif is essential for the life cycle of LTR retrotransposons/retroviruses. Intriguingly, frequent perinatal lethality, not early embryonic lethality, was observed with fetal and placental growth retardation starting mid-gestation. In the mutant placentas, severe defects were observed in the fetal vasculature, where PEG10 is expressed in the three trophoblast cell layers that surround fetal capillary endothelial cells. Thus, Peg10 has essential roles, not only in early placenta formation, but also in placental vasculature maintenance from mid- to late-gestation. This implies that along the feto-maternal placenta interface an interaction occurs between two retrovirus-derived genes, Peg10 and retrotransposon Gag like 1 (Rtl1, also called Peg11), that is essential for the maintenance of fetal capillary endothelial cells.

Keywords: Peg10, Rtl1, eutherian placenta evolution

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Okubo Y, Ohtake F*1, Igarashi K*2, Yasuhiko Y, Hirabayashi Y, Saga Y*3, Kanno J: Cleaved Delta like 1 intracellular domain regulates neural development via Notch signal dependent and independent pathways.
*Development*. 2021;148(19):dev193664. doi: 10.1242/dev.193664

Notch-Delta signaling regulates many developmental processes, including tissue homeostasis and maintenance of stem cells. Upon interaction of juxtaposed cells via Notch and Delta proteins, intracellular domains of both transmembrane proteins are cleaved and translocate to the nucleus. Notch intracellular domain activates target gene expression; however, the role of the Delta intracellular domain remains elusive. Here, we show the biological function of Delta like 1 intracellular domain (D1ICD) by modulating its production. We find that the sustained production of D1ICD abrogates cell proliferation but enhances neurogenesis in the developing dorsal root ganglia (DRG), whereas inhibition of D1ICD production promotes cell proliferation and gliogenesis. D1ICD acts as an integral component of lateral inhibition mechanism by inhibiting Notch activity. In addition, D1ICD promotes neurogenesis in a Notch signaling-independent manner. We show that D1ICD binds to Erk1/2 in neural crest stem cells and inhibits the phosphorylation of Erk1/2. In summary, our results indicate that D1ICD regulates DRG development by modulating not only Notch signaling but also the MAP kinase pathway.

Keywords: Notch-Delta signaling, DRG development, lateral inhibition

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Taquahashi Y, Saito H, Kuwagata M, Kitajima S: Development of an inhalation exposure system of a pressurized metered-dose inhaler (pMDI) formulation for small experimental animals. *Fundam Toxicol Sci*. 2021;8:169-175. doi: 10.2131/fts.8.169

Inhalation exposure systems for small experimental animals are necessary evaluation tools of efficacy, pharmacokinetics, and safety when developing inhaled drugs. However, the development of inhalants is characterized by high technical barriers and costs. This project aimed to develop an aerosol generator specialized for a pressurized metered-dose inhaler (pMDI) formulation of ciclesonide (CIC), a prodrug-type corticosteroid for asthma. Our results showed that the developed aerosol generator achieved approximately 160 mg/m$^3$ in mass concentration, by using 60 bottles of the pMDI within a one-hour

inhalation exposure study. The CIC used in the study was 672 mg in total. The mass median aerodynamic diameter (MMAD) was approximately 1 μm, with less than 2 in geometric standard deviation. Although the amount of test article used was less than 1 g, the aerosol generator achieved approximately 160 mg/$m^3$ in mass concentration, and enough of the CIC was delivered to the rat lungs to allow the visualization of its spatial localization by desorption electrospray ionization-time-of-flight mass spectrometry imaging. We concluded that (i) the aerosol generator was able to drive pMDI accurately, and (ii) the CIC aerosol was delivered to the rodent under appropriate MMAD and concentration; the device's performance as an excellent nonclinical inhalation exposure system was thus demonstrated. Furthermore, as the device is highly versatile, it would be possible to utilize it when conducting nonclinical inhalation studies at the optimal conditions for various pMDIs. In the future, aerosol generators could reduce costs and shorten the development period of inhaled drugs.

Keywords: inhalation exposure system, small experimental animals, ciclesonide

Igarashi T, Yasuhiko Y, Ono R, Tachihara E, Uchiyama M, Takagi A, Takahashi Y, Kuwagata M, Kitajima S: Diverse unintended on-target mutations induced by zygote genome-editing using CRISPR/Cas9 system.

*Fundam Toxicol Sci*. 2021;8(5):161-167. doi: 10.2131/fts.8.161

With the advent of the CRISPR/Cas9 system, genome editing in various fields is advancing. Unintended mutation in off-target regions is a major problem of genome editing using the CRISPR/Cas9 system, and it is being reviewed. However, we found a high frequency and various unintended mutations in the "on-target" region when we generated a "knock-in" mouse with point mutation using this technique to develop a supernumerary rib model. Additionally, an inserted sequence of unknown origin was observed. Furthermore, these mutations were transferred to the next generation, even if tandem knock-in or large deletions occurred. These strongly suggest that a proper selection that meets the purpose is essential when considering the safety of foods and medicines using the genome-editing technology.

Keywords: CRISPR/Cas9 system, genome editing, zygote electroporation

Harada T*, Tsuboi I*, Hino H*, Yuda M*, Hirabayashi Y, Hirai S*, Aizawa S*: Age-related exacerbation of hematopoietic organ damage induced by systemic hyper-inflammation in senescence-accelerated mice.

*Sci Rep*. 2021;11(1):23250. doi: 10.1038/s41598-021-02621-4

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening systemic hyper-inflammatory disorder. The mortality of HLH is higher in the elderly than in young adults. Senescence-accelerated mice (SAMP1/TA-1) exhibit characteristic accelerated aging after 30 weeks of age, and HLH-like features, including hematopoietic organ damage, are seen after lipopolysaccharide (LPS) treatment. Thus, SAMP1/TA-1 is a useful model of hematological pathophysiology in the elderly with HLH. In this study, dosing of SAMP1/TA-1 mice with LPS revealed that the suppression of myelopoiesis and B-lymphopoiesis was more severe in aged mice than in young mice. The bone marrow (BM) expression of genes encoding positive regulators of myelopoiesis (G-CSF, GM-CSF, and IL-6) and of those encoding negative regulators of B cell lymphopoiesis (TNF-alpha) increased in both groups, while the expression of genes encoding positive-regulators of B cell lymphopoiesis (IL-7, SDF-1, and SCF) decreased. The expression of the GM-CSF-encoding transcript was lower in aged mice than in young animals. The production of GM-CSF by cultured stromal cells after LPS treatment was also lower in aged mice than in young mice. The accumulation of the TNF-alpha-encoding transcript and the depletion of the IL-7-encoding transcript were prolonged in aged mice compared to young animals. LPS dosing led to a prolonged increase in the proportion of BM M1 macrophages in aged mice compared to young animals. The expression of the gene encoding p16 (INK4a) and the proportion of beta-galactosidase- and phosphorylated ribosomal protein S6-positive cells were increased in cultured stromal cells from aged mice compared to those from young animals, while the proportion of Ki67-positive cells was decreased in stromal cells from aged mice. Thus, age-related deterioration of stromal cells probably causes the suppression of hematopoiesis in aged mice. This age-

related latent organ dysfunction may be exacerbated in elderly people with HLH, resulting in poor prognosis.
Keywords: hemophagocytic, gene expression regulation, lipopolysaccharides

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Kuwagata M, Hasegawa T*1, Takashima H*1, Shimizu M*2, Kitajima S, Yamazaki H*2: Pharmacokinetics of primary metabolites 5-hydroxythalidomide and 5'-hydroxythalidomide formed after oral administration of thalidomide in the rabbit, a thalidomide-sensitive species.

*J Toxicol Sci*. 2021;46(12):553-560. doi: 10.2131/jts.46.553

The teratogenicity of the chemotherapeutic drug thalidomide is species-specific and affects humans, non-human primates, and rabbits. The primary oxidation of thalidomide in previously investigated rodents predominantly resulted in the formation of deactivated 5'-hydroxythalidomide. In the current study, similar *in vivo* biotransformations to 5-hydroxythalidomide and 5'-hydroxythalidomide were confirmed by the analysis of blood plasma from male rabbits, a thalidomide-sensitive species, after oral administration of thalidomide (2.0 mg/kg). Similar levels of thalidomide in seminal plasma and in blood plasma were detected using liquid chromatography-tandem mass spectrometry at 4 hr and 7 hr after oral doses in male rabbits. Seminal plasma concentrations of 5-hydroxythalidomide and 5'-hydroxythalidomide were also seen in male rabbits in a roughly similar time-dependent manner to those in the blood plasma after oral doses of thalidomide (2.0 mg/kg). Furthermore, the values generated by a simplified physiologically based pharmacokinetic rabbit model were in agreement with the measured *in vivo* blood plasma data under metabolic ratios of 0.01 for the hepatic intrinsic clearance of thalidomide to both unconjugated 5-hydroxythalidomide and 5'-hydroxythalidomide. These results suggest that metabolic activation of thalidomide may be dependent on rabbit liver enzymes just it was for cytochrome P450 enzymes in humanized-liver mice; in contrast, rodent livers predominantly mediate biotransformation of thalidomide to 5'-hydroxythalidomide. A developmental toxicity test system with experimental animals that involves intravaginal exposures to the chemotherapeutic drug thalidomide via semen should be considered in the future.
Keywords: 5-Hydroxythalidomide, PBPK modeling, seminal plasma

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Taquahashi Y, Tsuruoka S*, Morita K, Tsuji M, Suga K, Aisaki KI, Kitajima S: A novel high-purity carbon-nanotube yarn electrode used to obtain biopotential measurements in small animals: flexible, wearable, less invasive, and gel-free operation.

*Fundam Toxicol Sci*. 2022;9:17-21. doi: 10.2131/fts.9.17

Carbon-nanotube yarn (CNT-Y) made from high-purity, highly crystalized, double-walled carbon nanotubes is an advanced material with excellent electrical conductivity and flexibility; hence, it could potentially be used as a novel electrode for biopotential measurements. To our knowledge, the present study is the first in which CNT-Y electrodes were used to conduct electrocardiography (ECG) and electroencephalography (EEG) on experimental animals. All procedures and biopotential measurements were performed under isoflurane anesthesia. The CNT-Y electrodes were attached to the animals by creating a single interrupting suture on the skin. The lead II electrode configuration was used for ECG recording, i.e., the positive, negative, and body-earth electrodes were placed on the left apex of the auricular surface, the interscapular region, and the cervical region, respectively. The bipolar lead was used for EEG recording, with the exploring and reference electrodes on the bregma and base of the right auricular surface, respectively. Using CNT-Y electrodes, we obtained a clear ECG waveform from rats and a guinea pig; the QRS amplitude was ~1.4 mV. In rats, we obtained an EEG waveform with an amplitude of ~150 μV; the peak frequency was 0.8 Hz and the range was ~3 Hz according to power spectral density analysis. In the guinea pig, we obtained an EEG waveform with an amplitude of ~500 μV; the first peak was 0.1 Hz, the second peak was 1 Hz, and the range was ~3 Hz. These results show that CNT-Y could be used in toxicology studies to easily and inexpensively obtain high-resolution biological signals.

Keywords: biopotential measurements, carbon-nanotube, vital signs

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Kanno S*, Okubo Y, Kageyama T*, Yan L*, Kitajima S, Fukuda J*: Establishment of a Developmental Toxicity Assay based on Human iPSC Reporter to Detect Fibroblast Growth Factor Signal Disruption. *iScience*. 2022;25(2):103770. doi: 10.1016/j.isci.2022.103770

The number of man-made chemicals has increased exponentially recently, and exposure to some of them can induce fetal malformations. Because complex and precisely programmed signaling pathways play important roles in developmental processes, their disruption by external chemicals often triggers developmental toxicity. However, highly accurate and high-throughput screening assays for potential developmental toxicants are currently lacking. In this study, we propose a reporter assay that utilizes human-induced pluripotent stem cells (iPSCs) to detect changes in fibroblast growth factor signaling, which is essential for limb morphogenesis. The dynamics of this signaling after exposure to a chemical were integrated to estimate the degree of signaling disruption, which afforded a good prediction of the capacity of chemicals listed in the ECVAM International Validation Study that induce limb malformations. This study presents an initial report of a human iPSC-based signaling disruption assay, which could be useful for the screening of potential developmental toxicants.

Keywords: developmental toxicants, FGF-SRF signaling, *in vitro* screening

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Kanno S*, Okubo Y, Kageyama T*, Yan L*, Fukuda J*: Integrated fibroblast growth factor signal disruptions in human iPS cells for prediction of teratogenic toxicity of chemicals. *J Biosci Bioeng*. 2022;133(3):291-299. doi: 10.1016/j.jbiosc.2021.12.006

The number of man-made chemicals has increased rapidly in recent decades, with certain chemicals potentially causing malformations in fetuses. Although the toxicities of chemicals have been tested in animals, chemicals that are not teratogenic in rodents can cause severe malformations in humans, owing to the differences in the susceptibility to the teratogenicity of chemicals among species. One possible cause of such species differences, other than pharmacokinetics, could be the difference in sensitivity to such chemicals at the cellular level. Therefore, a human cell-based high-throughput assay system is needed for detecting potential teratogenic chemicals. In this study, we proposed a signal reporter assay using human induced pluripotent stem cells (iPSCs). Because developmental processes are governed by highly intricate and precisely programmed signaling pathways, external chemical-induced disruption of these pathways often triggers developmental toxicities. The reporter assay using hiPSCs was used to detect changes in the fibroblast growth factor (FGF) signaling pathway, a pathway essential for limb morphogenesis. The method was based on monitoring and time-accumulation of the signal disruption over time, rather than the classical endpoint detection of the signal disruption. This approach was useful for detecting signal disruptions caused by the malformation chemicals listed in the ICH S5 guideline, including thalidomide. The human iPSC-based signal disruption assay could be a promising tool for the initial screening of developmental toxicants.

