Morita T, Yoshida H, Abe Y, Tomita K[*1], Nakamura A[*2], Hada C[*3], Nakai C[*4], Kina K[*5], Takahashi M[*6], Uemura N[*7], Yoneda T[*8], Yasui M[*9], Shintani Y[*10], Tomita N, Inagaki A, Izutsu KI[*11], Sato Y: Analysis of factors related to variation in dissolution profiles estimated from continuously conducted dissolution tests of generic products.

Chem Pharm Bull. 2024;72(1):28-35. doi: 10.1248/cpb.c23-00647

The development of generic pharmaceuticals involves a bioequivalence study to ensure the therapeutic equivalence of the test formulation to the original innovative product. The formulation characteristics of generic products are expected to be maintained in the long term after approval. This study analyzed the factors contributing to the changes in the dissolution profiles of approved products during their life cycles. Cumulative data on the dissolution similarity of 1675 products of 127 ingredients tested by official laboratories in Japan were assessed according to Japanese bioequivalence guidelines with slight modifications. The products showing dissimilarities in dissolution profiles were analyzed for reporting year, therapeutic category, co-development, physical properties of the active pharmaceutical ingredient (API), and suspected reasons for dissolution change. The increase in the number of dissimilar products is related to the co-development of generic products. Although the solubility of the API was not associated with the dissolution change in the analysis of the total dissolution data, control of the API particle size is suggested to be important for drugs with poorly soluble APIs. Additionally, a risk factor for dissolution changes in the test solutions at a certain pH was the presence of acidic or basic residues. These results indicate the importance of proper development through a thorough evaluation of the formulation and process factors affecting the dissolution properties throughout the product lifecycle.

Keywords: bioequivalence, dissolution study, generic pharmaceutical

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Yoshida H, Morita T, Abe Y, Inagaki A, Tomita N, Izutsu KI*, Sato Y: Effects of apex size on dissolution profiles in the USP II paddle apparatus.

AAPS PharmSciTech. 2023;25(1):9. doi: 10.1208/s12249-023-02722-5

The use of apex vessels may solve coning problems associated with dissolution testing. However, excessive dissolution acceleration can reduce the discriminatory power. This study aimed to clarify how different apex vessel sizes affect the dissolution behavior of cone-forming formulations. Five apex vessels with different heights, centralities, and compendial vessels were used. The paddle rotation speed at which the coning phenomenon resolved was measured using standard particles of different densities. Three model formulations-USP prednisone tablets, atorvastatin calcium hydrate tablets, and levofloxacin fine granules-were selected, and dissolution tests were conducted at 30-100 revolutions per minute (rpm). Compared to the compendial vessels, the disappearance of standard particles at the apex base at lower paddle speeds in apex vessels was observed. Standard particles tended to remain in the center of the apex vessels and disappear at rotational speeds comparable to those of the compendial vessels. Dissolution increased in an apex height-dependent manner in the model formulations, except for the atorvastatin calcium hydrate tablets at 50 rpm. For levofloxacin fine granules, dissolution was also improved by reducing the paddle agitation speed to 30 rpm in the compendial vessels. Differences in apex centrality by 3 mm did not affect the dissolution rate. Our results indicate that apex vessels with low apex heights have a mount-resolving effect, but the degree of dissolution improvement by avoiding the coning phenomenon

depends on the formulation characteristics used in the dissolution tests.

Keywords: apex size, coning, dissolution testing

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Han H[*1], Akiyoshi T[*1,2], Morita T, Tsuchitani T[*1], Nabeta M[*1], Yajima K[*1], Imaoka A[*1], Ohtani H[*1,2,3]: The Effects of Jabara Juice on the Intestinal Permeation of Fexofenadine.

Bio Pharm Bull. 2023;46(12):1745-1752. doi: 10.1248/bpb.b23-00479.

Jabara juice and its component narirutin inhibit the activity of organic anion-transporting polypeptides (OATPs) 1A2 and OATP2B1, which are considered to play significant roles in the intestinal absorption of fexofenadine. In this study, we investigated the effects of jabara juice on the intestinal absorption of fexofenadine in mice and the inhibitory effects of jabara juice and narirutin on the permeation of fexofenadine using Caco-2 cell monolayers and LLC-GA5-COL300 cell monolayers. In the *in vivo* study, the area under the plasma concentration-time curve (AUC) of fexofenadine in mice was increased 1.8-fold by jabara juice. In the permeation study, 5% jabara juice significantly decreased the efflux ratio (ER) of fexofenadine for Caco-2 monolayers. Furthermore, the ERs of fexofenadine and digoxin, which is a typical substrate of P-glycoprotein (P-gp), for LLC-GA5-COL300 cell monolayers were decreased in a concentration-dependent manner by jabara juice extract, suggesting that jabara juice may increase the intestinal absorption of fexofenadine by inhibiting P-gp, rather than by narirutin inhibiting OATPs. The present study showed that jabara juice increases the intestinal absorption of fexofenadine both *in vivo* and *in vitro*. The intestinal absorption of fexofenadine may be altered by the co-administration of jabara juice in the clinical setting.

Keywords: jabara juice, P-glycoprotein, food–drug interaction

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Kondo A[*], Koide T, Fukami T[*]: Evaluation of the Effect of Disintegrant Distribution on the Dissolution Behavior of Pharmaceutical Tablets Using Raman Chemical Imaging.

Chem Pharm Bull. 2023;71:454-458. *doi:* 10.1248/cpb.c22-00924

In pharmaceutics, substandard drug manufacturing can sometimes occur. Usually, end-product release tests are conducted to detect defective products, but in many cases, they are not able to identify the root causes of quality defects. In recent years, chemical imaging techniques have been widely used to study quality defects by visualizing the distribution of components in solid dosage forms. However, in most studies, the causes are predicted from images of ingredients, and the impact of each factor is unclear. In this study, we prepared model tablets and intentionally changed only the distribution of disintegrants, and visualized this distribution using the Raman chemical imaging technique to evaluate the effect on the dissolution behavior of the tablets. We found that tablet disintegration occurs completely when the amount of disintegrant is sufficient to disintegrate the tablet and is distributed throughout the tablet, even if the distribution is not uniform. In contrast, if there was a large area where the disintegrant was not present, the tablet did not disintegrate sufficiently. This suggests that it is more important that a sufficient amount of disintegrant is present throughout the tablet rather than the degree of deviation of disintegrant distribution.

Keywords: chemical imaging, Raman spectroscopy, disintegrant distribution

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Yamamoto Y[*1], Kajita M[*2], Hirose Y[*2], Shimada N[*3], Fukami T[*3], Koide T: Pharmaceutical evaluation of Levofloxacin orally disintegrating tablet formulation using low frequency Raman spectroscopy.

Pharmaceutics. 2023;15:2041. doi: 10.3390/pharmaceutics15082041

We evaluated the pharmaceutical properties of levofloxacin (LV) in the form of an orally disintegrating tablet (LV_{ODT}) to find a new usefulness of low frequency (LF) Raman spectroscopy. LV_{ODT} contained dispersed granules with diameters in the

order of several hundred micrometers, which were composed of the active pharmaceutical ingredient (API), as confirmed by infrared (IR) microspectroscopy. On the contrary, the API and inactive pharmaceutical ingredients (non-APIs) were homogeneously distributed in LV tablet (LV_T) formulations. Microscopic IR spectroscopy and thermal analyses showed that LV_{ODT} and LV_T contained the API in different crystalline forms or environment around the API each other. Furthermore, powder X-ray diffraction showed that LV_T contained a hemihydrate of the API, while LV_{ODT} showed a partial transition to the monohydrate form. This result was confirmed by microscopic LF Raman spectroscopy. Moreover, this method confirmed the presence of thin layers coating the outer edges of the granules that contained the API. Spectra obtained from these thin layers indicated the presence of titanium dioxide, suggesting that the layers coexisted with a polymer that masks the bitterness of API. The microscopic LF Raman spectroscopy results in this study indicated new applications of this method in pharmaceutical science.

Keywords: low frequency, Raman spectroscopy, levofloxacin

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Ohashi R*1,2, Koide T, Fukami T*1: Effects of wet granulation process variables on the quantitative assay model of transmission Raman spectroscopy for pharmaceutical tablets.

Euro J Pharm Biopharm. 2023;191:276–289 doi: 10.1016/j.ejpb.2023.09.009

Transmission Raman spectroscopy (TRS) is a process analytical technology tool for nondestructive analysis of drug content in tablets. Although wet granulation is the most used tablet manufacturing method, most TRS studies have focused on tablets manufactured via direct compression. The effects of upstream process parameter variations, such as granulation, on the prediction performance of TRS quantitative models are unknown. We evaluated the effects of process parameter variations during granulation on the prediction performance of the TRS quantitative model. Tablets with a drug concentration of 1%w/w were used. We developed PLS calibration models for the drug concentration range of 70–130% label claims. Subsequently, we predicted the drug content of the tablets with different granulation parameters. The results of our study demonstrate that the variation in the predicted recovery due to the variation in granulation parameters was practically acceptable. The calibration model showed a good prediction performance for tablets manufactured at different granulation scales and thicknesses. Therefore, we conclude that TRS quantitative models are robust to variations in upstream processes, such as granulation and downstream variations in tableting parameters. These results suggest that TRS is a versatile non-destructive quantitative analysis method that can be applied in tablet manufacturing.

Keywords: wet granulation, transmission Raman spectroscopy, quantitative assay model

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Miyazaki T, Mizoguchi R*1, Ueda K*2, Shinozaki T*3, Kamoto M*4, Takeda Y*5, Sakuma S*6, Ito N*7, Momo M*8, Kawakami K*9: Crystallization of amorphous nifedipine under isothermal conditions: Inter-laboratory reproducibility and investigation of the factors affecting reproducibility.

J Pharm Sci. 2023;112(10):2703-2716. doi: 10.1016/j.xphs.2023.06.002

High inter-laboratory reproducibility is required for conducting collaborative experiments among several laboratories. The primary aim of our evaluation of the physical stability of amorphous drugs, conducted in co-operation with eight laboratories, was to establish a protocol for isothermal storage tests to obtain data of the same quality from all the participating laboratories. Sharing a protocol that contained the same level of detail as the experimental section of general papers was insufficient for high inter-laboratory reproducibility. We investigated the causes of variations in the data from the various laboratories and restricted the protocol step-by-step to achieve high inter-laboratory reproducibility. The various experimentalists had very different levels of awareness regarding how to control the temperature of a sample

as the samples were transferred into and out of thermostatic chambers. Specific instructions on how to conduct this operation, such as regarding the time required for the transfer and thermal protection of the container during the transfer, helped to reduce variation. Improved inter-laboratory reproducibility revealed that the physical stabilities of amorphous drugs differed when samples were prepared in differently shaped aluminum pans designed for various differential scanning calorimeters.

Keywords: amorphous, crystallization, reproducibility

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Ando D, Ozawa A*1, Sakaue M*1, Yamamoto E, Miyazaki T, Sato Y, Koide T, Izutsu K*2: Fabrication and Characterization of Dissolving Microneedles for Transdermal Drug Delivery of Apomorphine Hydrochloride in Parkinson's Disease.
Pharm Res. 2024;41(1):153-163. doi: 10.1007/s11095-023-03621-x

Purpose: We fabricated and characterized polyvinyl alcohol (PVA)-based dissolving microneedles (MNs) for transdermal drug delivery of apomorphine hydrochloride (APO), which is used in treating the wearing-off phenomenon observed in Parkinson's disease. Methods: We fabricated MN arrays with 11 × 11 needles of four different lengths (300, 600, 900, and 1200 μm) by micromolding. The APO-loaded dissolving MNs were characterized in terms of their physicochemical and functional properties. We also compared the pharmacokinetic parameters after drug administration using MNs with those after subcutaneous injection by analyzing the blood concentration of APO in rats.

Results: PVA-based dissolving MNs longer than 600 μm could effectively puncture the stratum corneum of the rat skin with penetrability of approximately one-third of the needle length. Although APO is known to have chemical stability issues in aqueous solutions, the drug content in APO-loaded MNs was retained at 25° C for 12 weeks. The concentration of APO after the administration of APO-loaded 600-μm MNs that dissolved completely in skin within 60 min was 81%. The absorption of 200-μg APO delivered by MNs showed a T_{max} of 20 min, C_{max} of 76 ng/mL, and $AUC_{0\text{-}120}$ min of 2,829 ng・min/mL, compared with a T_{max} of 5 min, C_{max} of 126 ng/mL, and $AUC_{0\text{-}120}$ min of 3,224 ng・min/mL for subcutaneous injection. The bioavailability in terms of $AUC_{0\text{-}120}$ min of APO delivered by MNs was 88%. Conclusion: APO-loaded dissolving MNs can deliver APO via skin into the systemic circulation with rapid absorption and high bioavailability.

Keywords: apomorphine hydrochloride, dissolving microneedle array, transdermal drug delivery system

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Ando D, Miyatsuji M, Sakoda H, Yamamoto E, Miyazaki T, Koide T, Sato Y, Izutsu K*: Mechanical Characterization of Dissolving Microneedles: Factors Affecting Physical Strength of Needles.
Pharmaceutics. 2024;16(2):200. doi: 10.3390/pharmaceutics16020200

Dissolving microneedles (MNs) are novel transdermal drug delivery systems that can be painlessly self-administered. This study investigated the effects of experimental conditions on the mechanical characterization of dissolving MNs for quality evaluation. Micromolding was used to fabricate polyvinyl alcohol (PVA)-based dissolving MN patches with eight different cone-shaped geometries. Axial force mechanical characterization test conditions, in terms of compression speed and the number of compression needles per test, significantly affected the needle fracture force of dissolving MNs. Characterization using selected test conditions clearly showed differences in the needle fracture force of dissolving MNs prepared under various conditions. PVA-based MNs were divided into two groups that showed buckling and unbuckling deformation, which occurred at aspect ratios (needle height/base

diameter) of 2.8 and 1.8, respectively. The needle fracture force of PVA-based MNs was negatively correlated with an increase in the needle's aspect ratio. Higher residual water or higher loading of lidocaine hydrochloride significantly decreased the needle fracture force. Therefore, setting appropriate methods and parameters for characterizing the mechanical properties of dissolving MNs should contribute to the development and supply of appropriate products.

Keywords: dissolving microneedles, mechanical characterization, quality control

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Takechi-Haraya Y, Ohgita T[*1], Usui A, Nishitsuji K[*2], Uchimura K[*3], Abe Y, Kawano R[*4], Konaklieva MI[*5], Reimund M[*6], Remaley AT[*6], Sato Y, Izutsu K[*7], Saito H[*1]: Structural flexibility of apolipoprotein E-derived arginine-rich peptides improves their cell penetration capability.

Scientific Reports. 2023;13:19396. doi: 10.1038/s41598-023-46754-0

Amphipathic arginine-rich peptide, A2-17, exhibits moderate perturbation of lipid membranes and the highest cell penetration among its structural isomers. We investigated the direct cell-membrane penetration mechanism of the A2-17 peptide while focusing on structural flexibility. We designed conformationally constrained versions of A2-17, stapled (StpA2-17) and stitched (StchA2-17), whose α-helical conformations were stabilized by chemical crosslinking. Circular dichroism confirmed that StpA2-17 and StchA2-17 had higher α-helix content than A2-17 in aqueous solution. Upon liposome binding, only A2-17 exhibited a coil-to-helix transition. Confocal microscopy revealed that A2-17 had higher cell penetration efficiency than StpA2-17, whereas StchA2-17 remained on the cell membrane without cell penetration. Although the tryptophan fluorescence analysis suggested that A2-17 and its analogs had similar membrane-insertion positions between the interface and hydrophobic core, StchA2-17 exhibited a higher membrane affinity than A2-17 or StpA2-17. Atomic force microscopy demonstrated that A2-17 reduced the mechanical rigidity of liposomes to a greater extent than StpA2-17 and StchA2-17. Finally, electrophysiological analysis showed that A2-17 induced a higher charge influx through transient pores in a planer lipid bilayer than StpA2-17 and StchA2-17. These findings indicate that structural flexibility, which enables diverse conformations of A2-17, leads to a membrane perturbation mode that contributes to cell membrane penetration.

Keywords: amphipathic arginine-rich peptide, cell penetration, membrane perturbation

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Inoue M[*1], Tsuji Y[*1], Ueno R[*1], Miyamoto D[*1], Tanaka K[*1], Moriyasu Y[*1], Shibata S[*1], Okuda M[*1], Ando D, Abe Y, Kamada H[*2], Tsunoda S[*1]: Bivalent structure of a TNFR2-selective and agonistic TNF-alpha mutein Fc-fusion protein enhances the expansion activity of regulatory T cells.