Keywords: teratogenic toxicity, FGF-SRF signaling, *in vitro* screening

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Hirata N, Yamada S, Yanagida S, Ono A*, Yasuhiko Y, Nishida M, Kanda Y: Lysophosphatidic Acid Promotes the Expansion of Cancer Stem Cells via TRPC3 Channels in Triple-Negative Breast Cancer. *Int J Mol Sci* 2022;23(4):1967 DOI: 10.3390/ijms23041967.

Triple-negative breast cancer (TNBC) is a highly aggressive cancer for which targeted therapeutic agents are limited. Growing evidence suggests that TNBC originates from breast cancer stem cells (BCSCs), and elucidation of the molecular mechanisms controlling BCSC proliferation will be crucial for new drug development. We have previously reported

that the lysosphingolipid sphingosine-1-phosphate mediates the CSC phenotype, which can be identified as the ALDH-positive cell population in several types of human cancer cell lines. In this study, we have investigated additional lipid receptors upregulated in BCSCs. We found that lysophosphatidic acid (LPA) receptor 3 was highly expressed in ALDH-positive TNBC cells. The LPAR3 antagonist inhibited the increase in ALDH-positive cells after LPA treatment. Mechanistically, the LPA-induced increase in ALDH-positive cells was dependent on intracellular calcium ion (Ca2+), and the increase in Ca2+ was suppressed by a selective inhibitor of transient receptor potential cation channel subfamily C member 3 (TRPC3). Moreover, IL-8 production was involved in the LPA response via the activation of the Ca2+-dependent transcriptional factor nuclear factor of activated T cells. Taken together, our findings provide new insights into the lipid-mediated regulation of BCSCs via the LPA-TRPC3 signaling axis and suggest several potential therapeutic targets for TNBC.

Keywords: cancer stem cells, lysophosphatidic acid, nuclear factor of activated T cells, transient receptor potential cation channel subfamily C member 3, triple-negative breast cancer

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Piantino M[*1], Louis F[*2], Shigemoto-Mogami Y, Kitamura K, Sato K, Yamaguchi T[*3], Kawabata K[*3], Yamamoto S[*4], Iwasaki S[*4], Hirabayashi H[*4], Matsusaki M[*1,2]: Brain microvascular endothelial cells derived from human induced pluripotent stem cells as *in vitro* model for assessing blood-brain barrier transferrin receptor-mediated transcytosis.

*Mater Today Bio*. 2022 Mar 10;14:100232. doi: 10.1016/j.mtbio.2022.100232. eCollection 2022 Mar
PMID: 35308041

The blood-brain barrier (BBB), a selective barrier formed by brain microvascular endothelial cells (BMEC), represents a major challenge for the efficient accumulation of pharmaceutical drugs into the brain. The receptor-mediated transcytosis (RMT) has recently gained increasing interest for pharmaceutical industry as it shows a great potential to shuttle large-sized therapeutic cargos across the BBB. Confirming the presence of the RMT pathway by BMEC is therefore important for the screening of peptides or antibody libraries that bind RMT receptors. Herein, a comparative study was performed between a human cell line of BMEC (HBEC) and human induced pluripotent stem cells-derived BMEC-like cells (hiPS-BMEC). The significantly higher gene and protein expressions of transporters and tight junction proteins, excepting CD31 and VE-cadherin were exhibited by hiPS-BMEC than by HBEC, suggesting more biomimetic BBB features of hiPS-BMEC. The presence and functionality of transferrin receptor (TfR), known to use RMT pathway, were confirmed using hiPS-BMEC by competitive binding assays and confocal microscopy observations. Finally, cysteine-modified T7 and cysteine modified-Tfr-T12 peptides, previously reported to be ligands of TfR, were compared regarding their permeability using hiPS-BMEC. The hiPS-BMEC could be useful for the identification of therapeutics that can be transported across the BBB using RMT pathway.

Keywords: blood-brain barrier, *in vitro* model, receptor-mediated transcytosis

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Yoshihara A[*1], Kawasaki H[*1], Masuno H[*2], Takada K[*2], Numoto N[*2], Ito N[*2], Hirata N, Kanda Y, Ishizawa M[*3], Makishima M[*3], Kagechika H[*2], Tanatani A[*1]: Lithocholic Acid Amides as Potent Vitamin D Receptor Agonists.

*Biomolecules*. 2022;12(1):130. DOI: 10.3390/biom12010130.

1α,25-Dihydroxyvitamin D3 [1α,25(OH)2D3, 1] is an active form of vitamin D3 and regulates various biological phenomena, including calcium and phosphate homeostasis, bone metabolism, and immune response via binding to and activation of vitamin D receptor (VDR). Lithocholic acid (LCA, 2) was identified as a second endogenous agonist of VDR, though its potency is very low. However, the lithocholic acid derivative 3 (Dcha-20) is a more potent agonist than 1α,25(OH)2D3, (1), and its carboxyl group has similar interactions to the 1,3-dihydroxyl groups of 1 with amino acid residues in the VDR ligand-binding pocket.

Here, we designed and synthesized amide derivatives of 3 in order to clarify the role of the carboxyl group. The synthesized amide derivatives showed HL-60 cell differentiation-inducing activity with potency that depended upon the substituent on the amide nitrogen atom. Among them, the N-cyanoamide 6 is more active than either 1 or 3.

Keywords: amide, cell differentiation, lithocholic acid, nuclear receptor, vitamin D

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Tsuji K, Yamada S, Hirai K, Asakura H, Kanda Y: Development of alveolar and airway cells from human iPS cells: toward SARS-CoV-2 research and drug toxicity testing.
*J Toxicol Sci*. 2021;46(9):425-435. DOI: 10.2131/jts.46.425

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19). SARS-CoV-2 enters host cells by binding with the receptor angiotensin-converting enzyme 2 (ACE2). While ACE2 is expressed in multiple cell types, it has been implicated in the clinical progression of COVID-19 as an entry point for SARS-CoV-2 into respiratory cells. Human respiratory cells, such as airway and alveolar epithelial type II (ATII) cells, are considered essential for COVID-19 research; however, primary human respiratory cells are difficult to obtain. In the present study, we generated ATII and club cells from human induced pluripotent stem cells (hiPSCs) for SARS-CoV-2 infection and drug testing. The differentiated cells expressed ATII markers (SFTPB, SFTPC, ABCA3, SLC34A2) or club cell markers (SCGB1A1 and SCGB3A2). Differentiated cells, which express ACE2 and TMPRSS2, were infected with SARS-CoV-2. Remdesivir treatment decreased intracellular SARS-CoV-2 viral replication and, furthermore, treatment with bleomycin showed cytotoxicity in a concentration-dependent manner. These data suggest that hiPSC-derived AT2 and club cells provide a useful *in vitro* model for drug development.

Keywords: alveolar epithelial cell, bleomycin, club cell, human iPS cell, SARS-CoV-2

Nishimura Y*1, Kanda Y, Sone H*2, Aoyama H*3: Oxidative Stress as a Common Key Event in Developmental Neurotoxicity.
*Oxid Med Cell Longev*. 2021;6685204. DOI: 10.1155/2021/6685204

The developing brain is extremely sensitive to many chemicals. Perinatal exposure to neurotoxicants has been implicated in several neurodevelopmental disorders, including autism spectrum disorder, attention-deficit hyperactive disorder, and schizophrenia. Studies of the molecular and cellular events related to developmental neurotoxicity have identified a number of "adverse outcome pathways," many of which share oxidative stress as a key event. Oxidative stress occurs when the balance between the production of free oxygen radicals and the activity of the cellular antioxidant system is dysregulated. In this review, we describe some of the developmental neurotoxins that target the antioxidant system and the mechanisms by which they elicit stress, including oxidative phosphorylation in mitochondria and plasma membrane redox system in rodent models. We also discuss future directions for identifying adverse outcome pathways related to oxidative stress and developmental neurotoxicity, with the goal of improving our ability to quickly and accurately screen chemicals for their potential developmental neurotoxicity.

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Yanagida S, Satsuka A, Hayashi S, Ono A*, Kanda Y: Chronic cardiotoxicity assessment of BMS-986094, a guanosine nucleotide analogue, using human iPS cell-derived cardiomyocytes.
*J Toxicol Sci*. 2021;46(8):359-369. DOI: 10.2131/jts.46.359

Predicting drug-induced side effects in the cardiovascular system is very important because it can lead to the discontinuation of new drugs/candidates or the withdrawal of marketed drugs. Although chronic assessment of cardiac contractility is an important issue in safety pharmacology, an *in vitro* evaluation system has not been fully developed. We previously developed an imaging-based contractility

assay system to detect acute cardiotoxicity using human iPS cell-derived cardiomyocytes (hiPSC-CMs). To extend the system to chronic toxicity assessment, we examined the effects of the anti-hepatitis C virus (HCV) drug candidate BMS-986094, a guanosine nucleotide analogue, which was withdrawn from phase 2 clinical trials because of unexpected contractility toxicities. Additionally, we examined sofosbuvir, another nucleotide analogue inhibitor of HCV that has been approved as an anti-HCV drug. Motion imaging analysis revealed the difference in cardiotoxicity between the cardiotoxic BMS-986094 and the less toxic sofosbuvir in hiPSC-CMs, with a minimum of 4 days of treatment. In addition, we found that BMS-986094-induced contractility impairment was mediated by a decrease in calcium transient. These data suggest that chronic treatment improves the predictive power for the cardiotoxicity of anti-HCV drugs. Thus, hiPSC-CMs can be a useful tool to assess drug-induced chronic cardiotoxicity in non-clinical settings.

Keywords: cardiomyocyte, chronic cardiotoxicity, contractility, nucleotide analogue, iPS cell

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Kanda Y, Satsuka A, Hayashi S, Hagiwara-Nagasawa M, Sugiyama A*: Assessment of contractility in human iPS cell-derived cardiomyocytes using motion vector analysis.

*Methods in Molecular Biology*. 2021;2320:151-160. DOI: 10.1007/978-1-0716-1484-6_15

Human-induced pluripotent stem cell (iPSC) technology paves the way for next-generation drug-safety assessment. In particular, human iPSC-derived cardiomyocytes, which exhibit electrical activity, are useful as a human cell model for assessing QT-interval prolongation and the risk of the lethal arrhythmia Torsade de Pointes (TdP). In addition to proarrhythmia assay, contractile behavior has received increased attention in drug development. In this study, we developed a novel high-throughput *in vitro* assay system using motion vectors to evaluate the contractile activity of iPSC-derived cardiomyocytes as a physiologically relevant human platform. The methods presented here highlight the use of commercially available iPSC-derived cardiomyocytes, iCell cardiomyocytes, for contractility evaluation recorded by the motion vector system.

Keywords: cardiomyocyte, contractility, human iPS cell, motion vector, multielectrode array

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Kato-Hayashi M*[1,2], Sato K, Sekino Y*[3]: Neurons induce tiled astrocytes with branches that avoid each other.

*Int J Mol Sci*. 2022 23(8):4161. doi: 10.3390/ijms23084161

Neurons induce astrocyte branches that approach synapses. Each astrocyte tiles by expanding branches in an exclusive territory, with limited entries for the neighboring astrocyte branches. However, how astrocytes form exclusive territories is not known. For example, the extensive branching of astrocytes may sterically interfere with the penetration of other astrocyte branches. Alternatively, astrocyte branches may actively avoid each other or remove overlapped branches to establish a territory. Here, we show time-lapse imaging of the multi-order branching process of GFP-labeled astrocytes. Astrocyte branches grow in the direction where other astrocyte branches do not exist. Neurons that had just started to grow dendrites were able to induce astrocyte branching and tiling. Upon neuronal loss by glutamate excitotoxicity, astrocytes' terminal processes retracted and more branches went over other branches. Our results indicate that neurons induce astrocyte branches and make them avoid each other.

Keywords: astrocyte, glutamate transporter, tiling

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Hirata N, Yamada S, Yanagida S, Ono A*, Kanda Y: FTY720 Inhibits Expansion of Breast Cancer Stem Cells via PP2A Activation.

*Int J Mol Sci*. 2021;22(14):7259. DOI: 10.3390/ijms22147259

Growing evidence suggests that breast cancer originates from a minor population of cancer cells termed cancer stem cells (CSCs), which can be identified by aldehyde dehydrogenase (ALDH) activity-based flow cytometry analysis. However, novel

therapeutic drugs for the eradication of CSCs have not been discovered yet. Recently, drug repositioning, which finds new medical uses from existing drugs, has been expected to facilitate drug discovery. We have previously reported that sphingosine kinase 1 (SphK1) induced proliferation of breast CSCs. In the present study, we focused on the immunosuppressive agent FTY720 (also known as fingolimod or Gilenya), since FTY720 is known to be an inhibitor of SphK1. We found that FTY720 blocked both proliferation of ALDH-positive cells and formation of mammospheres. In addition, we showed that FTY720 reduced the expression of stem cell markers such as Oct3/4, Sox2 and Nanog via upregulation of protein phosphatase 2A (PP2A). These results suggest that FTY720 is an effective drug for breast CSCs *in vitro*.

Keywords: ALDH, FTY720, PP2A, SphK1, cancer stem cells, drug repositioning

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Aghasafari P[*1], Yang PC[*1], Kernik DC[*2], Sakamoto K[*3], Kanda Y, Kurokawa J[*3], Vorobyov I[*1], Clancy CE[*1]: A deep learning algorithm to translate and classify cardiac electrophysiology.

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The development of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) has been a critical *in vitro* advance in the study of patient-specific physiology, pathophysiology, and pharmacology. We designed a new deep learning multitask network approach intended to address the low throughput, high variability, and immature phenotype of the iPSC-CM platform. The rationale for combining translation and classification tasks is because the most likely application of the deep learning technology we describe here is to translate iPSC-CMs following application of a perturbation. The deep learning network was trained using simulated action potential (AP) data and applied to classify cells into the drug-free and drugged categories and to predict the impact of electrophysiological perturbation across the continuum of aging from the immature iPSC-CMs to the adult ventricular myocytes. The phase of the AP extremely sensitive to perturbation due to a steep rise of the membrane resistance was found to contain the key information required for successful network multitasking. We also demonstrated successful translation of both experimental and simulated iPSC-CM AP data validating our network by prediction of experimental drug-induced effects on adult cardiomyocyte APs by the latter.

Keywords: arrhythmias, artificial intelligence, computational biology, deep learning, human, machine learning, pharmacology, regenerative medicine, stem cells, systems biology

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Yanagida S, Satsuka A, Hayashi S, Ono A*, Kanda Y: Comprehensive Cardiotoxicity Assessment of COVID-19 Treatments Using Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes.

*Toxicol Sci*. 2021;30:183(1):227-239. DOI: 10.1093/toxsci/kfab079

Coronavirus disease 2019 (COVID-19) continues to spread across the globe, with numerous clinical trials underway seeking to develop and test effective COVID-19 therapies, including remdesivir. Several ongoing studies have reported hydroxychloroquine-induced cardiotoxicity, including development of torsade de pointes (TdP). Meanwhile, human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are expected to serve as a tool for assessing drug-induced cardiotoxicity, such as TdP and contraction impairment. However, the cardiotoxicity of COVID-19 treatments has not been fully assessed using hiPSC-CMs. In this study, we focused on drug repurposing with various modes of actions and examined the TdP risk associated with COVID-19 treatments using field potential using multi-electrode array system and motion analysis with hiPSC-CMs. Hydroxychloroquine induced early after depolarization, while remdesivir, favipiravir, camostat, and ivermectin had little effect on field potentials. We then analyzed electromechanical window, which is defined as the difference between field potential and contraction-relaxation durations. Hydroxychloroquine decreased electromechanical window of hiPSC-CMs in a concentration-dependent manner. In contrast, other drugs had little effect. Our data suggest that hydroxychloroquine has proarrhythmic risk and other

drugs have low proarrhythmic risk. Thus, hiPSC-CMs represent a useful tool for assessing the comprehensive cardiotoxicity caused by COVID-19 treatments in nonclinical settings.

Keywords: COVID-19, contractility, electromechanical window, hiPSC-CMs, proarrhythmia

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Yamada S, Kanda Y: Evaluation of Barrier Functions in Human iPSC-Derived Intestinal Epithelium.

*Methods Mol Biol*. 2021;2367:27-35. DOI: 10.1007/7651_2021_346

The small intestine plays roles in the absorption and metabolism of orally administered drugs and chemicals. Tight junctions between intestinal epithelial cells, which form a tight barrier preventing the invasion of pathogens and toxins, are essential components of the intestinal defense system. These intestinal functions have generally been evaluated using established cell lines or primary cells in two-dimensional culture. However, these culture systems have not shown the complexity of the three-dimensional structure and diversity of cell types comprising the intestinal epithelial tissue. Here, we report the generation of intestinal organoids using human induced pluripotent stem cells subjected to sequential treatment with different cytokines and compounds. We further describe the tool for evaluating intestinal barrier functions using organoids as a physiologically relevant human platform.

Keywords: barrier, human iPS cells, intestine, organoid, tight junction

Irie T: Essential Role of Somatic Kv2 Channels in High-Frequency Firing in Cartwheel Cells of the Dorsal Cochlear Nucleus.

*eNeuro* 8:1-17, 2021.

Among all voltage-gated potassium (Kv) channels, Kv2 channels are the most widely expressed in the mammalian brain. However, studying Kv2 in neurons has been challenging because of a lack of high-selective blockers. Recently, a peptide toxin, guangxitoxin-1E (GxTX), has been identified as a specific inhibitor of Kv2, thus facilitating the study of Kv2 in neurons. The mammalian dorsal cochlear nucleus (DCN) integrates auditory and somatosensory information. In the DCN, cartwheel inhibitory interneurons receive excitatory synaptic inputs from parallel fibers conveying somatosensory information. The activation of parallel fibers drives action potentials in the cartwheel cells up to 130 Hz *in vivo*, and the excitation of cartwheel cells leads to the strong inhibition of principal cells. Therefore, cartwheel cells play crucial roles in monaural sound localization and cancelling detection of self-generated sounds. However, how Kv2 controls the high-frequency firing in cartwheel cells is unknown. In this study, we performed immunofluorescence labeling with anti-Kv2.1 and anti-Kv2.2 antibodies using fixed mouse brainstem slice preparations. The results revealed that Kv2.1 and Kv2.2 were largely present on the cartwheel cell body membrane but not on the axon initial segment (AIS) nor the proximal dendrite. Whole-cell patch-clamp recordings using mouse brainstem slice preparation and GxTX demonstrated that blockade of Kv2 induced failure of parallel fiber-induced action potentials when parallel fibers were stimulated at high frequencies (30-100 Hz). Thus, somatic Kv2 in cartwheel cells regulates the action potentials in a frequency-dependent manner and may play important roles in the DCN function.

Keywords: Kv2 channels, cartwheel cells, dorsal cochlear nucleus, guangxitoxin-1E, sustained firing

Satoh A*, Fujimoto S*, Irie T, Suzuki T*, Miyazaki Y*, Tanaka K*, Usami M*, and Takizawa T*: Valproic acid promotes differentiation of adipose tissue-derived stem cells to neuronal cells selectively expressing functional N-type voltage-gated Ca(2+) channels.

*Biochem Biophys Res Commun* 589:55-62, 2021.

The differentiation of adipose tissue-derived stem cells (ASCs) to neuronal cells is greatly promoted by valproic acid (VPA), and is synergistically enhanced by the following treatment with neuronal induction medium (NIM) containing cAMP-elevating agents. In the present study, we investigated the synergism between VPA and NIM in neuronal differentiation of ASCs, assessed by the expression of neurofilament medium polypeptide (NeFM), with respect to Ca2þ entry. VPA (2 mM) treatment for 3 days followed by NIM for 2 h synergistically increased the incidence of neuronal cells differentiated from ASCs to an extent more than VPA alone treatment for 6 days,

shortening the time required for the differentiation. VPA increased intracellular Ca2+ and the mRNAs of voltage-gated Ca2+ channels, Cacna1b (Cav2.2) and Cacna1h (Cav3.2), in ASCs. Inward currents through Ca2+ channels were evoked electrophysiologically at high voltage potential in ASCs treated with VPA. NIM reduced the mRNAs of NeFM and Cacna1b in VPA promoted neuronal differentiation of ASCs. It was concluded that functional N-type voltage-gated Ca2+ channels (Cav2.2) are selectively expressed in VPA-promoted neuronal differentiation of ASCs. NIM seems to enhance the mRNA translation of molecules required for the differentiation. Neuronal cells obtained from ASCs by this protocol will be used as a cell source for regenerative therapy of neurological disorders associated with altered Cav2.2 activity.

Keywords: adipose tissue-derived stem cell, Ca2+ channel, cAMP, valproic acid, neuronal differentiation, neuronal induction

* Azabu University

Matsushita K, Takasu T, Ishii Y, Toyoda T, Yamada T, Morikawa T, Ogawa K: *In vivo* mutagenicity and tumor-promoting activity of 1,3-dichloro-2-propanol in the liver and kidneys of *gpt* delta rats.

*Arch Toxicol*. 2021;95:3117-31. doi: 10.1007/s00204-021-03120-1.

1,3-Dichloro-2-propanol (1,3-DCP), a food contaminant, exerts carcinogenic effects in multiple organs, including the liver and kidneys, in rats. However, the underlying mechanisms of 1,3-DCP-induced carcinogenesis remain unclear. Here, the *in vivo* mutagenicity and tumor-promoting activity of 1,3-DCP in the liver and kidneys were evaluated using medium-term *gpt* delta rat models previously established in our laboratory (GPG and GNP models). Six-week-old male F344 *gpt* delta rats were treated with 0 or 50 mg/kg body weight/day 1,3-DCP by gavage for 4 weeks. After 2 weeks of cessation, partial hepatectomy or unilateral nephrectomy was performed to collect samples for *in vivo* mutation assays, followed by single administration of diethylnitrosamine (DEN) for tumor initiation. One week after DEN injection, 1,3-DCP treatment was resumed, and tumor-promoting activity was evaluated in the residual liver or kidneys by histopathological analysis of preneoplastic lesions. gpt mutant frequencies increased in excised liver and kidney tissues following 1,3-DCP treatment. 1,3-DCP did not affect the development of glutathione S-transferase placental form-positive foci in residual liver tissues, but enhanced atypical tubule hyperplasia in residual kidney tissues. Detailed histopathological analyses revealed glomerular injury and increased cell proliferation of renal tubular cells in residual kidney tissues of rats treated with 1,3-DCP. These results suggested possible involvement of genotoxic mechanisms in 1,3-DCP-induced carcinogenesis in the liver and kidneys. Additionally, we found that 1,3-DCP exhibited limited tumor-promoting activity in the liver, but enhanced clonal expansion in renal carcinogenesis via proliferation of renal tubular cells following glomerular injury.

Keywords: 1,3-dichloro-2-propanol, *gpt* delta rat, *in vivo* mutagenicity

Nakamura K, Ishii Y, Takasu S, Nohmi T, Shibutani M*, Ogawa K: Chromosome aberrations induced by the non-mutagenic carcinogen acetamide involve in rat hepatocarcinogenesis through micronucleus formation in hepatocytes.

*Arch Toxicol*. 2021;95:2851-65. doi: 10.1007/s00204-021-03099-9.

Chromosome aberrations (CAs), i.e. changes in chromosome number or structure, are known to cause chromosome rearrangements and subsequently tumorigenesis. However, the involvement of CAs in chemical-induced carcinogenesis is unclear. In the current study, we aimed to clarify the possible involvement of CAs in chemical carcinogenesis using a rat model with the non-mutagenic hepatocarcinogen acetamide. In an *in vivo* micronucleus (MN) test, acetamide was revealed to induce CAs specifically in rat liver at carcinogenic doses. Acetamide also induced centromere-positive large MN (LMN) in hepatocytes. Immunohistochemical and electron microscopic analyses of the LMN, which can be histopathologically detected as basophilic cytoplasmic inclusion, revealed abnormal expression of nuclear envelope proteins, increased heterochromatinization, and massive DNA damage. These molecular pathological features in LMN progressed with acetamide exposure in a time-dependent manner, implying that LMN formation can lead to chromosome rearrangements. Overall, these

data suggested that CAs induced by acetamide play a pivotal role in acetamide-induced hepatocarcinogenesis in rats and that CAs can cause chemical carcinogenesis in animals via MN formation.

Keywords: acetamide, hepatocarcinogenesis, micronucleus test

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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, madder color, rubiadin

Matsushita K, Toyoda T, Yamada T, Morikawa T, Ogawa K: Specific expression of survivin, SOX9, and CD44 in renal tubules in adaptive and maladaptive repair processes after acute kidney injury in rats.

*J Appl Toxicol*. 2021;41:607-17. doi: 10.1002/jat.4069.

Acute kidney injury (AKI) is thought to be a reversible condition; however, growing evidence has suggested that AKI may be associated with subsequent development of chronic kidney disease. Although renal tubules have intrinsic regeneration capacity, disruption of the regeneration mechanisms leads to irreversible interstitial fibrosis. In this study, we investigated immunohistochemical markers of renal tubules in adaptive and maladaptive repair processes to predict AKI reversibility. Histopathological analysis demonstrated that regenerative tubules and dilated tubules were observed in the kidneys of AKI model rats after ischemia/reperfusion (I/R). Regenerative tubules gradually redifferentiated after I/R, whereas dilated tubules exhibited no tendency for redifferentiation. In fibrotic areas of the kidney in renal fibrosis model rats subjected to I/R, renal tubules were dilated or atrophied. There results suggested that the histopathological features of renal tubules in the maladaptive repair were dilation or atrophy. From microarray data of regenerative tubules, survivin, SOX9, and CD44 were extracted as candidate markers. Immunohistochemical analysis demonstrated that survivin and SOX9 were expressed in regenerative tubules, whereas SOX9 was also detected in renal tubules in fibrotic areas. These findings indicated that survivin and SOX9 contributed to renal tubular regeneration, whereas sustained SOX9 expression may be associated to fibrosis. CD44 was expressed in dilated tubules in the kidneys of AKI model rats and in the tubules of fibrotic areas of renal fibrosis model rats, suggesting that CD44 was expressed in renal tubules in maladaptive repair. Thus, these factors could be useful markers for detecting disruption of the regenerative mechanisms of renal tubules.