Sci Rep. 2023;13(1):13762. doi: 10.1038/s41598-023-40925-9

Recently, TNF receptor type 2 (TNFR2) signaling was found to be involved in the proliferation and activation of regulatory T cells (Tregs), a subpopulation of lymphocytes that suppress immune responses. Tregs mediate peripheral immune tolerance, and the disruption of their functions causes autoimmune diseases or allergy. Therefore, cell expanders or regulators of Tregs that control immunosuppressive activity can be used to treat these diseases. We focused on TNFR2, which is preferentially expressed on Tregs, and created tumor necrosis factor-α (TNF-α) muteins that selectively activate TNFR2 signaling in mice and humans, termed R2agoTNF and R2-7, respectively. In this study, we attempted to optimize the structure of muteins to enhance their TNFR2 agonistic activity and stability *in vivo* by IgG-Fc fusion following single-chain homo-trimerization. The fusion protein, scR2agoTNF-Fc, enhanced the expansion of $CD4^+CD25^+$ Tregs and $CD4^+Foxp3^+$ Tregs and contributed to their immunosuppressive activity *ex vivo* and *in vivo* in mice. The prophylactic administration of scR2agoTNF-

Fc suppressed inflammation in contact hypersensitivity and arthritis mouse models. Furthermore, scR2-7-Fc preferentially expanded Tregs in human peripheral blood mononuclear cells via TNFR2. These TNFR2 agonist-Fc fusion proteins, which have bivalent structures, are novel Treg expanders.
Keywords: biologics, inflammation, immunotherapy

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Inoue M*1, Tsuji Y*1, Kashiwada A*1, Yokoyama A*1, Iwata A*1, Abe Y, Kamada H*2, Tsunoda S*1: An immunocytokine consisting of a TNFR2 agonist and TNFR2 scFv enhances the expansion of regulatory T cells through TNFR2 clustering.
Biochem Biophys Res Commun. 2024;697:149498. doi: 10.1016/j.bbrc.2024.149498

Regulatory T cells (Tregs) are lymphocytes that play a central role in peripheral immune tolerance. Tregs are promising targets for the prevention and suppression of autoimmune diseases, allergies, and graft-versus-host disease, and treatments aimed at regulating their functions are being developed. In this study, we created a new modality consisting of a protein molecule that suppressed excessive immune responses by effectively and preferentially expanding Tregs. Recent studies reported that tumor necrosis factor receptor type 2 (TNFR2) expressed on Tregs is involved in the proliferation and activation of Tregs. Therefore, we created a functional immunocytokine, named TNFR2-ICK-Ig, consisting of a fusion protein of an anti-TNFR2 single-chain Fv (scFv) and a TNFR2 agonist TNF-α mutant protein, as a new modality that strongly enhances TNFR2 signaling. The formation of agonist-receptor multimerization (TNFR2 cluster) is effective for the induction of a strong TNFR2 signal, similar to the TNFR2 signaling mechanism exhibited by membrane-bound TNF. TNFR2-ICK-Ig improved the TNFR2 signaling activity and promoted TNFR2 cluster formation compared to a TNFR2 agonist TNF-α mutant protein that did not have an immunocytokine structure. Furthermore, the Treg expansion efficiency was enhanced. TNFR2-ICK-Ig promotes its effects via scFv, which crosslinks receptors whereas the agonists transmit stimulatory signals. Therefore, this novel molecule expands Tregs via strong TNFR2 signaling by the formation of TNFR2 clustering.
Keywords: immunocytokine, regulatory T cell, TNF receptor type 2

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Shibata H, Nishimura K, Saito Y, Ishii-Watabe A: Comparison of Immunochemical Reactions of Infliximab Innovator and Biosimilars on an Infliximab Detection Kit Used for Therapeutic Drug Monitoring.
Biol Pharm Bull. 2023;46(4):621-629. doi: 10.1248/bpb.b22-00830

Monitoring serum infliximab (INF) concentrations is crucial for designing appropriate doses for patients with rheumatoid arthritis. It is recommended to maintain the serum trough INF level at least 1.0 μg/mL. In Japan, an *in vitro* diagnostic kit using immunochromatography has been approved to determine whether the serum INF concentration is over 1.0 μg/mL or not, and to support the determination of the necessity of increasing the dose or switching to another drug. Biosimilars (BS) of INF may have immunochemical properties different from those of its innovator product, which may show different reactivities on the diagnostic kit. In this study, the responses of the innovator and five BS products on the kit were compared. Based on visually comparing the intensity of color development between the test and control samples, differences were found in the judgment results depending on the analyst. In particular, 1.0 μg/mL was not determined as positive in some cases, whereas 2.0 μg/mL was reliably determined as positive. Overall, no significant difference in reactivity was found between the innovator and five BS products. To further compare the differences in immunochemical properties, the reactivity of these products with three enzyme-linked immunosorbent assay (ELISA) kits was compared. The results confirmed that there were no significant differences among the innovator and BS products in reactivity with the examined kits. When using that diagnostic kit, the users need to be aware that the judgement around 1.0 μg/mL INF may differ depending on the test conditions, including the analyst.
Keywords: Infliximab biosimilar, therapeutic drug

monitoring, immunochromatography

Tada M, Aoyama M, Ishii-Watabe A: Characterization of anti-SARS-CoV-2 monoclonal antibodies focusing on antigen binding, neutralization, and FcγR activation via formation of immune complex.

MAbs. 2023;15(1):2222874. doi: 10.1080/19420862.2023.2222874

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19). Antibodies induced by SARS-CoV-2 infection or vaccination play pivotal roles in the body's defense against the virus; many monoclonal antibodies (mAbs) against SARS-CoV-2 have been cloned, and some neutralizing mAbs have been used as therapeutic drugs. In this study, we prepared an antibody panel consisting of 31 clones of anti-SARS-CoV-2 mAbs and analyzed and compared their biological activities. The mAbs used in this study were classified into different binding classes based on their binding epitopes and showed binding to the SARS-CoV-2 spike protein in different binding kinetics. A multiplex assay using the spike proteins of Alpha, Beta, Gamma, Delta, and Omicron variants clearly showed the different effects of variant mutations on the binding and neutralization activities of different binding classes of mAbs. In addition, we evaluated Fcγ receptor (FcγR) activation by immune complexes consisting of anti-SARS-CoV-2 mAb and SARS-CoV-2 pseudo-typed virus, and revealed differences in the FcγR activation properties among the binding classes of anti-SARS-CoV-2 mAbs. It has been reported that FcγR-mediated immune-cell activation by immune complexes is involved in the promotion of immunopathology of COVID-19; therefore, differences in the FcγR-activation properties of anti-SARS-CoV-2 mAbs are among the most important characteristics when considering the clinical impacts of anti-SARS-CoV-2 mAbs.

Keywords: biological activities, Fcγ receptor, anti-SARS-CoV-2 mAb

Tada M, Aoyama M, Ishii-Watabe A: Target-independent Immune-cell Activation by Aggregates of T Cell-redirecting Bispecific Antibodies.

J Pharm Sci. 2023; 112(9): 2419-2426. doi: 10.1016/j.xphs.2023.06.016

T cell-redirecting bispecific antibodies (bsAbs) have been under development as a new class of biotherapeutics for cancer immunotherapy. T cell-redirecting bsAbs simultaneously bind tumor-associated antigens on tumor cells and CD3 on T cells, resulting in T cell-mediated cytotoxicity against tumor cells. In this study, we prepared a tandem scFv-typed bsAb targeting HER2 and CD3 (HER2-CD3), and evaluated the impact of aggregation of HER2-CD3 on the *in vitro* immunotoxicity. A cell-based assay using CD3-expressing reporter cells revealed that the aggregates of HER2-CD3 directly activated CD3-expressing immune cells in the absence of target antigen (HER2)-expressing cells. Comparison of the aggregates generated under various stress conditions indicated the possibility that insoluble protein particles, which were detected by qLD analysis and contained non-denatured functional domains, contributed to the activation of CD3-expressing immune cells. In addition, HER2-CD3 aggregates stimulated hPBMCs and strongly induced the secretion of inflammatory cytokines and chemokines. The cytokine/chemokine-release profiles suggested that the aggregates could induce inflammatory responses not only by CD3-mediated T cell activation but also by other immune cell activations. These results indicated the potential risk of aggregation of T cell-redirecting bsAbs, which could induce unwanted immune cell activation and inflammation and thereby immune-mediated adverse reactions.

Keywords: antibody drug, protein aggregation, immune response

Miyajima R[*1], Manaka H[*1], Honda T[*2], Hashii N, Suzuki M[*1], Komeno M[*1], Takao K[*3], Ishii-Watabe A, Igarashi K[*2], Toida T[*2], Higashi K[*1]: Intracellular polyamine depletion induces N-linked galactosylation of the monoclonal antibody produced by CHO DP-12 cells.

J Biotechnol. 2023;378:1-10. doi: 10.1016/j.jbiotec.2023.10.008

The heterogeneity of the N-linked glycan profile of therapeutic monoclonal antibodies (mAbs) derived from animal cells affects therapeutic efficacy and, therefore, needs to be appropriately controlled during the manufacturing process. In this study, we examined

the effects of polyamines on the N-linked glycan profiles of mAbs produced by CHO DP-12 cells. Normal cell growth of CHO DP-12 cells and their growth arrest by α-difluoromethylornithine (DFMO), an inhibitor of the polyamine biosynthetic pathway, was observed when 0.5% fetal bovine serum was added to serum-free medium, despite the presence of cadaverine and aminopropylcadaverine, instead of putrescine and spermidine in cells. Polyamine depletion by DFMO increased IgG galactosylation, accompanied by β1,4-galactosyl transferase 1 (B4GAT1) mRNA elevation. Additionally, IgG production in polyamine-depleted cells was reduced by 30% compared to that in control cells. Therefore, we examined whether polyamine depletion induces an ER stress response. The results indicated increased expression levels of chaperones for glycoprotein folding in polyamine-depleted cells, suggesting that polyamine depletion causes ER stress related to glycoprotein folding. The effect of tunicamycin, an ER stress inducer that inhibits N-glycosylation, on the expression of B4GALT1 mRNA was examined. Tunicamycin treatment increased B4GALT1 mRNA expression. These results suggest that ER stress caused by polyamine depletion induces B4GALT1 mRNA expression, resulting in increased IgG galactosylation in CHO cells. Thus, introducing polyamines, particularly SPD, to serum-free CHO culture medium for CHO cells may contribute to consistent manufacturing and quality control of antibody production.