Keywords: acute kidney injury, SOX9, CD44

Toyoda T, Matsushita K, Akane H, Morikawa T, Ogawa K: A 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol in F344 rats.

*J Toxicol Pathol*. 2021;34:309-17. doi.org/10.1293/tox.2020-0091.

2-(*l*-Menthoxy)ethanol has been used as a flavoring agent. Despite its frequent use, there are limited toxicity data for 2-(*l*-menthoxy)ethanol. We performed a 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol in male and female F344 rats. Doses of 0, 15, 60, or 250 mg/kg body weight (BW)/day of 2-(*l*-menthoxy)ethanol were given orally by gavage using corn oil as the vehicle. No significant toxicological changes in general condition, body weight, or food intake were observed in any groups. Hematological assessment showed decreases in hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin and increases in platelet count in the male 250 mg/kg group. Serum biochemistry showed increases in total cholesterol in the 250 mg/kg group for both sexes, decreases in triglyceride in the female 250 mg/kg group, and increases in total protein in the male 250 mg/kg group, suggesting effects on lipid metabolism and protein synthesis. For organ weights, increases in absolute and relative weights of the liver and adrenal glands were observed in the 250 mg/kg group of both sexes and the male 250 mg/kg group, respectively. Histopathological analysis showed chronic nephropathy was observed in the male 15 mg/kg or higher groups, and related changes including increases in absolute and relative kidney weight and serum creatinine in the male 60 and 250 mg/kg groups. However, eosinophilic granules containing $\alpha_{2u}$-globulin were identified in proximal tubules, suggesting $\alpha_{2u}$-globulin nephropathy that is specific to male rats and without toxicological significance. These results indicated that no-observed-adverse-effect level of 2-(*l*-menthoxy)ethanol was 60 mg/kg BW/day for both sexes.

Keywords: food additive, flavoring agent, subchronic toxicity

Ide T, Cho YM, Oishi Y*, Ogawa K: Spontaneous adenolipoma of the mammary gland in the male F344 rat.

*J Toxicol Pathol*. 2021;34:231-4. doi: 10.1293/tox.2021-0012.

A 110-week-old male F344 rat from the high-dose group of a 104-week carcinogenicity study, exhibited a spontaneously occurring subcutaneous mass in the left axilla extending to the chest. Histologically, the mass was well-demarcated from the adjacent mammary tissue and slightly encapsulated without evidence of infiltration into the surrounding tissues. The mass contained both epithelial and adipose components. The epithelial component consisted of ductal structures of various sizes lined by a single layer of flattened to cuboidal epithelial cells with relatively clear or vacuolated cytoplasm. These ductal structures were well-intermingled with an adipose component that consisted of a uniform monomorphic cell population of mature adipocytes. Both cell types were well-differentiated and did not exhibit cellular atypia. Within the mass, fibrous connective tissue was found in the stroma with infiltration of numerous mast cells. Based on these findings, the mass was diagnosed as an adenolipoma of the mammary gland.

Keywords: adenolipoma, mammary gland, spontaneous tumor

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Yamashita S*, Ogawa K, Hirata T*: Quantitative imaging analysis of nanoparticles and dissolved forms using laser ablation-single particle-ICP-mass spectrometry.

*Metallomics Res*. 2021;1:MR202106.

Laser ablation-single particle-ICP-mass spectrometry (LA-spICP-MS) was applied to define the size, position of silver nanoparticles (Ag NPs), and the concentration of ionic Ag (dissolved Ag) on a frozen section of mouse liver (6 hours after intraperitoneal administered 60 nm Ag NPs (0.2 mg per mouse)). For the accurate size calibration of Ag NPs and quantitative analysis of ionic Ag, a cellulose filter paper doped with Ag NPs suspension and a custom-made photocurable resin reference material containing ionic Ag were prepared in this study. From the imaging results of liver sample, preferential accumulation of the Ag NPs in certain regions was observed. Ionic Ag was also accumulated at regions where Ag NPs are. This suggests that there is a possible contribution of dissolution of Ag NPs through cell activity. This is supported by the detection of small Ag NPs (8-20 nm). The simultaneous imaging analyses of both Ag NPs and ionic Ag can become a useful tool to understand the mechanism of incorporation or metabolism of the NPs.

Keywords: nanoparticle, imaging analysis, laser

ablation

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Ishii Y, Takasu S, Grúz P, Masumura K, Ogawa K, Nohmi T, Umemura T: The role of DNA polymerase ζ in benzo[*a*]pyrene-induced mutagenesis in the mouse lung.

*Mutagenesis*. 2021;36:155-64. doi: 10.1093/mutage/geab007.

DNA polymerase zeta (Pol ζ) is a heterotetramer composed of the catalytic subunit Rev3l, Rev7 and two subunits of Pol δ (PolD2/Pol31 and PolD3/Pol32), and this polymerase exerts translesion DNA synthesis (TLS) in yeast. Because Rev3l knockout results in embryonic lethality in mice, the functions of Pol ζ need further investigation *in vivo*. Then, we noted the two facts that substitution of leucine 979 of yeast Rev3l with methionine reduces Pol ζ replication fidelity and that reporter gene transgenic rodents are able to provide the detailed mutation status. Here, we established *gpt* delta mouse knocked in the constructed gene encoding methionine instead of leucine at residue 2610 of Rev3l (Rev3l L2610M *gpt* delta mice), to clarify the role of Pol ζ in TLS of chemical-induced bulky DNA adducts *in vivo*. Eight-week-old *gpt* delta mice and Rev3l L2610M *gpt* delta mice were treated with benzo[a]pyrene (BaP) at 0, 40, 80, or 160 mg/kg via single intraperitoneal injection. At necropsy 31 days after treatment, lungs were collected for reporter gene mutation assays. Although the *gpt* mutant frequency (MF) was significantly increased by BaP in both mouse genotypes, it was three times higher in Rev3l L2610M *gpt* delta than *gpt* delta mice after treatment with 160 mg/kg BaP. The frequencies of G:C base substitutions and characteristic complex mutations were significantly increased in Rev3l L2610M *gpt* delta mice compared with *gpt* delta mice. The BaP dose-response relationship suggested that Pol ζ plays a central role in TLS when protective mechanisms against BaP mutagenesis, such as error-free TLS, are saturated. Overall, Pol ζ may incorporate incorrect nucleotides at the sites opposite to BaP-modified guanines and extend short DNA sequences from the resultant terminal mismatches only when DNA is heavily damaged.

Keywords: *gpt* delta mouse, polymerase zeta, benzo[*a*]pyrene

Mitsumoto T, Ishii Y, Namiki M, Nakamura K, Takasu S, Ogawa K: A 90-day subchronic toxicity study of Myrrh in F344 rats.

*Regul Toxicol Pharmacol*. 2021;127:105076. doi: 10.1016/j.yrtph.2021.105076.

Myrrh is a flavoring agent and food additive. Here, we performed a subchronic toxicity study of Myrrh in male and female F344 rats by feeding at 5,000, 15,000 and 50,000 ppm for 90 days. No deaths or clinical signs were observed. Suppression of body weight gain was observed from the early phase of administration in both males and females in the 50,000 ppm group. Because there were no obvious changes in food intake in any of the Myrrh groups compared with the control group, suppression of body weight gain was considered an adverse effect of Myrrh. Hematology and serum biochemistry parameters with significant changes observed in the Myrrh groups were considered to have no toxicological significance. We observed a significant increase in relative kidney weight in male rats treated with 50,000 ppm Myrrh; this effect was considered to be related to the appearance of hyaline droplets in the epithelium of the proximal tubules histopathologically observed in this group. Immunohistochemical staining with anti-$\alpha_{2u}$-globulin antibodies suggested that these hyaline droplets were caused by factors other than $\alpha_{2u}$-globulin deposition. Thus, the no-observed-adverse-effect level of Myrrh was determined to be 15,000 ppm (males: 0.85 g/kg/day, females: 0.95 g/kg/day).

Keywords: flavoring agent, food additive, subchronic toxicity

Sugiyama K, Kinoshita M, Furusawa H, Sato K, Honma M: Epigenetic effect of the mycotoxin fumonisin B1 on DNA methylation.

*Mutagenesis*. 2021;36:295-301. doi: 10.1093/mutage/geab019

Mycotoxin fumonisin B1 ($FB_1$) is a secondary metabolite that is produced by certain *Fusarium* species. Although numerous studies demonstrate toxic and carcinogenic effects of FB1, the underlying mechanisms have not been fully elucidated. In this study, we evaluated the epigenetic effects of $FB_1$ for the first time using FLO assays, which detect epigenetic changes that affect the flocculation gene

(*FLO1*) promoter activity in budding yeast. FLO assays showed increased reporter activities of the *FLO1* promoter in the presence of 10 and 20 μM $FB_1$. $FB_1$ (20 μM) treatments also promoted flocculation. In subsequent *in vitro* methylation assays of a bacterial DNA methyltransferase (DNMT), $FB_1$ treatments increased DNMT activities. Moreover, global DNA methylation was significantly increased in HEK293 cells treated with 100 μM $FB_1$. Taken together, these results suggest that $FB_1$ exposure leads to unique epigenetic alterations due to increased DNMT activities and demonstrate that $FB_1$ may be an important risk factor for epigenetic dysfunction-associated human diseases including cancer.

Keywords: FLO assay, DNA methylation, fumonisin B1

Sassa A[*1], Fukuda T[*2], Ukai A, Nakamura M[*2], Sato R[*2], Fujiwara S[*2], Hirota K[*3], Takeda S[*4], Sugiyama K, Honma M, Yasui M: Follow-up genotoxicity assessment of Ames-positive/equivocal chemicals using the improved thymidine kinase gene mutation assay in DNA repair-deficient human TK6 cells.

*Mutagenesis*. 2021;36:331-338. doi: 10.1093/mutage/geab025

Genotoxicity testing plays an important role in the safety assessment of pharmaceuticals, pesticides and chemical substances. Among the guidelines for various genotoxicity tests, the *in vitro* genotoxicity test battery comprises the bacterial Ames test and mammalian cell assays. Several chemicals exhibit conflicting results for the bacterial Ames test and mammalian cell genotoxicity studies, which may stem from the differences in DNA repair capacity or metabolism, between different cell types or species. For better understanding the mechanistic implications regarding conflict outcomes between different assay systems, it is necessary to develop *in vitro* genotoxicity testing approaches with higher specificity towards DNA-damaging reagents. We have recently established an improved thymidine kinase (*TK*) gene mutation assay (TK assay) i.e. deficient in DNA excision repair system using human lymphoblastoid TK6 cells lacking *XRCC1* and *XPA* (*XRCC1*$^{-/-}$/*XPA*$^{-/-}$), the core factors of base excision repair (BER) and nucleotide excision repair (NER), respectively. This DNA repair-deficient TK6 cell line is expected to specifically evaluate the genotoxic potential of chemical substances based on the DNA damage. We focussed on four reagents, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDA), *p*-phenylenediamine (PPD), auramine and malachite green (MG) as the Ames test-positive chemicals. In our assay, assessment using *XRCC1*$^{-/-}$/*XPA*$^{-/-}$ cells revealed no statistically significant increase in the mutant frequencies after treatment with NEDA, PPD and MG, suggesting the chemicals to be non-genotoxic in humans. The observations were consistent with that of the follow-up *in vivo* studies. In contrast, the mutant frequency was markedly increased in *XRCC1*$^{-/-}$/*XPA*$^{-/-}$ cells after treatment with auramine. The results suggest that auramine is the genotoxic reagent that preferentially induces DNA damages resolved by BER and/or NER in mammals. Taken together, BER/NER-deficient cell-based genotoxicity testing will contribute to elucidate the mechanism of genotoxicity and therefore play a pivotal role in the accurate safety assessment of chemical substances.

Keywords: TK6 cells, base excision repair, nucleotide excision repair

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Grúz P, Yasui M, Ukai A, Horibata K, Honma M, Sugiyama K: Potent mutagenicity of an azide, 3-azido-1,2-propanediol, in human TK6 cells.

*Mutat Res Genet Toxicol Environ Mutagen*. 2022;876-877:503475. doi: 10.1016/j.mrgentox.2022.503475

Sodium azide is a strong mutagen that has been successfully employed in mutation breeding of crop plants. In biological systems, it is metabolically converted to the proximate mutagen azidoalanine, which requires further bioactivation to a putative ultimate mutagen that remains elusive. The nature of the DNA modifications induced by azides leading to mutations is also unknown. Other mutagenic organic azido compounds seem to share the same bioactivation pathway to the ultimate mutagenic species as they induce point mutations dependent on the same DNA repair pathways. We investigated mutations induced by the representative mutagen 3-azido-1,2-propanediol (azidoglycerol, AZG) in the human TK6 cell line. Until

now, azides have been considered to be non-mutagens and non-carcinogens in mammals, including humans, as judged only by the conventional clastogenicity chromosomal aberration types of bioassays. Here, we show the potent mutagenicity of AZG in cultured human cells, comparable to alkylating agents such as methyl methanesulfonate at concentrations with similar lethality. The potent ability of an organic azide to induce base substitutions in a mammalian system raises an alert with respect to human exposure to organic and inorganic azido compounds.

Keywords: $NaN_3$, azidoglycerol, TK6

Suzuki T[*1], Sassa A[*2], Grúz P, Gupta RC[*3], Johnson F[*3], Adachi N[*4], Nohmi T: Error-prone bypass patch by a low-fidelity variant of DNA polymerase zeta in human cells.

*DNA Repair*. 2021;100:103052. doi: 10.1016/j.dnarep.2021.103052

DNA polymerase ζ (Pol ζ) is a specialized Pol that is involved in translesion DNA synthesis (TLS), in particular, in the extension of primer DNA after bypassing DNA lesions. Previously, we established human cells that express a variant form of Pol ζ with an amino acid change of leucine 2618 to methionine (L2618M) in the catalytic subunit REV3L (DNA Repair, 45, 34-43, 2016). This amino acid change made the cells more sensitive to the mutagenicity of benzo[a]pyrene diol epoxide (BPDE). In this study, we embedded BPDE-$N^2$-guanine at a defined position in the supF gene on the shuttle plasmid and introduced it to REV3 L2618M cells or the wild-type (WT) cells to examine how far Pol ζ L2618M extends the primer DNA after bypassing the lesion. The adduct induced primarily G to T and G to C at the adducted site in both cell lines, but generated additional sequence changes such as base substitutions, deletions and additions in the extension patch much more often in REV3 L2618M cells than in the WT cells. Mutations in the extension patch in REV3 L2618M cells occurred most often within 10 bps from the adducted site. Then, the number of mutations gradually decreased and no mutations were observed between 30 and 40 bps from the lesion. We concluded that human Pol ζ L2618M and perhaps WT Pol ζ extend the primer DNA up to approximately 30 bps from the lesion *in vivo*. The possibility of involvement of Pol ζ L2618M in the insertion step of TLS is discussed.

Keywords: DNA polymerase ζ, REV3L, translesion DNA synthesis

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Honma M, Yamada M[*], Yasui M, Horibata K, Sugiyama K, Masumura K: *In vivo* and *in vitro* mutagenicity of perillaldehyde and cinnamaldehyde.

*Genes Environ*. 2021;43:30. doi: 10.1186/s41021-021-00204-3

Background: Perillaldehyde and cinnamaldehyde are natural substances found in plants that are used as flavoring ingredients. Due to the α,β-unsaturated aldehydes in their structures, these compounds are expected to be DNA reactive. Indeed, several reports have indicated that perillaldehyde and cinnamaldehyde show positive in *in vitro* and *in vivo* genotoxicity tests. However, their genotoxic potentials are currently disputed. To clarify the mutagenicity of perillaldehyde and cinnamaldehyde, we conducted in silico quantitative structure-activity relationship (QSAR) analysis, *in vitro* Ames tests, and *in vivo* transgenic rodent gene mutation (TGR) assays.

Results: In Ames tests, perillaldehyde was negative and cinnamaldehyde was positive; these respective results were supported by QSAR analysis. In TGR assays, we treated Muta™ Mice with perillaldehyde and gpt-delta mice with cinnamaldehyde up to the maximum tested doses (1000 mg/kg/day). There was no increase in gene mutations in the liver, glandular stomach, or small intestine following all treatments except the positive control (*N*-ethyl-*N*-nitrosourea at 100 mg/kg/day).

Conclusions: These data clearly show no evidence of *in vivo* mutagenic potentials of perillaldehyde and cinnamaldehyde (administered up to 1000 mg/kg/day) in mice; however, cinnamaldehyde is mutagenic *in vitro*.

Keywords: quantitative structure-activity relationship, Ames test, transgenic rodent gene mutation assay

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Kasamatsu T, Kitazawa A, Tajima S[*1], Kaneko M[*1], Sugiyama K, Yamada M[*2], Yasui M, Masumura K, Horibata K, Honma M: Development of a new quantitative structure-activity relationship model for predicting Ames mutagenicity of food flavor chemicals using StarDrop™ auto-Modeller™.
*Genes Environ*. 2021;43:16. doi: 10.1186/s41021-021-00182-6

Background: Food flavors are relatively low molecular weight chemicals with unique odor-related functional groups that may also be associated with mutagenicity. These chemicals are often difficult to test for mutagenicity by the Ames test because of their low production and peculiar odor. Therefore, application of the quantitative structure-activity relationship (QSAR) approach is being considered. We used the StarDrop™ Auto-Modeller™ to develop a new QSAR model.
Results: In the first step, we developed a new robust Ames database of 406 food flavor chemicals consisting of existing Ames flavor chemical data and newly acquired Ames test data. Ames results for some existing flavor chemicals have been revised by expert reviews. We also collected 428 Ames test datasets for industrial chemicals from other databases that are structurally similar to flavor chemicals. A total of 834 chemicals' Ames test datasets were used to develop the new QSAR models. We repeated the development and verification of prototypes by selecting appropriate modeling methods and descriptors and developed a local QSAR model. A new QSAR model "StarDrop NIHS 834_67" showed excellent performance (sensitivity: 79.5%, specificity: 96.4%, accuracy: 94.6%) for predicting Ames mutagenicity of 406 food flavors and was better than other commercial QSAR tools.
Conclusions: A local QSAR model, StarDrop NIHS 834_67, was customized to predict the Ames mutagenicity of food flavor chemicals and other low molecular weight chemicals. The model can be used to assess the mutagenicity of food flavors without actual testing.
Keywords: food flavor, Ames test, quantitative structure-activity relationship (QSAR)

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Masumura K, Ando T, Ukai A, Fujiwara S[*1], Yokose S[*1], You X[*2], Suzuki T, Hayashi H[*3], Nohmi T, Takagi H[*1], Honma M: New homozygous *gpt* delta transgenic rat strain improves an efficiency of the *in vivo* mutagenicity assay.
*Genes Environ*. 2021;43:25. doi: 10.1186/s41021-021-00195-1

Background: Gene mutation assays in transgenic rodents are useful tools to investigate *in vivo* mutagenicity in a target tissue. Using a lambda EG10 transgene containing reporter genes, *gpt* delta transgenic mice and rats have been developed to detect point mutations and deletions. The transgene is integrated in the genome and can be rescued through an *in vitro* packaging reaction. However, the packaging efficiency is lower in *gpt* delta rats than in mice, because of the transgene in *gpt* delta rats being heterozygous and in low copy number. To improve the packaging efficiency, we herein describe a newly developed homozygous *gpt* delta rat strain.
Results: The new *gpt* delta rat has a Wistar Hannover background and has been successfully maintained as homozygous for the transgene. The packaging efficiency in the liver was 4 to 8 times higher than that of existing heterozygous F344 *gpt* delta rats. The frequency of *gpt* point mutations significantly increased in the liver and bone marrow of *N*-nitroso-*N*-ethylurea (ENU)- and benzo[*a*]pyrene (BaP)-treated rats. $Spi^-$ deletion frequencies significantly increased in the liver and bone marrow of BaP-treated rats but not in ENU-treated rats. Whole genome sequencing analysis identified ≥ 30 copies of lambda EG10 transgenes integrated in rat chromosome 1.
Conclusions: The new homozygous *gpt* delta rat strain showed a higher packaging efficiency, and could be useful for *in vivo* gene mutation assays in rats.
Keywords: *gpt* delta transgenic rat, mutant frequency, mutation spectrum

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Aoki Y[*1], Ohno M[*2], Matsumoto M[*1], Matsumoto M[*1], Masumura K, Nohmi T, Tsuzuki T[*2]: Characteristic mutations induced in the small intestine of *Msh2*-knockout *gpt* delta mice.

*Genes Environ*. 2021;43:27. doi: 10.1186/s41021-021-00196-0

Background: Base pair mismatches in genomic DNA can result in mutagenesis, and consequently in tumorigenesis. To investigate how mismatch repair deficiency increases mutagenicity under oxidative stress, we examined the type and frequency of mutations arising in the mucosa of the small intestine of mice carrying a reporter gene encoding guanine phosphoribosyltransferase (*gpt*) and in which the *Msh2* gene, which encodes a component of the mismatch repair system, was either intact (*Msh2*+/+::*gpt*/0; *Msh2*-bearing) or homozygously knockout (KO) (*Msh2*−/−::*gpt*/0; *Msh2*-KO).

Results: *Gpt* mutant frequency in the small intestine of *Msh2*-KO mice was about 10 times that in *Msh2*-bearing mice. Mutant frequency in the *Msh2*-KO mice was not further enhanced by administration of potassium bromate, an oxidative stress inducer, in the drinking water at a dose of 1.5 g/L for 28 days. Mutation analysis showed that the characteristic mutation in the small intestine of the *Msh2*-KO mice was G-to-A transition, irrespective of whether potassium bromate was administered. Furthermore, administration of potassium bromate induced mutations at specific sites in *gpt* in the *Msh2*-KO mice: G-to-A transition was frequently induced at two known sites of spontaneous mutation (nucleotides 110 and 115, CpG sites) and at nucleotides 92 and 113 (3'-side of 5'-GpG-3'), and these sites were confirmed to be mutation hotspots in potassium bromate-administered *Msh2*-KO mice. Administration of potassium bromate also induced characteristic mutations, mainly single-base deletion and insertion of an adenine residue, in sequences of three to five adenine nucleotides (A-runs) in *Msh2*-KO mice, and elevated the overall proportion of single-base deletions plus insertions in *Msh2*-KO mice.

Conclusions: Our previous study revealed that administration of potassium bromate enhanced tumorigenesis in the small intestine of *Msh2*-KO mice and induced G-to-A transition in the *Ctnnb1* gene. Based on our present and previous observations, we propose that oxidative stress under conditions of mismatch repair deficiency accelerates the induction of single-adenine deletions at specific sites in oncogenes, which enhances tumorigenesis in a synergistic manner with G-to-A transition in other oncogenes (e.g., *Ctnnb1*).

Keywords: mismatch repair, oxidative stress, potassium bromate

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Masumura K, Ando T. Toyoda-Hokaiwado N, Ukai A, Nohmi T, Honma M: Comparison of the frequencies of ENU-induced point mutations in male germ cells and inherited germline mutations in their offspring.

*Genes Environ*. 2021;43:43. doi: 10.1186/s41021-021-00216-z

Background: Gene mutations induced in germ cells may be transmitted to the next generation and cause adverse effects such as genetic diseases. Certain mutations may result in infertility or death in early development. Thus, the mutations may not be inheritable. However, the extent to which point mutations in male germ cells are transmitted to the next generation or eliminated during transmission is largely unknown. This study compared mutation frequencies (MFs) in sperm of *N*-ethyl-*N*-nitrosourea (ENU)-treated *gpt* delta mice and *de novo* MFs in the whole exome/genome of their offspring.

Results: Male *gpt* delta mice were treated with 10, 30, and 85 mg/kg of ENU (i.p., weekly × 2) and mated with untreated females to generate offspring. We previously reported a dose-dependent increase in *de novo* MFs in the offspring estimated by whole exome sequencing (WES) (*Mutat. Res.*, 810, 30-39, 2016). In this study, *gpt* MFs in the sperm of ENU-treated mice were estimated, and the MFs per reporter gene were converted to MFs per base pair. The inherited *de novo* MFs in the offspring (9, 26 and 133 × $10^{-8}$ /bp for 10, 30, and 85 mg/kg ENU-treated groups, respectively) were comparable to those of the converted *gpt* MFs in the sperm of ENU-treated fathers (6, 16, and 69 × $10^{-8}$ /bp). It indicated that the *gpt* MFs in the ENU-treated father's sperm were comparable to the inherited *de novo* MFs in the offspring as estimated by WES. In addition, *de novo* MFs in the offspring of 10 mg/kg

ENU-treated and control fathers were estimated by whole genome sequencing (WGS), because WES was not sufficiently sensitive to detect low background MF. The *de novo* MF in the offspring of the ENU-treated fathers was $6 \times 10^{-8}$ /bp and significantly higher than that of the control ($2 \times 10^{-8}$ /bp). There were no significant differences in *de novo* MFs between gene-coding and non-coding regions. WGS analysis was able to detect ENU-induced characteristic *de novo* base substitutions at a low dose group.

Conclusions: Despite a difference between exome/genome and exogenous reporter genes, the results indicated that ENU-induced point mutations in male germ cells could be transmitted to the next generation without severe selection.

Keywords: *gpt* delta transgenic mouse, whole genome sequencing, germline mutation

Gajewicz-Skretna A[*1], Furuhama A, Yamamoto H[*2], Suzuki N[*2]: Generating accurate *in silico* predictions of acute aquatic toxicity for a range of organic chemicals: Towards similarity-based machine learning methods.