Keywords: ER stress, galactosylation, monoclonal antibody

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橋井則貴，蛭田葉子，林真由美*，海老澤亜樹子*，中川ゆかり*，石井明子: 日局医薬品各条合成グルカゴンの定量法等に関する研究.
医薬品医療機器レギュラトリーサイエンス. 2023;54:428-38. doi: 10.51018/pmdrs.54.5_428

In Japan, two types of glucagon products, one containing recombinant glucagon and the other containing synthetic glucagon, have been approved and marketed. On June 7th, 2021, the Glucagon (Genetical Recombination) monograph was newly listed in the eighteenth edition of the Japanese Pharmacopeia (JP). In addition, Synthetic Glucagon has been listed as a new candidate monograph of JP. For the approval of synthetic glucagon, *in vivo* bioassay has been adopted as an assay method, although high-performance liquid chromatography (HPLC) has been designated for the assay and purity test in the JP Glucagon (Genetical Recombination) monograph. The HPLC method has also been adopted in the Glucagon, Human monograph in the European Pharmacopeia, and the Glucagon monograph in the United States Pharmacopeia. Therefore, an HPLC method for synthetic glucagon is required as an alternative to bioassay from the viewpoints of animal welfare and harmonization of analytical methods between the Synthetic Glucagon and Glucagon (Genetical Recombination) monographs in JP. In this study, we demonstrate that the HPLC assay method described in the Glucagon (Genetical Recombination) monograph is applicable to synthetic glucagon.

Keywords: synthetic glucagon, Glucagon (Genetical recombination), glucagon assay

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Yamaguchi K*1, 2, †, Nakayama J*1, 3, 4, †, Yamamoto T†, Semba K*4, 5, Shirota T*2, Yamamoto Y*1: Collagen induction of immune cells in the mammary glands during pregnancy.
Physiol Genomics., 2024;56:128-135. doi: 10.1152/physiolgenomics.00098.2023

The mammary glands are dynamic tissues affected by pregnancy-related hormones during the pregnancy-lactation cycle. Collagen production and its dynamics are essential to the remodeling of the mammary glands. Alterations of the mammary microenvironment and stromal cells during the pregnancy-lactation cycle are important for understanding the physiology of the mammary glands and the development of breast tumors. In this study, we performed an evaluation of collagen dynamics in the mammary fat pad during the pregnancy-lactation cycle. Reanalysis of single-cell RNA-sequencing (scRNA-Seq) data showed the ectopic collagen expression in the immune cells and cell-cell interactions for collagens with single-cell resolution. The scRNA-Seq data showed that type I

and type III collagen were produced not only by stromal fibroblasts but also by lymphoid and myeloid cell types in the pregnancy phase. Furthermore, the total cell-cell interaction score for collagen interactions was dramatically increased in the pregnancy tissue. The data presented in this study provide evidence that immune cells contribute, at least in part, to mammary collagen dynamics. Our findings suggest that immune cells, including lymphoid and myeloid cells, might be supportive members of the extracellular matrix orchestration in the pregnancy-lactation cycle of the mammary glands.

Keywords: collagen, immune cells, mammary gland,

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Kiyoshi M, Nakakido M*1, Rafique A*2, Tada M, Aoyama M, Terao Y*3, Nagatoishi S*1,4, Shibata H, Ide T*3, Tsumoto K*1,4, Ito Y*2 & Ishii-Watabe A: Specific peptide conjugation to a therapeutic antibody leads to enhanced therapeutic potency and thermal stability by reduced Fc dynamics.

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Antibody–drug conjugates are powerful tools for combatting a wide array of cancers. Drug conjugation to a therapeutic antibody often alters molecular characteristics, such as hydrophobicity and effector function, resulting in quality deterioration. To develop a drug conjugation methodology that maintains the molecular characteristics of the antibody, we engineered a specific peptide for conjugation to the Fc region. We used trastuzumab and the chelator (DOTA) as model antibody and payload, respectively. Interestingly, peptide/DOTA-conjugated trastuzumab exhibited enhanced antibody-dependent cellular cytotoxicity (ADCC) and increased thermal stability. Detailed structural and thermodynamic analysis clarified that the conjugated peptide blocks the Fc dynamics like a "wedge." We revealed that (1) decreased molecular entropy results in enhanced ADCC, and (2) blockade of Fc denaturation results in increased thermal stability. Thus, we believe that our methodology is superior not only for drug conjugation but also as for reinforcing therapeutic antibodies to enhance ADCC and thermal stability.

Keywords: Antibody drug conjugates, peptide, engineering

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Ogihara T*1,2, Kagawa M*1,2, Yamanaka R*1,2, Imai S*1,2, Itohara K*1,2, Hira D*1, Nakagawa S*1, Yonezawa A*1,2, Ito M, Nakagawa T*1, Terada T*1, Matsubara K*1,3:: Content and distribution of prunasin in *Perilla frutescens*

J Nat Med. 2023;77:207-218. DOI: 10.1007/s11418-022-01654-x

Chemotherapy-induced oral mucositis (COM) is a common adverse effect of cancer chemotherapy. Several clinical studies reported that repetitive use of mouthwashes containing 2.5-6.25% Hangeshashinto (HST), a Kampo formula, relieves COM, but the effect is insufficient. To solve this problem, we produced an oral ointment of 12% HST extract (considered quantitatively equivalent to 20% commercially available HST), which will increase the local concentrations of its active ingredients and prolong the contact time with COM. In this study, we evaluated the pharmaceutical properties (spreadability and stability) of HST oral ointment. In addition, its safety (oral mucosal irritation) and therapeutic effects on 5-fluorouracil-induced oral mucositis were evaluated in male Syrian hamsters. The HST ointment showed good spreadability and stability for more than 8 weeks at 4℃. In the oral mucosal irritation test, topical application of HST ointment (0.2 g) three times per day for 14 days had no adverse effect on the oral mucosa of hamsters. In hamsters treated with 5-fluorouracil (60 mg/kg) twice, COM was induced by a submucosal injection of 5% acetic acid into the cheek pouch. When HST ointment (50 μg) was topically applied to the mucositis area once per day for 12 days, the area and macroscopic score of mucositis were significantly decreased, and the depth of the wound

tended to be reduced compared with the lactose ointment-treated control animals. These findings suggest that HST oral ointment shows good properties in spreadability, stability, and safety, and elicits a therapeutic effect in an animal model of COM.

Keywords: 5-fluorouracil, chemotherapy-induced oral mucositis, Hangeshashinto

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Akatsuka R*1, 2, Ito M: Content and distribution of prunasin in *Perilla frutescens*

J Nat Med. 2023;77:207-218. DOI: 10.1007/s11418-022-01654-x

Perilla frutescens var. *crispa* (Lamiaceae) is an annual plant that is the botanical origin of the natural medicine "Soyo" listed in the Japanese Pharmacopoeia and is also used as a fragrant vegetable. Its characteristic components are essential oils and anthocyanins. Cyanogenic glycosides have also been isolated from perilla, but no reports have clarified which cyanogenic glycosides are abundant or differences in cyanogenic glycoside content according to the extent of perilla leaf growth or growth stage. Here, for the first time we determined the content and distributions of cyanogenic glycosides in perilla. The picric acid test, a common qualitative test for cyanogenic compounds, was used to quickly and semi-quantitatively detect cyanogenic compounds in perilla. Prunasin was the most abundant cyanogenic glycoside. The prunasin content per unit mass of perilla leaves varied by strain, regardless of leaf color or the main compound in the essential oils of each strain. Prunasin was higher in fresh leaves than in dried leaves and higher in young leaves than in mature leaves. When perilla was cultivated in an outdoor field, the prunasin content was initially high during the vegetative stage in summer before decreasing and then increasing until flower buds were beginning to form, and then gradually decreased again after flowering.

Keywords: *Perilla frutescens*, cyanogenic glycoside, HPLC analysis

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Deguchi Y*1, 2, Ito M: Investigation of microsatellite loci for the identification of registered varieties of *Perilla frutescens* and a discussion on the ancestor species of *P. frutescens*.