*Chemosphere*. 2021;280:130681. doi: 10.1016/j.chemosphere.2021.130681

There has been an increase in the use of non-animal approaches, such as *in silico* and/or *in vitro* methods, for assessing the risks of hazardous chemicals. A number of machine learning algorithms link molecular descriptors that interpret chemical structural properties with their biological activity. These computer-aided methods encounter several challenges, the most significant being the heterogeneity of datasets; more efficient and inclusive computational methods that are able to process large and heterogeneous chemical datasets are needed. In this context, this study verifies the utility of similarity-based machine learning methods in predicting the acute aquatic toxicity of diverse organic chemicals on *Daphnia magna* and *Oryzias latipes*. Two similarity-based methods were tested that employ a limited training dataset, most similar to a given fitting point, instead of using the entire dataset that encompasses a wide range of chemicals. The kernel-weighted local polynomial approach had a number of advantages over the distance-weighted *k*-nearest neighbor (*k*-NN) algorithm. The results highlight the importance of lipophilicity, electrophilic reactivity, molecular polarizability, and size in determining acute toxicity. The rigorous model validation ensures that this approach is an important tool for estimating toxicity in new or untested chemicals.

Keywords: chemical risk assessment, ecotoxicity, *in silico* methods

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Petkov PI[*], Ivanova H[*], Honma M, Yamada T, Morita T, Furuhama A, Kotov S[*], Kaloyanova E[*], Dimitrova G[*], Mekenyan O[*]: Differences between *in vitro* and *in vivo* genotoxicity due to metabolism: The role of kinetics.

*Computat Toxicol*. 2022;22:100222. doi: 10.1016/j.comtox.2022.100222

Traditional QSAR models predict mutagenicity solely based on structural alerts for the interaction of parent chemicals or their metabolites with target macromolecules. In the present work, it is demonstrated that the presence of an alert is necessary to identify damage but it is not always sufficient to assess mutagenic potential. This is addressed by accounting for the kinetics of simulating metabolism and formation of adducts with macromolecules. The mutagenic potential of chemicals is related to the degree to which selected macromolecules are altered. This extent is estimated by the amount of formed DNA/protein adducts. Here the effect of modelling kinetic factors is investigated for chemicals having documented *in vitro* negative and *in vivo* positive data in mutagenicity and clastogenicity tests of similar capacity - *in vitro* Ames vs *in vivo* TGR and *in vitro* CA vs *in vivo* MN tests. Two factors justify the conflict in mutagenicity data: the differences in enzyme expression in the *in vitro* vs *in vivo* metabolism and the difference in exposure time for *in vitro* and *in vivo* tests. Addressing these factors required simulating the formation of DNA/protein adducts and introducing empirically-defined thresholds for the amounts of the adducts leading to mutagenic potential.

Keywords: metabolism, *in vitro* genotoxicity, *in vivo* genotoxicity

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Furuhama A, Hayashi TI*, Yamamoto H*: Strategy for development of quantitative structure-activity-activity relationship models for chronic fish toxicity: prediction of early-life stage toxicity to *Oryzias latipes* from acute *Daphnia magna* toxicity.
*Jpn J Environ Toxicol*. 2021;24:33-42. doi: 10.11403/jset.24.33

We examined two groups of quantitative structure-activity-activity relationship (QSAAR) models for predicting Japanese medaka (*Oryzias latipes*) early-life stage (ELS) toxicities of chemicals for the purpose of chronic hazard and environmental risk assessments. The models included not only typical molecular descriptors but also acute *Daphnia magna* toxicity data, ELS test conditions, and information about chemical categories (e.g., pesticides). We found that acute Daphnia magna toxicity was an important descriptor for predicting fish ELS toxicity, along with molecular descriptors. The group II models, which were based on 119 training data for three warm freshwater species (fathead minnow, Japanese medaka, and zebrafish) had higher predictivity than the group I models, which were based on a 172 training data for four freshwater species (fathead minnow, Japanese medaka, zebrafish, and rainbow trout). In addition, the group II models had higher predictivity than the QSAAR models we reported previously (*SAR QSAR Environ. Res.* 29:9, 725-742 and 30:11, 825-846). Models developed by means of the strategy used to develop the group II models would be usable for estimating chronic fish toxicity in screening assessments such as those required under the Japanese Chemical Substances Control Law.
Keywords: acute *Daphnia magna* toxicity, fish ELS toxicity, QSAAR

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Tanabe S, Quader S*1, Ono R, Cabral H*2, Aoyagi K*3, Hirose A, Yokozaki H*4, Sasaki H*3: Molecular network analysis of RNA viral infection pathway in diffuse- and intestinal-type gastric cancer.
*Fundam Toxicol Sci*. 2022;9(2):37-46. doi: 10.2131/fts.9.37

There are several subtypes of gastric cancer, such as diffuse-type gastric cancer (GC) and intestinal-type GC. Diffuse-type GC is known to be more malignant than intestinal-type GC, showing high metastasis, recurrence and anti-cancer drug resistance. The malignant phenotype of diffuse-type GC includes cancer stem cell (CSC)-like features and epithelial-mesenchymal transition (EMT). By analyzing the molecular network in these tumors, it is possible to reveal the mechanisms of anti-cancer drug resistance, therapeutic targets and drug safety. Upon the analyses of the molecular network in diffuse- and intestinal-type GC, a regulatory network for RNA virus infection was obtained. This study aims to reveal the relationship between cancer and RNA virus infection in detail. RNA virus infection-related molecules and cancer-related molecules were analyzed using network analysis tools, such as Ingenuity Pathway Analysis (IPA), and molecular networks related to RNA virus infection mechanisms. Regulator effect analysis revealed the involvement of RNA virus infection network in diffuse-type GC. c-Jun N-terminal kinase (JNK) and BCL2 like 11 (BCL2L11) in the Coronavirus Pathogenesis Pathway were activated. In conclusion, this research suggested the relationship between the mechanisms of RNA virus infection and diffuse-type GC. This study may be useful for virus infection control and cancer drug discovery by clarifying the relationship between the mechanism of RNA virus infection and cancer.
Keywords: epithelial-mesenchymal transition, gastric cancer, molecular network analysis

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Iso T, Natsume M*, Murata Y, Shigeta Y, Hirose N, Umano T, Horibata K, Masumura K, Sugiyama K, Matsumoto M, Hirose A: Absence of *in vivo* mutagenicity of 4,4'-oxybis(benzenesulfonohydrazide) in liver and glandular stomach of Muta™ mice.
*Fundam Toxicol Sci*. 2022;9(2):31-36. doi: 10.2131/fts.9.31

4,4'-Oxybis(benzenesulfonohydrazide) (OBSH) is a blowing agent widely used in the manufacture of porous plastics and rubber. OBSH was notified as an additive in the Japanese positive list system

for food utensils, containers and packaging. The *in vitro* mutagenicity of OBSH was shown extensively in bacterial reverse mutation assays, a DNA repair test, and a chromosomal aberration test. Few studies exist on *in vivo* genotoxic evaluation on OBSH apart from an *in vivo* micronuclei test. To clarify *in vivo* mutagenicity, we conducted a transgenic rodent gene mutation (TGR) assay (OECD TG 488). We dosed male Muta™ mice with OBSH by oral gavage at 0 (negative control), 25, 50, and 100 mg/kg/day for 28 consecutive days, and evaluated mutant frequencies (MFs) of *lacZ* in the liver and glandular stomach (5 mice/group). We observed two deaths and a reduction in body weight gain at 100 mg/kg/day. Although we exposed Muta™ mice to OBSH orally for 28 days up to the maximum tolerated dose, we did not detect *in vivo* mutagenicity in the liver and glandular stomach. In contrast, in the positive control we detected significantly increased MFs. The results of this study suggest that OBSH is not mutagenic *in vivo*.

Keywords: 4,4'-oxybis(benzenesulfonohydrazide), *in vivo* mutagenicity, transgenic rodent gene mutation assay

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Ohno A, Okiyama Y, Hirose A, Fukuhara K*: The Position of the Nitro Group Affects the Mutagenicity of Nitroarenes.

*Toxicol Appl Pharmacol*. 2022;441;115974. doi: 10.1016/j.taap.2022.115974

The ease with which a nitrated polyaromatic hydrocarbon ($NO_2$PAH) is activated by reductive metabolism is an important factor in determining mutagenicity. However, the mutagenicity of 3-nitrobenzo[a]pyrene (3-$NO_2$BaP) is stronger than that of 1-NO2BaP despite similar reduction properties, and the more potent mutagenicity of 3,6-di$NO_2$BaP relative to that of 1,6-di$NO_2$BaP cannot be explained by relative reducibility. Here, we investigated structural factors leading to the mutagenicity of these compounds by synthesizing 1- and 3-$NO_2$BaP derivatives with C6-position substituents that affect reduction properties and testing the mutagenicity of the compounds and their derivatives against Salmonella typhimurium TA98 and TA98NR. The LUMO and LUMO+1 energies of 6-substituted 3-$NO_2$BaPs were found to correlate with mutagenicity, but such correlations were much weaker with 6-substituted 1-$NO_2$BaPs, indicating that the mutagenicity of 3-$NO_2$BaPs is influenced by the ease of reductive metabolic activation. *In silico* structural analyses demonstrated that the distances between the nitrogen of the N-acetoxyamino group in reductive metabolites and a DNA alkylation target were longer for 1-$NO_2$BaPs than for 3-$NO_2$BaPs. Therefore, the active metabolites of 6-substituted 3-$NO_2$BaPs intercalate with DNA at a distance where they can readily form adducts with guanine. Conversely, the unfavorable position of intercalated active metabolites of 1-$NO_2$BaPs relative to guanine leads to difficult adduct formation despite the facile formation of the active metabolite due to a low LUMO energy. Therefore, the chemical reducibility of the nitro group and, more importantly, the ease of adduct formation between an active metabolite and DNA are essential for the prediction of the mutagenicity of $NO_2$PAHs.

Keywords: mutagenicity, nitroarene, *in silico* study

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Watanabe-Matsumoto S, Yoshida K, Meiseki Y, Ishida S, Hirose A, Yamada T: A physiologically based kinetic modeling of ethyl tert-butyl ether in humans - An illustrative application of quantitative structure-property relationship and Monte Carlo simulation.

*J Toxicol Sci*. 2022;47(2):77-87. doi: 10.2131/jts.47.77

Although physiologically based kinetic (PBK) modeling is informative for the risk assessment of industrial chemicals, chemical-specific input values for partition coefficients and metabolic parameters, including Vmax and Km are mostly unavailable; however, *in silico* methods, such as quantitative structure-property relationship (QSPR) could fill the absence. To assess the PBK model validity using necessary toxicokinetic (TK) parameters predicted by QSPR, the PBK model of ethyl tert-butyl ether (ETBE) as a model substance was constructed, in which the values of the partition coefficients, Vmax, and Km of ETBE were predicted using those of the related chemicals previously reported in the literature, and toxicokinetics of inhaled ETBE were stochastically estimated using the Monte Carlo

simulation. The calculated ETBE concentrations in venous blood were comparable to the measured values in humans, implying that the reproducibility of ETBE toxicokinetics in humans was established in this PBK model. The Monte Carlo simulation was used to conduct uncertainty and sensitivity analyses of the dose metrics in terms of maximum blood concentration (Cmax) and area under the blood concentration-time curve (AUC) and the estimated Cmax and AUC were highly and moderately reliable, respectively. Conclusively, the PBK model validity combined with *in silico* methods of QSPR was demonstrated in an ETBE model substance. QSPR-PBK modeling coupled with the Monte Carlo simulation is effective for estimating chemical toxicokinetics for which input values are unavailable and for evaluating the estimation validity.
Keywords: ethyl tert-butyl ether, Monte Carlo simulation, PBK modeling

Fujita M[*1], Yamamoto Y[*1], Wanibuchi S[*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]: The within- and between-laboratories reproducibility and predictive capacity of Amino acid Derivative Reactivity Assay using 4 mM test chemical solution: Results of ring study implemented at five participating laboratories.
*J Appl Toxicol*. 2022;42(2):318-333. doi: 10.1002/jat.4268

Amino acid derivative reactivity assay (ADRA) for skin sensitization was adopted as an alternative method in the 2019 OECD Guideline for the Testing of Chemicals (OECD TG 442C). The molar ratio of the nucleophilic reagent to the test chemicals in the reaction solution was set to 1:50. Imamura et al. reported that changing this molar ratio from 1:50 to 1:200 reduced in false negatives and improved prediction accuracy. Hence, a ring study using ADRA with 4 mM of a test chemical solution (ADRA, 4 mM) was conducted at five different laboratories to verify within- and between-laboratory reproducibilities (WLR and BLR, respectively). In this study, we investigated the WLR and BLR using 14 test chemicals grouped into three classes: (1) eight proficiency substances, (2) four test chemicals that showed false negatives in the ADRA with 1 mM test chemical solution (ADRA, 1 mM), but correctly positive in ADRA (4 mM), and (3) current positive control (phenylacetaldehyde) and a new additional positive control (squaric acid diethyl ester). The results showed 100% reproducibility and 100% accuracy for skin sensitization. Hence, it is clear that the ADRA (4 mM) is an excellent test method in contrast to the currently used ADRA (1 mM). We plan to resubmit the ADRA (4 mM) test method to the OECD Test Guideline Group in the near future so that OECD TG 442C could be revised for the convenience and benefit of many ADRA users.
Keywords: ADRA, ring study, skin sensitization

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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(6):1078-1090. 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, NAC, NAL

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Imamura M*1, Yamamoto Y*1, Fujita M*1, Wanibuchi S*1, Nakashima N*1, Kojima H, Ono A*2, Kasahara T*1: Applicability of ADRA (4 mM) for the prediction of skin sensitization by combining multiple alternative methods to evaluate key events. *J Appl Toxicol*. 2022; *in press*. doi: 10.1002/jat.4283

The amino acid derivative reactivity assay (ADRA) is an alternative method for evaluating key event 1 (KE-1) in the skin sensitization mechanism included in OECD TG442C (OECD, 2021). Recently, we found that ADRA with a 4-mM test chemical solution had a higher accuracy than the original ADRA (1 mM). However, ADRA (4 mM) has yet to be evaluated using integrated approaches to testing and assessment (IATA), a combination of alternative methods for evaluating KE. In this study, the sensitization potency of three defined approaches (DAs) using ADRA (4 mM) as KE-1 was predicted and compared with those of two additional ADRAs or direct peptide reactivity assay (DPRA): (i) "2 out of 3" approach, (ii) "3 out of 3" approach, and (iii) integrated testing strategy (ITS). In the hazard identification of chemical sensitizers, the accuracy of human data and local lymph node assay (LLNA) remained almost unchanged among the three approaches evaluated. Potency classifications for sensitization were predicted with the LLNA and human data sets using ITS. The potency classifications for the sensitization potency prediction accuracy of LLNA data using any alternative method were almost unchanged, at approximately 70%, and those with ITS were not significantly different. When ITS was performed using DPRA, the prediction accuracy was approximately 73% for human data, which was similar to that of the LLNA data; however, the accuracy tended to increase for all ADRA methods. In particular, when ITS was performed using ADRA (4 mM), the prediction accuracy was approximately 78%, which proved to be a practical level.