J Nat Med. 2023;77:412-420. DOI: 10.1007/s11418-022-01676-5

Techniques for identifying varieties of crops used as spices and food additives have important implications for the safety of food production, prevention of false labeling, protection of breeders' rights, and prevention of theft or outflow to other countries. Presently, there are 16 varieties of *Perilla frutescens* in the variety registration system of the Ministry of Agriculture, Forestry and Fishes in Japan (Ministry of Agriculture, Forestry and Fisheries. Variety registration data search. http://www.hinshu2.maff.go.jp/. Accessed 03 Nov 2022). One such variety is "Shimoadachi," which contains citral as a main essential oil component and has a lemon-like smell. To our knowledge, no other cultivars with similar characteristics in P. frutescens have been identified. Additionally, the registered variety "per-001" contains high contents of perillaldehyde and rosmarinic acid, with practical applications for herbal medicines and functional foods. Therefore, the development of variety identification techniques is necessary for stable production and protection. In this study, we investigated microsatellite loci for the accurate identification of registered varieties of red perilla. These loci provide a basis for breeding superior varieties of medicinal plants.

Keywords: microsatellite, *Perilla frutescens*, variety identification

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Dougnon G*, Ito M: Molecular descriptors and QSAR models for sedative activity of sesquiterpenes administered to mice via inhalation.

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

Keywords: QSAR, essential oil, inhalation

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中永絵理*，石原理恵*，居村克弥*，大井逸輝*，岡坂衛*，河端昭子*，寒川訓明*，嶋田康男*，田上貴臣*，西尾雅世*，野村涼坪*，山本豊*，横倉胤夫*，伊藤美千穂，酒井英二*，松田久司*：オンジについて：HPLC によるテヌイホリン分析法の検討と市場品の分析.

生薬学雑誌 2023;77:57-68.

POLYGALAE RADIX is defined as root or root bark of *Polygala tenuifolia* Willdenow (*Polygalaceae*) in Japanese Pharmacopoeia Eighteenth Edition (JP18). POLYGALAE RADIX is dispensed in Lampo prescriptions as Kihito, Kamiuntanto, and Ninjinyoeito. A method for analysis of tenuifolin, a marker compound of POLYGALAE RADIX was elaborated based on a method described in Hong Kong Chinese Materia Media Standards. Tenuifolin contents of market samples were measured using this method. Different parts of the sample, i.e. root bark and xylem and samples of different horizontal-section diameters were compared for their tenuifolin contents.

Keywords: POLYGALAE RADIX, tenuifolin, quantitative analysis

* 生薬品質集談会

大島朋子*，伊藤美千穂：五苓散エキスの異なる製法による成分組成の比較.

生薬学雑誌 2023;77:83-89.

Kampo dry extracts are made by mixing crude drugs as prescribed prior to decoction, while European dry extracts are made by mixing after separately decocting more than one crude drug. Since there is no definitive known difference in the quality of the two extract production methods, we investigated differences between methods in resultant component content by comparing these two types of extract using HPLC. In this study, we adopted Goreisan as the Kampo formula to be investigated. We found that some components of Goreisan differed significantly between the two production methods. In addition, the content of cinnamic acid, the quantitative index method are changed.

Keywords: Kampo extract, Goreisan, decocting method

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Mulyaningsih T*[1], Sunarwidhi AL*[2], Febrianti V*[1], Sari Bq. P*[1], Muspiah A*[1], Sukenti K*[1], Hadi S*[3], Ito M, Yamada I*[4]: Leaf morphoanatomical character variation of *Gyrinops* and *Aquilaria* (Thymelaeaceae) in Indonesia region at east Wallace line.

Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology. 2023;157:473-486.

DOI: 10.1080/11263504.2023.2165560

There are seven species of *Gyrinops* and two species of *Aquilaria* (Thymelaeaceae), which are distributed in Eastern Indonesia at the east of the Wallace line. The anatomical character of the paradermal tissue, midrib and petiole were the important characteristics possessed by plants that can be used as data for identification. The purpose of this article was to study leaf morphoanatomical character variation of *Gyrinops* and *Aquilaria* in Indonesia Region at East Wallace Line. The *Gyrinops* specimen used in the research was the collection of the Agarwood Study Center, Universitas Mataram, Mataram, Indonesia. The paradermal slide was made with the whole-mount slide method and the slide cross-section of midrib and petiole was made by using a hand-free section. Their preparation process used a permanent slide method and mounted with glycerine jelly. Based on the results of the research conducted, it can be seen that the anatomical characters of paradermal tissues, midrib and petiole of *Gyrinops* and *Aquilaria* have general characteristics such as hypostomatic leaves, stomata type anomocytic, non-glandular and unicellular trichome. While other characters of structure paradermal, midrib and petiole could be used as a taxonomy diagnostic marker to identify at the level genus and species on *Gyrinops* and *Aqilaria*.

Keywords: *Aquilaria*, *Gyrinops*, Indonesia

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Ito H*, Ito M: Genetic diversity of *Panax ginseng* cultivated in Japan and its relation with some plant characteristics.

J. Nat. Med. 2024;78:91-99. DOI: 10.1007/s11418-023-01747-1

In East Asia, *Panax ginseng* is one of the most important medicinal plants and has been used in traditional medicines from ancient times. Today, *P. ginseng* is cultivated in Korea, China, and Japan. Although the genetic diversity of *P. ginseng* in Korea and China has been reported previously, that of *P. ginseng* cultivated in Japan is largely unknown. In the present study, genetic diversity of *P. ginseng* cultivated in Japan was analyzed using eight simple sequence repeat markers that have been used in other studies, and the results were compared with previous results for Korea and China. The correlation between genetic diversity and plant characteristics, such as ginsenoside contents, were also examined. The genetic diversity of *P. ginseng* in Japan was substantially different from that in Korea and China, probably due to Japan's history of cultivation and the ginseng reproduction system of agamospermy. The genetic analysis indicated that *P. ginseng* cultivated in Japan could be classified into two clusters. The classification was related to the contents of ginsenosides Re and Ro in the main root but not to the cultivation region of the samples. These results may be useful for the cultivation and quality control of *P. ginseng* in Japan.

Keywords: genetic diversity, ginsenoside, *Panax ginseng*

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伊藤ほのか*，伊藤美千穂：オタネニンジンの主根に含まれるギンセノシド類の局在と経年劣化.

生薬学雑誌 2024;78:115-119.

Several researchers have reported changes in the ginsenoside content of *Panax ginseng* during its cultivation period, but their results were not consistent. This may be explained in part by root diameter or localization of ginsenosides in the main root. In the present study, the ginsenoside content in the periderm and inner part of the main root were analyzed and compared between different ages. The results showed higher ginsenoside content in the periderm than in the inner part of the main root and the compositions of ginsenosides were different between these parts. Ginsenosides content was the highest in 1-year-old root and lowest in 2-year-old root. In 3- to 6-year-old roots, the ginsenoside content was almost the same.

Keywords: *Panax ginseng*, ginsenoside, root diameter

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Tsuge A, Goto Y, Masada S, Ito M: Resultant compound from sublimation test for Gentianae Radix in Japanese Pharmacopoeia was 5-(hydroxymethyl) furfural.

J. Nat. Med. 2024;78:799-802. doi:10.1007/s11418-024-

01802-5.

Gentianae Radix, an herbal medicine, has been used as a gastrointestinal drug in Japan. In the Japanese Pharmacopoeia 18th Revision, the sublimation test is specified as an identification test for Gentianae Radix. The compound obtained in this sublimation test was believed to be gentisin, a xanthone family compound. However, the compound we identified using liquid chromatography-high-resolution mass spectrometry (LC-HRMS) and 1H- and 13C-NMR was 5-(hydroxymethyl)furfural (5-HMF). The same compound was found to be a sublimate of Gentianae Scabrae Radix and Gentianae Macrophyllae Radix, belonging to the same genus as Gentianae Radix. These results indicate the necessity to revise the identification test for Gentianae Radix to a more unique method.

Keywords: Gentianae Radix, sublimation test, 5-(hydroxymethyl)furfural

Dong Y*1, Toume K*1, Kimijima S*1, Zhang H*1, Zhu S*1,2, He Y*1,3, Cai S*4, Maruyama T, Komatsu K*1:
Metabolite profiling of Drynariae Rhizoma using 1H NMR and HPLC coupled with multivariate statistical analysis

J. Nat. Med. 2023;77:839-857. doi: 10.1007/s11418-023-01726-6

Drynariae Rhizoma has been used to treat bone diseases and kidney deficiency in traditional medicine. Recently its aqueous extract was reported to enhance memory function. Although the Japanese standards for non-Pharmacopoeial crude drugs 2022 prescribed *Drynaria roosii* as the botanical origin, some counterfeits and both raw and stir-fired crude drugs are available in markets. To distinguish Drynariae Rhizoma derived from *D. roosii* appropriately from others and verify the validity of uses of stir-fried ones, ^{1}H NMR-based metabolite profiling coupled with HPLC were performed. Raw samples derived from *D. roosii* contained naringin (**1**), neoeriocitrin (**2**), 5,7-dihydroxychromone-7-*O*-neohesperidoside (**3**), caffeic acid 4-*O*-β-D-glucoside (**4**), protocatechuic acid (**5**), trans-*p*-coumaric acid 4-*O*-β-D-glucoside (**6**), and kaempferol 3-*O*-α-L-rhamnoside 7-*O*-β-D-glucoside (**8**). Stir-fried samples were characterized by presence of 5-hydroxymethyl-2-furaldehyde (**13**), and were divided into two types; one possessing similar composition to raw samples (Type I) and another without above components except **5** (Type II). Quantitative analyses using qHNMR and HPLC, followed by principal component analysis demonstrated that the raw samples had higher contents of **1** (0.93–9.86 mg/g), **2** (0.74–7.59 mg/g), **3**(0.05–2.48 mg/g), **4**(0.27–2.51 mg/g), **6** (0.14–1.26 mg/g), and **8** (0.04–0.52 mg/g), and Type II had a higher content of **5** (0.84–1.32 mg/g). The counterfeit samples derived from Araiostegia divaricate var. formosana were characterized by higher content of (−)-epicatechin 3-*O*-β-D-allopyranoside (**10**) (1.44–11.49 mg/g) without **1** and **2**. These results suggested that Drynariae Rhizoma samples derived from other botanical origins and Type II stir-fried samples cannot substitute for *D. roosii* rhizome.

Keywords: Drynariae Rhizoma, HPLC, multivariate analysis

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Kitazoe T*1, Usui C*1, Kodaira E*1, Maruyama T, Kawano N*2, Fuchino H.*2, Yamamoto K.*2, Kitano Y.*3, Kawahara N.*2,4, Yoshimatsu K.*2, Shirahata T*1, Kobayashi Y*1:
Improved quantitative analysis of tenuifolin using hydrolytic continuous-flow system to build prediction models for its content based on near-infrared spectroscopy

J. Nat. Med. 2024;78:296-311. doi: 10.1007/s11418-023-01764-0

This study used two types of analyses and statistical calculations on powdered samples of Polygala root (PR) and Senega root (SR): (1) determination of saponin content by an independently developed quantitative analysis of tenuifolin content using a flow reactor, and (2) near-infrared spectroscopy (NIR) using crude drug powders as direct samples for metabolic profiling. Furthermore, a prediction model for tenuifolin content was developed and validated using multivariate analysis based on the results of (1) and (2). The goal of this study was to develop a rapid

analytical method utilizing the saponin content and explore the possibility of quality control through a wide-area survey of crude drugs using NIR spectroscopy. Consequently, various parameters and appropriate wavelengths were examined in the regression analysis, and a model with a reasonable contribution rate and prediction accuracy was successfully developed. In this case, the wavenumber contributing to the model was consistent with that of tenuifolin, confirming that this model was based on saponin content. In this series of analyses, we have succeeded in developing a model that can quickly estimate saponin content without post-processing and have demonstrated a brief way to perform quality control of crude drugs in the clinical field and on the market.

Keywords: crude drug, Near-infrared spectroscopy, metabolic profiling

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生薬学雑誌 2023;77:69-75.

In order to search for marker compounds to evaluate the bioequivalence of Kampo formulations, a clinical trial was conducted of 20 patients who received Kakkonto formulation and decoction. Five compounds were set as measurement targets, and it was possible to measure the plasma concentration transition of puerarian (PU) derived from Pueraria root, and glycyrrhizic acid derived from glycyrrhiza. PU met the bioequivalence in C_{max} pof PU may be insufficient, and the blood sampling time of 24 hours may also be insufficient. Thus, it was considered necessary to examine the design (number of subjects and blood sampling time) of the clinical trial. In this study, the possibility of utilizing PU, which is a compound contained om Pueraria root, as a marker compound of bioequivalence was shown. In the three Kampo formulas that contain peueraria root among the currently approved ethical Kampo formulations, an evaluation of the bioequivalence of PU as a marker compound was expected to lead to additional dosage forms of ethical Kampo formulations. In addition, similar to PU, other *C*-glycoside compounds may also serve as marker compounds for evaluating bioequivalence

Keywords: bioequivalence test, Kampo medicine, marker compound

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Masumoto N, Ito M: Genetic identification of the original plant species for Mentha Herb listed in the Japanese Pharmacopoeia and analyses of their essential oil composition.
*J. Nat. Med.*2023;77:489-495. doi: 10.1007/s11418-023-01690-1

Mentha arvensis Linné var. *piperascens* Malinvaud is an original plant species for "Mentha Herb (Hakka, ハッカ)" and "Mentha Oil (Hakka-yu, ハッカ)" listed in the Japanese Pharmacopoeia, whereas *Mentha canadensis* L. is that of "Mint oil, partly dementholised" listed in the European Pharmacopoeia. Although these two species are thought to be taxonomically identical, there are no data on whether the source plants of the Mentha Herb products distributed in the Japanese market are actually *M. canadensis* L. This is an important issue for international harmonization of the Japanese Pharmacopoeia and European Pharmacopoeia. In this study, 43 Mentha Herb products collected from the Japanese market and two plant samples of the original species of Japanese Mentha Herb harvested in China were identified by sequence analyses of the rpl16 regions in the chloroplast DNA, and the composition of their ether extracts was analyzed by GC-MS. Almost all samples were identified as *M. canadensis* L., and the main component of their ether extracts was menthol, although there were variations in their composition. However, there were some samples thought to be derived from other Mentha species, even though their main component was menthol. For quality control of Mentha Herb, it is important to be sure of not only the original plant species but also the composition of the essential oil and amount of menthol as the characteristic compound.

Keywords: mentha herb, *Mentha canadensis*, chloroplast rpl16

Tanaka R., Kawamura M., Mizutani S., Kikura-Hanajiri R：Identification of LSD analogs, 1cP-AL-LAD, 1cP-MIPLA, 1V-LSD and LSZ in sheet products.

Forensic Toxicol. 2023;41:294-303

Many analogs of lysergic acid diethylamide (LSD) have recently appeared as designer drugs around the world. These compounds are mainly distributed as sheet products. In this study, we identified three more newly distributed LSD analogs from paper sheet products. The structures of the compounds were determined by gas chromatography–mass spectrometry (GC-MS), liquid chromatography–photodiode array–mass spectrometry (LC-PDA-MS), liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy. From the NMR analysis, the compounds in the four products were identified as 4-(cyclopropanecarbonyl)-*N,N*-diethyl-7-(prop-2-en-1-yl)-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1cP-AL-LAD), 4-(cyclopropanecarbonyl)-N-methyl-N-isopropyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo-[4,3-*fg*]quinoline-9-carboxamide (1cP-MIPLA), *N,N*-diethyl-7-methyl-4-pentanoyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1V-LSD) and (2'*S*,4'*S*)-lysergic acid 2,4-dimethylazetidide (LSZ). In comparison with the structure of LSD, 1cP-AL-LAD was converted at the positions at N1 and N6, and 1cP-MIPLA was converted at the positions at N1 and N18. The metabolic pathways and biological activities of 1cP-AL-LAD and 1cP-MIPLA have not been reported. This is the first report showing that LSD analogs that were converted at multiple positions have been detected in sheet products in Japan. There are concerns about the future distribution of sheet drug products containing new LSD analogs. Therefore, the continuous monitoring for newly detected compounds in sheet products is important.

Keywords: lysergic acid diethylamide, LSD, lysergamide

Tanaka R., Kawamura M., Mizutani S., Kikura-Hanajiri R：Characterization of the lysergic acid diethylamide analog, 1-(thiophene-2-carbonyl)-*N,N*-diethyllysergamide (1T-LSD) from a blotter product.

*Drug Test Anal.*2024;16:482-488

Recently, lysergic acid diethylamide (LSD) analogs have appeared worldwide as designer drugs. In this study, we identified a distributed LSD analog from a paper-sheet product. Gas chromatography–mass spectrometry (GC–MS), liquid chromatography–photodiode array–mass spectrometry (LC–PDA–MS), and liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) were used to analyze the sheet product. The sheet product claimed to contain 1-(1,2-dimethylcyclobutanoyl)-*N,N*-diethyllysergamide (1D-LSD). However, an unknown compound was detected in the product together with tryptamine and L-tryptophan methyl ester. This compound was isolated from the sheets and identified as 1-(thiophene-2-carbonyl)-*N,N*-diethyl-6-methyl-9,10-didehydroergoline-8β-carboxamide (1-thiophenoyl LSD; 1-(2-thienoyl)-LSD, 1T-LSD), using ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectroscopy and various two-dimensional NMR techniques. 1T-LSD was shown to have the thiophene-2-carbonyl group at the N^{1} position instead of the 1,2-dimethylcyclobutanecarbonyl group as claimed. The amount of 1T-LSD (free base) in three individual unit from one sheet was determined to be 87–100 μg per unit using a proton-specific quantitative NMR (^{1}H-qNMR) method. Deacylation of 1T-LSD to LSD was also observed to occur in methanol-d^{4} during NMR analysis. The UV spectrum of 1T-LSD differed from that of other LSD analogs, and the fluorescence sensitivity was much lower. Because of concerns about the future distribution of products containing new LSD analogs, continued monitoring of newly detected compounds in sheet products is encouraged.