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

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Ashikaga T, Ambe K*, Suzuki M*, Kurimoto M, Yamada T, Tohkin M*. Establishment of a Threshold of Toxicological Concern Concept for Skin Sensitization by *in Vitro/in Silico* Approaches *日本香粧品学会誌*. 2021;45(4):331-5.

Recently, multiple *in vitro* skin sensitization tests have been listed in the Organisation for Economic

Co-operation and Development (OECD) guidelines. The threshold of toxicological concern (TTC) is a threshold for human exposure when chemicals do not show any obvious adverse effects at lower doses. In this study, we aimed to develop a quantitative prediction model using an *in vitro/in silico* dataset and to establish a TTC concept for skin sensitization. The EC3 value, the endpoint of the local lymph node assay (LLNA), was used as the objective variable and data on 120 substances were extracted from the dataset published by Cosmetics Europe. *In vitro* tests (DPRA, KeratinoSens™ and h-CLAT) data and physico-chemical properties were used as explanatory variables. A quantitative prediction model for EC3 was developed using support vector regression (SVR), a machine learning approach. Predicted EC3 values were used to establish a no expected sensitization induction level (NESIL), and the acceptable exposure level (AEL) for each chemical was calculated by dividing NESIL by the sensitization assessment factor (SAF). Then, by fitting the gamma distribution of AELs using a negative log (10) scale, the 95th and 99th percentile probability were calculated as the dermal sensitization threshold (DST) value. Finally, the conversion of the DST to the threshold concentration of a women's face cream was performed as an example of the application of this concept.

This prediction model was validated by a three-fold cross-validation, and the accuracy of prediction of potency class in five categories was 45.8%. Assuming 20% of all chemicals are skin sensitizers, the DST ($mg/cm^2$) for women's face cream was 0.129 (99th percentile) and 3.99 (95th percentile). Furthermore, the threshold concentration of this type of products was 0.008% (for DST 99th percentile) and 0.26% (for DST 95th percentile). The TTC concept for skin sensitization can be applied as a non-animal approach in evaluating the safety profile of cosmetic ingredients.

Keywords: TTC, DST, skin sensitization

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Kojima H: Alternatives to animal testing.
*Impact*. 2021;44-45. doi: 10.21820/23987073.2021.8.44

Scientists are working to develop new and innovative alternatives to animal testing that don't rely on the use of animals. Takao Ashikaga, Hajime Kojima and Yoko Hirabayashi are part of JaCVAM which works to promote the use of alternatives to animal testing. The goal is to replace, reduce or refine (3Rs) the use of animal under International harmonization. Hirabayashi is also the representative of a research group that is funded by the AMED and the representative of a research group funded by the MHLW. A challenge the researchers are facing in their quest to ensure the welfare of experimental animals and also ensure the safety of various pharmaceutical and chemicals is the lack of biomarkers to more accurately predict toxicity for regulatory acceptance. This means that without animal testing more costly and complex non-animal methods are required and presents a barrier to the adoption of non-animal methods for international standerisation. As such, there is a need to develop an easy way to obtain a lot of information. Hirabayashi and the team are working on the development of AI that can be used to evaluate the safety of different compounds. The researchers are developing *in vitro* assays such as ordinary 2-dimensional culture, 3-dimensional culture including organoids or spheroids, reporter gene assay and organ-on-a chip; and *in silico* assays such as computer toxicology using QSAR and Read Across. The researchers hope that their innovative work will contribute to the 3Rs, benefiting animal welfare for regulatory use.

Keywords: alternative, JaCVAM, regulatory acceptance

Narita K[*1], Okutomi H[*1], Kawakami K[*1], Sui H[*1], Basketter D[*2], Ashikaga T: Behavior of Chemical Respiratory Sensitizers in *in Vitro* Methods for Skin Sensitization.
*AATEX*. 2021;26(1):9-18.

Respiratory sensitization induced by chemicals is an important occupational and public health issue because it is associated with allergic asthma or other pulmonary symptoms. However, there are no validated test methods to identify respiratory sensitizers. In this study, to investigate the behavior of respiratory sensitizers in a skin sensitization test method, 14 respiratory sensitizers were tested with the human cell line activation test (h-CLAT). h-CLAT (an *in vitro* skin sensitization test - OECD test guideline 442E) evaluates dendritic cell activation by measuring the expression of cell surface CD86 and CD54 antigens in THP-1 cells. h-CLAT was positive for 7 of the 14

respiratory sensitizers, including all four diisocyanates used industrially as polyurethane materials. Furthermore, for three acid anhydrides negative in h-CLAT, we also performed a modified h-CLAT (a short-time exposure method using liquid paraffin) and phthalic anhydride then proved positive. Among the seven h-CLAT negatives, at least four chemicals are known to be positive in the direct peptide reactivity assay (DPRA - *in chemico* skin sensitization test method listed in OECD TG 442C). These results suggest that h-CLAT could be a useful non-animal test method for respiratory sensitizers when combined with other *in vitro* test methods like DPRA.

Keywords: respiratory sensitization, h-CLAT, test battery

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Kojima H, Nakada T*1, Yagami A*2, Todo H*3, Nishimura J*4, Yagi M*4, Sugiyama M*5, Yamamoto K*4, Ikarashi Y, Sakaguchi H*6, Yamaguchi M*6, Hirota M*6, Ikeda H*6, Imai N*6, Hatao M*6: A Step-by-Step Approach for Assessing Human Skin Irritation Without Animal Testing for Quasi-Drugs and Cosmetic Products.

*Applied in vitro Toxicology*. 2021;7(3). doi: 10.1089/aivt.2021.0016

Introduction: Animal tests of cosmetic ingredients and products have been banned in the EU since 2013. However, in Japan, the application of new quasi-drugs requires the generation of 24-hour data on primary and cumulative skin irritation by animal testing. Such data are unreliable because an ingredient predicted as nonirritating after short exposure (4 hours), based on the Organization for Economic Co-operation and Development (OECD)test guidelines (TG)404, may cause irritation after a longer application period in human skin irritation tests. Within sufficient data to draw conclusions about the irritation potential of an ingredient, there remains a high probability of skin irritation occurrence after extended exposure to the ingredient.

Materials and Methods: This study assessed whether the skin irritation caused by quasi-drugs and cosmetic products can be evaluated in a step-by-step manner.

Results: A workflow was developed considering several key steps such as the component characteristics based on physicochemical properties or the ingredient category based on existing information from animal tests and human patch test results, and its utility was assessed using the reconstructed human epidermis (RhE) test (OECD TG439), animal testing, the human patch test, and the human cumulative skin irritation test.

Conclusion: The RhE test and the aforementioned human skin tests can be employed to evaluate test substances that cause weak or nonskin irritation categorized as ''harmless ingredients''—thereby avoiding animal testing.

Keywords: cumulative skin irritation test, harmless ingredients, primary skin irritation

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Nishida H*1, Ohtake T*1, Ashikaga T, Hirota M*1, Onoue S*2, Seto Y*2, Tokura Y*3, Kouzuki H*1: *In chemico* sequential testing strategy for assessing the photoallegic potential.

*Toxicol In Vitro*. 2021;77:105245. doi: 10.1016/j.tiv.2021.105245

Several non-animal testing methods to assess photoallergic potential have been developed so far, while none of them have yet to be validated and regulatory accepted. Currently, some photoreactivity assays such as UV-VIS spectral analysis and ROS assay are generally used for initial photosafety assessments because of their high sensitivity. However, they have a low specificity, generating a high percentage of false positive results, and the development of a follow-up assessment method is desired. Therefore, this study aimed to develop an in chemico photoallergy testing method, photo-direct peptide reactivity assay (photo-DPRA). Based on photosafety information, 34 photoallergens and 16 non-photoallergens were selected and subjected to UV-VIS spectral analysis, ROS/micellar ROS assays, photo-DPRA, sequential testing strategy (STS) consisting

of all three methods, and 3T3 neutral red uptake phototoxicity testing (3T3 NRU PT). Combination of the methods addressing the key events of photoallergy exhibited high prediction performance. Our results showed the proposed strategy would be useful to predict the photoallergic potential of chemicals as the follow-up assessment for false positive chemicals by UV/VIS spectral analysis and ROS assay.

Keywords: photoallergy, ROS assay, photo-DPRA

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Dent MP*1, Vaillancourt E*2, Thomas RS*3, Carmichael PL*1, Ouedraogo G*4, Kojima H, Barroso J*5, Ansell J*6, Barton-Maclaren TS*2, Bennekou SH*7, Boekelheide K*8, Ezendam J*9, Field J*2, Fitzpatrick S*10, Hatao M*11, Kreiling R*12, Lorencini M*13, Mahony C*14, Montemayor B*15, Mazaro-Costa R*16, Oliveira J*17, Rogiers V*18, Smegal D*10, Taalman R*19, Tokura Y*20, Verma R*10, Willett C*21, Yang C*22: Paving the way for application of next generation risk assessment to safety decision-making for cosmetic ingredients.
*Regul Toxicol Pharmacol*. 2021;125:105026. doi: 10.1016/j.yrtph.2021.105026

Next generation risk assessment (NGRA) is an exposure-led, hypothesis-driven approach that has the potential to support animal-free safety decision-making. However, significant effort is needed to develop and test the *in vitro* and *in silico* (computational) approaches that underpin NGRA to enable confident application in a regulatory context. A workshop was held in Montreal in 2019 to discuss where effort needs to be focussed and to agree on the steps needed to ensure safety decisions made on cosmetic ingredients are robust and protective. Workshop participants explored whether NGRA for cosmetic ingredients can be protective of human health, and reviewed examples of NGRA for cosmetic ingredients. From the limited examples available, it is clear that NGRA is still in its infancy, and further case studies are needed to determine whether safety decisions are sufficiently protective and not overly conservative. Seven areas were identified to help progress application of NGRA, including further investments in case studies that elaborate on scenarios frequently encountered by industry and regulators, including those where a 'high risk' conclusion would be expected. These will provide confidence that the tools and approaches can reliably discern differing levels of risk. Furthermore, frameworks to guide performance and reporting should be developed.

Keywords: non-animal approaches, next generation risk assessment

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Ambe K*, Suzuki M*, Ashikaga T, Tohkin M*: Development of quantitative model of a local lymph node assay for evaluating skin sensitization potency applying machine learning CatBoost

*Regul Toxicol Pharmacol*. 2021;125:105019. doi: 10.1016/j.yrtph.2021.105019

The estimated concentrations for a stimulation index of 3 (EC3) in murine local lymph node assay (LLNA) is an important quantitative value for determining the strength of skin sensitization to chemicals, including cosmetic ingredients. However, animal testing bans on cosmetics in Europe necessitate the development of alternative testing methods to LLNA. A machine learning-based prediction method can predict complex toxicity risks from multiple variables. Therefore, we developed an LLNA EC3 regression model using CatBoost, a new gradient boosting decision tree, based on the reliable Cosmetics Europe database which included data for 119 substances. We found that a model using *in chemico/in vitro* tests, physical properties, and chemical information associated with key events of skin sensitization adverse outcome pathway as variables showed the best performance with a coefficient of determination ($R^2$) of 0.75. In addition, this model can indicate the variable importance as the interpretation of the model, and the most important variable was associated with the human cell line activation test that evaluate dendritic cell activation. The good performance and interpretability of our LLNA EC3 predictable regression model suggests that it could serve as a useful approach for quantitative assessment of skin sensitization.

Keywords: integrated approaches to testing and assessment, machine learning, skin sensitization

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Liao CC*1, Wu CY*1, Lin MH*1, Hsieh FK*1, Hsu LT*1, Chang SY*1, Chen KJ*1, Huang HT*1, Hsu HC*1, Lin CH*1, Lin PJ*1, Lai HM*1, Kojima H, Todo H*2, Lin SJ*3, Li JH*4, Chen W*1: Validation study of a new reconstructed human epidermis model EPiTRI for *in vitro* skin irritation test according to OECD guidelines.

*Toxicol In Vitro*. 2021;75:105197. doi: 10.1016/j.tiv.2021.105197

Following the global trend of reducing animal testing, various reconstructed human epidermis (RHE) models for skin irritation test (SIT) have been developed, verified, validated and included in OECD TG 439. We developed a new RHE called EPiTRI and a SIT method using EPiTRI (EPiTRI-SIT model) following the OECD guidelines. EPiTRI possesses morphological, biochemical and physiological properties similar to human epidermis with well-differentiated multilayered viable cells with barrier function. The EPiTRI-SIT model was tested for 20 reference chemicals in Performance Standard of OECD TG 439 (GD 220), showing good predictive capacity with 100% sensitivity, 70% specificity and 85% accuracy. EPiTRI had sensitivity in detecting di-n-propyl disulphate, as an irritant chemical (UN GHS Category 2), whereas most validated reference methods detected it as a non-irritant. An international validation study of EPiTRI-SIT was conducted in four laboratories to confirm the within- and between-laboratory reproducibility, as well as predictive capacity. The phase I/II within-laboratory and between-laboratory reproducibility was 100%/95% and 95%, respectively. The overall sensitivity, specificity and accuracy of EPiTRI-SIT was 96%, 70% and 83%, respectively, which fulfilled the OECD criteria. Thus, EPiTRI, meets the criteria of Performance Standards of OECD TG 439 (GD 220) and is suitable for screening irritating chemicals *in vitro*.

Keywords: reconstructed human epidermis (RHE), *in vitro* skin irritation test (SIT), validation study

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Lee B-M*1, Lee SH*2, Yamada T, Park S*3, Wang Y*4, Kim K-B*5, Kwon S*6: Read-across approaches: Current applications and regulatory acceptance in Korea, Japan, and China.

*J Toxicol Environ Health A*. 2022;85(5):184-197. doi: 10.1080/15287394.2021.1992323

The aim of this paper was to investigate the current status of read-across approaches in the Republic of Korea, Japan, and China in terms of applications and regulatory acceptance. In the Republic of Korea, over the last 6 years, approximately 8% of safety data records used for chemical registrations were based upon read-across, and a guideline published on the use of read-across results in 2017. In Japan, read-across is generally accepted for screening hazard classification of toxicological endpoints according to the Chemical Substances Control Law (CSCL). In China, read-across data, along with data from other animal alternatives are accepted as a data source for chemical registrations, but could be only considered when testing is not technically feasible. At present, read-across is not widely used for chemical registrations and regulatory acceptance of read-across may differ among countries in Asia. With consideration of the advantages and limitations of read-across, it is expected that read-across may soon gradually be employed in Asian countries. Thus, regulatory agencies need to prepare for this progression.

Keywords: read-across, regulatory acceptance, toxicity prediction

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Yamada T, Kawamura T, Maruyama T, Kurimoto M, Yamamoto H, Katsutani N, Hirose A: Quantitative structure-activity relationship and a category approach to support algal toxicity assessment of human pharmaceuticals.

*Fundam Toxicol Sci*. 2021;8:195-204. doi: 10.2131/fts.8.195

Releasing human pharmaceuticals to the environment is an emerging ecotoxicological concern. In this study, we examine the feasibility of evaluating the algal chronic toxicity of human pharmaceuticals using quantitative structure-activity relationship (QSAR) models and a category approach. We constructed an ecotoxicology database of human pharmaceuticals using publicly available information, such as regulatory agency reports and scientific papers. We created an algal chronic toxicity dataset using this database, and predicted the No Observed Effect Concentrations (NOEC) of human pharmaceuticals using ECOlogical Structure-Activity Relationship (ECOSAR) and KAshinhou Tool for Ecotoxicity (KATE) QSAR models. Almost half of query substances were applicable to the QSAR models, and the feasibility was confirmed with high concordant predictions-predicted/measured ratios were in the range of 0.01-100 in 92.9% and 79.1% of applicable substances in ECOSAR and KATE, respectively-and false predictions (predicted/measured ratios > 100) that could lead to significant underestimation of toxicity were rarely observed. Two case studies of diphenhydramine and lamotrigine demonstrated that detailed evaluation of target and reference substances in the corresponding chemical class could increase the reliability and accuracy of prediction results of KATE. Grouping of substances based on pharmacology revealed some category classes with a toxicological concern. Finally, a workflow model to assess algal toxicity of human pharmaceuticals was proposed based on these evaluations including QSAR predictions and category approach.

Keywords: algal toxicity, *in silico* approaches, pharmaceuticals

Yamada T, Miura M, Kawamura T, Ushida K, Inoue K, Kuwagata M, Katsutani N, Hirose A: Constructing a developmental and reproductive toxicity database of chemicals (DART NIHS DB) for integrated approaches to testing and assessment.

*J Toxicol Sci*. 2021;46:531-538. doi: 10.2131/jts.46.531

Developmental and reproductive toxicity (DART) is an important endpoint, and databases (DBs) are essential for evaluating the risk of untested substances using alternative methods. We have constructed a reliable and transparent DART DB, which we named DART NIHS DB, using the publicly available datasets of DART studies of industrial chemicals conducted by Japanese government ministries in accordance with the corresponding OECD test guidelines (OECD TG421 and TG422). This DB is unique because its

dataset chemicals have little overlap with those of ToxRefDB, which compiles large-scale DART data, and it is reliable because the included datasets were created after reviewing the individual study reports. In DART NIHS DB, 171 of 404 substances exhibited signs of DART, which occurred during fertility and early embryonic development (49 substances), organogenesis (59 substances), and the perinatal period (161 substances). When the lowest-observed-adverse-effect level (LOAEL) of DART was compared with that of repeated-dose toxicity (RDT), 15 substances (12%) had a lower LOAEL for DART than for RDT. Of these, five substances displayed significant DART at doses of ≤ 50 mg/kg bw/day. The chemical and toxicity information in this DB will be useful for the development of stage-specific adverse outcome pathways (AOPs) via integration with mechanistic information. The whole datasets of the DB can be implemented in read-across support tools such as the OECD QSAR Toolbox, which will further lead to future integrated approaches to testing and assessment based on AOPs.

Keywords: DART database, industrial chemical, developmental and reproductive toxicity

Tanabe S, Quader S[*1], Ono R, Cabral H[*2], Aoyagi K[*3], Hirose A, Yokozaki H[*4], Sasaki H[*3]: Cell cycle regulation and DNA damage response networks in diffuse- and intestinal-type gastric cancer.

*Cancers*. 2021;13:5786. doi: 10.3390/cancers13225786

Dynamic regulation in molecular networks including cell cycle regulation and DNA damage response play an important role in cancer. To reveal the feature of cancer malignancy, gene expression and network regulation were profiled in diffuse- and intestinal-type gastric cancer (GC). The results of the network analysis with Ingenuity Pathway Analysis (IPA) showed that the activation states of several canonical pathways related to cell cycle regulation were altered. The $G_1$/S checkpoint regulation pathway was activated in diffuse-type GC compared to intestinal-type GC, while canonical pathways of the cell cycle control of chromosomal replication, and the cyclin and cell cycle regulation, were activated in intestinal-type GC compared to diffuse-type GC. A canonical pathway on the role of BRCA1 in the DNA damage response was activated in intestinal-type GC compared to diffuse-type GC, where gene expression of BRCA1, which is related to $G_1$/S phase transition, was upregulated in intestinal-type GC compared to diffuse-type GC. Several microRNAs (miRNAs), such as mir-10, mir-17, mir-19, mir-194, mir-224, mir-25, mir-34, mir-451 and mir-605, were identified to have direct relationships in the $G_1$/S cell cycle checkpoint regulation pathway. Additionally, cell cycle regulation may be altered in epithelial-mesenchymal transition (EMT) conditions. The alterations in the activation states of the pathways related to cell cycle regulation in diffuse- and intestinal-type GC highlighted the significance of cell cycle regulation in EMT.

Keywords: cancer malignancy, epithelial-mesenchymal transition (EMT), molecular network

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Matsumoto M, Takano M[*], Takabe M[*], Yamaguchi N[*], Iso T, Shigeta Y, Murata Y, Hirose N, Inoue K, Hirose A: Initial hazard assessment of ethyl (dimethyl) (tetradecyl)ammonium ethyl sulfate: Genotoxicity tests and combined repeated-dose and reproductive/developmental toxicity screening in rats.

*Regul Toxicol Pharmacol*. 2021;122:104914. doi: 10.1016/j.yrtph.2021.104914

Ethyl(dimethyl)(tetradecyl)ammonium ethyl sulfate, used in laundry detergents, shampoos, and body soaps, is classified by the Japanese Chemical Substances Control Law as a priority assessment chemical substance for environmental effects. However, its toxicity data for human health are insufficient. This study evaluated this chemical under the Safety Examination of Existing Chemicals and Safety Programmes of the Ministry of Health, Labour and Welfare (MHLW). The MHLW conducted bacterial reverse mutation (Ames test), *in vitro* chromosomal aberration, and combined repeated-dose and reproductive/developmental toxicity screening tests. We performed a screening assessment of ethyl (dimethyl) (tetradecyl)ammonium ethyl sulfate for human health. The chemical showed a negative reaction in the Ames test and a positive reaction in the

*in vitro* chromosomal aberration test with metabolic activation in rats. The combined repeated-dose and reproductive/developmental toxicity screening test showed significantly decreased food consumption at 50 mg/kg body weight/day, but no reproductive and developmental toxicity was observed. The no-observed-effect level of 15 mg/kg/day was obtained as a screening value. Therefore, this chemical was classified as hazard class 3, with a derived-no-effect level of 0.025 mg/kg/day. The results of this study will be useful for risk assessment of groups of structurally similar alkyl quaternary ammonium surfactants.

Keywords: quaternary ammonium compound, surfactant, chemical substances control law

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Matsumoto M, Fujii S*, Hirose N, Iso T, Shigeta Y, Murata Y, Inoue K. Hirose A. Repeated-dose and reproductive/developmental toxicity screening of polyoxymethylene in rats.

*Fundam Toxicol Sci*. 2021;8(4):103-116. doi: 10.2131/fts.8.103

The Japanese government requires risk assessment of chemicals under the Chemical Substances Control Law (CSCL). Toxicity data for polyoxymethylene (paraformaldehyde; CAS No.: 30525-89-4) for human health are insufficient though the chemical needs a screening assessment under the CSCL. Thus, polyoxymethylene was selected by the Safety Examination of Existing Chemicals and Safety Programmes of the Ministry of Health, Labour and Welfare (MHLW) to assess repeated-dose and reproductive/developmental toxicity. A combined toxicity screening was conducted following the OECD TG422. Male and female rats were administered the test chemical once daily by gavage at doses of 0 (control), 20, 60, or 200 mg/kg bw from 14 days before mating for a total of 28 to 61 days. The 200 mg/kg bw/day dose caused a significant decrease in food consumption. Histopathological examination found ulcers in the forestomach and glandular stomach, and erosion and inflammatory cell infiltration in the submucosa of the glandular stomach at the end of dosing in both sexes. Inflammatory cell infiltration in the submucosa of the glandular stomach was also observed in both sexes after the recovery period. No reproductive and developmental toxicity was observed even at the highest dose. A no-observed-adverse-effect level (NOAEL) for repeated-dose toxicity was 60 mg/kg bw/day, and a NOAEL for reproductive and developmental toxicity was 200 mg/kg bw/day, the highest dose tested.

Keywords: polyoxymethylene, paraformaldehyde, chemical substances control law

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Inoue K, Shigeta Y, Umemura T*1, Nishiura H*2, Hirose A. Application of the benchmark dose method to the incidence data for various pathological findings and its validation analysis.

*Shokuhin Eiseigaku Zasshi*. 2021;62(2):56-64. doi: 10.3358/shokueishi.62.56

Benchmark dose (BMD) method have been used in the toxicological assessment of chemical substances so that the point of departure can be derived, as an alternative to the use of no observable adverse effect level (NOAEL), and the method is often applied to the incidence data of histopathological findings in the toxicity studies. In the present study, the BMD method was applied to various patterns of incidence data derived from some toxicity studies as case studies, and the validity of each application was discussed. Five independent applications including toxicity studies of madder color or semicarbazide hydrochloride were prepared and model averaging over the three models with the lowest three AIC (Akaike information criteria) values (MA-3), a recently proposed model averaging method, was employed. The series of case studies indicated, for the better application of the BMD method to histopathological findings, the following points: (i) If there are incidence data with severity grading of pathologically significant lesions, we must discuss whether the BMD method should be applied to the total incidence data or the incidence data above certain grade with or without data aggregation. (ii) If a lesion of interest had higher toxicological significance rather than the secondary lesions with higher severity, the BMD method should be applied to the incidence data of the lesion of interest. (iii) If it is highly necessary to apply the BMD method to obtained incidence data without toxicological

and statistical validity, toxicological pathologists are advised to review individual datasets of histopathology and associated data, and provide new incidence data of comprehensive findings (diagnostic name) such as hepatocellular injury or nephropathy, if possible. In all cases, toxicological significance and mechanism of a lesion of interest need to be considered in light of the dose-dependence. In view of both toxicology and statistics, sufficient discussions must be made on the validity of applying BMD method and its estimate.
Keywords: benchmark dose method, pathological finding, case study

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