Keywords: lysergic acid diethylamide, LSD, lysergamides

Tanaka R., Kikura-Hanajiri R：Identification of hexahydrocannabinol (HHC), dihydro-iso-tetrahydrocannabinol (dihydro-iso-THC) and hexahydrocannabiphorol (HHCP) in electronic cigarette cartridge products.

Forensic Toxicol. 2024;42:71-81.

Since 2021, products claiming to contain hexahydrocannabinol (HHC) and hexahydrocannabiphorol (HHCP), which are tetrahydrocannabinol (THC) analogs, have been distributed via the Internet. Owing to the presence of three asymmetric carbons in their structure, HHC and HHCP have multiple stereoisomers. This study aimed to identify the actual stereoisomers of HHC and HHCP isolated from electronic cigarette cartridge products using nuclear magnetic resonance (NMR) spectroscopy. Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–photodiode array–mass spectrometry (LC–PDA–MS) were used for the analyses of two major peaks and one minor peak in product A and two major peaks in product B. These five compounds were isolated by silica gel column chromatography, and their structures were analyzed by ^{1}H, ^{13}C-NMR and various two-dimensional NMR techniques, i.e., H–H correlation spectroscopy, heteronuclear multiple quantum coherence, heteronuclear multiple-bond correlation, and nuclear Overhauser effect spectroscopy. Three compounds isolated from product A were identified as *rel*-(6a*R*,9*R*,10a*R*)-hexahydrocannabinol (11β-hexahydrocannabinol; 11β-HHC), rel-(6a*R*,9*S*,10a*R*)-hexahydrocannabinol (11α-hexahydrocannabinol, 11α-HHC), and a minor compound (2R,5S,6R)-dihydro-iso-tetrahydrocannabinol (dihydro-*iso*-THC). Meanwhile, the structural isomers of the major compound isolated from product B were identified as rel-(6a*R*,9*R*,10a*R*)-hexahydrocannabiphorol (11β-hexahydrocannabiphorol; 11β-HHCP) and rel-(6aR,9S,10aR)-hexahydrocannabiphorol (11α-hexahydrocannabiphorol; 11α-HHCP). The presence of both 11β-HHC and 11α-HHC in the HHC products analyzed in this study suggests that they were most likely synthesized via the reduction reaction of Δ^{8}-THC or Δ^{9}-THC. Dihydro-*iso*-THC was probably obtained as a byproduct of the synthesis of Δ^{8}-THC or Δ^{9}-THC from cannabidiol. Similarly, 11β-HHCP and 11α-HHCP in the HHCP product could stem from Δ^{9}-tetrahydrocannabiphorol.

Keywords: Cananbis, Hexahydrocannabinol (HHC), Hexahydrocannabiphorol (HHCP)

Miura T, Kouno T[*1], Takano M, Kuroda T, Yamamoto Y[*1], Kusakawa S, Morioka MS[*1], Sugawara T[*2], Hirai T, Yasuda S, Sawada R, Matsuyama S, Kawaji H[*3], Kasukawa T[*1], Itoh M[*1], Matsuyama A[*4], Shin JW[*5], Umezawa A[*6], Kawai J[*7], Sato Y: Single-Cell RNA-Seq Reveals LRRC75A-Expressing Cell Population Involved in VEGF Secretion of Multipotent Mesenchymal Stromal/Stem Cells Under Ischemia.

Stem Cells Translational Medicine. 2023; 12:379-390. doi: 10.1093/stcltm/szad029.

Human multipotent mesenchymal stromal/stem cells (MSCs) have been utilized in cell therapy for various diseases and their clinical applications are expected to increase in the future. However, the variation in MSC-based product quality due to the MSC heterogeneity has resulted in significant constraints in the clinical utility of MSCs. Therefore, we hypothesized that it might be important to identify and ensure/enrich suitable cell subpopulations for therapies using MSC-based products. In this study, we aimed to identify functional cell subpopulations to predict the efficacy of angiogenic therapy using bone marrow-derived MSCs (BM-MSCs). To assess its angiogenic potency, we observed various levels of vascular endothelial growth factor (VEGF) secretion among 11 donor-derived BM-MSC lines under *in vitro* ischemic culture conditions. Next, by clarifying the heterogeneity of BM-MSCs using single-cell RNA-sequencing analysis, we identified a functional cell subpopulation that contributed to the overall VEGF production in BM-MSC lines under ischemic conditions. We also found that leucine-rich repeat-containing 75A (LRRC75A) was more highly expressed in this cell subpopulation than in the others. Importantly, knockdown of LRRC75A using small interfering RNA resulted in significant inhibition of VEGF secretion in ischemic BM-MSCs, indicating that LRRC75A regulates VEGF secretion under ischemic conditions. Therefore, LRRC75A may be a useful biomarker to identify cell subpopulations that contribute to the angiogenic effects of BM-MSCs. Our work provides evidence that a strategy based on single-cell transcriptome profiles is effective for identifying functional cell subpopulations in heterogeneous MSC-based products.

Keywords: multipotent mesenchymal stromal/stem cell, single-cell transcriptome analysis, vascular endothelial growth factor

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Kuroda T, Yasuda S, Matsuyama S, Miura T, Sawada R, Matsuyama A*1, Yamamoto Y*2, Morioka MS*2, Kawaji H*2,3, Kasukawa T*2, Itoh M*2, Akutsu H*4, Kawai J*2,5, Sato Y: *ROR2* expression predicts human induced pluripotent stem cell differentiation into neural stem/progenitor cells and GABAergic neurons.

Scientific Reports, 2024; 14:690. doi: 10.1038/s41598-023-51082-4.

Despite the development of various *in vitro* differentiation protocols for the efficient derivation of specific cell types, human induced pluripotent stem cell (hiPSC) lines have varing ability to differentiate into specific lineages. Therefore, surrogate markers for accurately predicting the differentiation propensity of hiPSC lines may facilitate cell-based therapeutic product development and manufacture. We attempted to identify marker genes that could predict the differentiation propensity of hiPSCs into neural stem/progenitor cells (NS/PCs). Using Spearman's rank correlation coefficients, we investigated genes in the undifferentiated state, the expression levels of which were significantly correlated with the neuronal differentiation propensity of several hiPSC lines. Among genes significantly correlated with NS/PC differentiation ($P < 0.01$), we identified *ROR2* as a novel predictive marker. *ROR2* expression in hiPSCs was negatively correlated with NS/PC differentiation tendency, regardless of the differentiation method, whereas its knockdown enhanced differentiation. *ROR2* regulates NS/PC differentiation, suggesting that *ROR2* is functionally essential for NS/PC differentiation. Selecting cell lines with relatively low *ROR2* expression facilitated identification of hiPSCs that can differentiate into NS/PCs. Cells with *ROR2* knockdown showed increased efficiency of differentiation into forebrain GABAergic neurons compared to controls. These findings suggest that *ROR2* is a surrogate marker for selecting hiPSC lines appropriate for NS/PC and GABAergic neuronal differentiations.

Keywords: pluripotent stem cell, differentiation propensity, neural differentiation

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Hirai T, Kataoka K, Yuan Y*, Yusa K*, Sato Y, Uchida K*, Kono K: Evaluation of next-generation sequencing performance for *in vitro* detection of viruses in biological products.

Biologicals. 2024; 85:101739. doi: 10.1016/j.biologicals.2023.101739

Next-Generation Sequencing (NGS) can detect nucleic acid sequences in a massively parallel sequencing. This technology is expected to be widely applied for the detection of viral contamination in biologics. The recently published ICH-Q5A (R2) draft indicates that NGS could be an alternative or supplement to *in vitro* viral tests. To examine the performance of NGS for the *in vitro* detection of viruses, adenovirus type 5 (Ad5), a model virus, was inoculated into Vero cells, which are the most popular indicator cells for the detection of adventitious viruses in the *in vitro* test. Total RNA extracted from the Vero cells infected with Ad5 was serially diluted with that from non-infected Vero cells, and each sample was analyzed using short- or long-read NGSs. The limits of detection of both NGS methods were almost the same and both methods were sensitive enough to detect viral sequences as long as there was at least one copy in one assay. Although the multiplexing in NGS carries the risk of cross-contamination among the samples, which could lead to false positives, this technology has the potential to become a rapid and sensitive method for detecting adventitious agents in biologics.

Keywords: ICH-Q5A, *in vitro* viral tests, next-generation sequencing

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築茂由則，吉田徳幸，大岡伸通，内田恵理子，山本真梨子，野口耕司*，鈴木孝昌，本間正充，合田幸広，井上貴雄：早期に使用許可を受けたCOVID-19診断用核酸増幅検査薬の構成に関する調査と考察.
医薬品医療機器レギュラトリーサイエンス 2023;54:148-161. doi: 10.51018/pmdrs.54.2_148

With the spread of a novel coronavirus disease COVID-19 in early 2020, dozens of nucleic acid amplification test (NAT) kits were developed in Japan and used for the diagnosis of COVID-19. Because of the urgent need to supply the kits to testing facilities, they were developed over a short time period and had to be urgently approved for use. In this study, we investigated the composition and specifications of COVID-19 diagnostic NAT kits that were approved for emergency use in Japan and the USA to see if there are any distinctive features that might be related to the urgency of development. With regard to primer design, we found that several NAT kits had adopted primers designed by public organizations such as the National Institute of Infectious Diseases in Japan and the USA Centers for Disease Control and Prevention (CDC), without designating manufacturer's original primers. In addition, some of the NAT kits had been developed to detect a single region of the viral genome, rather than multiple regions. A comparison between the NAT kits approved in Japan and the USA, showed that a higher percentage of NAT kits had adopted newer methods for specimen processing and nucleic acid amplification in Japan than in the USA. In addition, some NAT kits developed in Japan did not use an internal standard, which is a positive control nucleotide sequence to ensure valid amplification reaction and/or RNA extraction, whereas all kits in the USA used an internal standard. Based on the results of this survey, we discuss how NAT kits should be developed for a future pandemic, as well as the points to be considered in order to ensure the reliability of NAT kits.

Keywords: COVID-19, SARS-CoV-2, nucleic acid amplification test

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医薬品医療機器レギュラトリーサイエンス 2023;54:439-454. doi: 10.51018/pmdrs.54.5_439

In evaluating the quality of oligonucleotide therapeutics, it is necessary to pay close attention to the presence of oligonucleotide impurities. As an analysis method, LC/MS, combining liquid chromatography (LC) and mass spectrometry (MS), is generally used for quality assessment. However, since the physicochemical properties of active pharmaceutical ingredient (API)-derived impurities are likely to be similar to those of the API, there are technical

limitations on their separation and purification. Therefore, it is important to be aware of such limitations and to understand the capabilities of the analytical techniques used in evaluating the quality of oligonucleotide therapeutics with

due consideration of the characteristics of the oligonucleotides and their manufacturing processes. In order to achieve this, we analyzed model oligonucleotides using several different types of commercial liquid chromatograph-mass spectrometers (LC-MS). We investigated the LC separation of impurities from the parent oligonucleotide, the relative quantification of impurities, the characterization of the parent oligonucleotide, and the identification of impurities. In the LC separation of the impurities from the parent oligonucleotide, none of the LC-MS analyses achieved complete separation (Rs > 1.5) of all the major impurities. However, almost complete separations were observed between the parent oligonucleotide and three or more nucleotide-deleted oligonucleotide impurities. The relative

quantification of impurities was not consistent among the LC-MS instruments tested here, and inconsistent ion suppressions or enhancements were observed. For the characterization of the parent oligonucleotide, the accuracy of deconvoluted mass was demonstrated to be within 3 ppm for all LC-MS analyses, and MS/MS sequence analysis showed 100% coverage in more than half of the cases. For the structural estimation of impurities, the mass accuracy was within 2 ppm for all LC-MS analyses when

impurities were spiked at 0.1% or more, and the MS/MS sequence coverage was 76% or more when the spiked amounts of impurities were 1% or more. These results indicate that generally used LC/MS methods could provide reliable information for estimating the composition of impurities present at a level of 1% or more.
Keywords: oligonucleotide therapeutics, quality evaluation, impurities

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Adeno-associated virus (AAV) vectors are produced as a mixture of the desired particle (full particle, FP), which is filled with the designed DNA, product-related impurities such as particle without DNA (empty particle, EP), and aggregates. Cesium chloride or iodixanol equilibrium density gradient ultracentrifugation (DGE-UC) has been used for the purification of AAV vectors. DGE-UC can separate FP from impurities based on the difference in their buoyant densities. Here, we report the applications and limitations of equilibrium density gradient analytical ultracentrifugation (DGE-AUC) using a modern AUC instrument that employs DGE-UC principles for the characterization and quantitation of AAV vectors. We evaluated the quantitative ability of DGE-AUC in comparison with sedimentation velocity AUC (SV-AUC) or band sedimentation AUC (BS-AUC) using AAVs with different DNA lengths and different serotypes. DGE-AUC enabled the accurate quantification of the ratio of FP to EP when the AAV vector primarily contains these particles. Furthermore, we developed a new workflow to identify the components of separated peaks in addition to FP and EP. Ultraviolet absorption spectra obtained by multiwavelength detection can also support peak assignment following component identification. DGE-AUC experiments for AAV vectors have limitations with regard to minor components with low absorption at the detected wavelength or those with a density similar to that of major components of AAV vectors. DGE-AUC is the only analytical method that can evaluate particle density heterogeneity; therefore, SV-AUC or BS-AUC and DGE-AUC are complementary methods for reliable assessment of the purity of AAV vectors.
Keywords: gene therapy, AAV, AUC

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Okamoto Y, Fukui C, Kobayashi T*1, Morioka H*1, Mizumachi H*2, Inomata Y*3, Kaneki A*3, Okada M*3, Haishima Y, Yamamoto E, Nomura Y: Proof of concept testing of a positive reference material for *in vivo* and *in vitro* sensitization testing of medical devices.
JBMR Part B. 2024;112(2):e35386.doi: 10.1002/jbm.b.35386.

In vivo skin sensitization tests are required to evaluate the biological safety of medical devices in contact with living organisms to provide safe medical care to patients. Negative and positive reference materials have been developed for biological tests of cytotoxicity, implantation, hemolysis, and *in vitro* skin irritation. However, skin sensitization tests are lacking. In this study, polyurethane sheets containing 1 w/w% 2,4-dinitrochlorobenzene (DNCB-PU) were developed

and evaluated as a positive reference material for skin sensitization tests. DNCB-PU sheet extracts prepared with sesame oil elicited positive sensitization responses for *in vivo* sensitization potential in the guinea pig maximization test and the local lymph node assay. Furthermore, DNCB-PU sheet extracts prepared with water and acetonitrile, 10% fetal bovine serum-containing medium, or sesame oil elicited positive sensitization responses as alternatives to animal testing based on the amino acid derivative reactivity assay, human cell line activation test, and epidermal sensitization assay, respectively. These data suggest that the DNCB-PU sheet is an effective extractable positive reference material for *in vivo* and *in vitro* skin sensitization testing in medical devices. The formulation of this reference material will lead to the development of safer medical devices that contribute to patient safety.

Keywords: alternatives to animal testing, biological safety, material, medical device, skin sensitization

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Tamai Y*, Noda A*, Yamamoto E. Estimation of confidence intervals for quantitation of coeluted peaks in liquid chromatography–Photodiode array detection through a combination of multivariate curve resolution-alternating least-square and Bayesian inference techniques.
J Chromatogr A. 2023;1704:464136. doi:10.1016/j.chroma.2023.464136.

There is a dramatic increase in drug candidates that exhibit complex structures and do not comply with Lipinski's rule of five. One of the most critical and complex technical challenges in the quality control of such drug candidates is the control of analogous substances contained in active pharmaceutical ingredients and related formulations. Although the development of ultrahigh-performance liquid chromatography and high-performance columns has improved efficiency per unit time, the difficulty of peak separation to quantify impurities with similar structures and physicochemical properties continues to rise, and so does the probability of failure to achieve the necessary separation. Coeluting peaks observed in the case of high-performance liquid chromatography (HPLC) with photodiode array detection can be separated using the multivariate curve resolution-alternating least-square (MCR-ALS) method exploiting differences in analyte UV spectra. However, relatively large quantitation errors have been observed for coeluting analogous substances, and the reliability of the corresponding quantitative data requires improvement. Herein, Bayesian inference is applied to separation by the MCR-ALS method to develop an algorithm assigning a confidence interval to the quantitative data of each analogous substance. The usefulness and limitations of this approach are tested using two analogs of telmisartan as models. For this test, a simulated two-component HPLC-UV dataset with an intensity ratio (relative to the main peak) of 0.1–1.0 and a resolution of 0.5–1.0 is used. The developed algorithm allows the prediction confidence interval, including the true value, to be assigned to the peak area in almost all cases, even when the intensity ratio, resolution, and signal-to-noise ratio are changed. Finally, the developed algorithm is also evaluated on a real HPLC-UV dataset to confirm that reasonable prediction confidence intervals including true values are assigned to peak areas. In addition to allowing the separation and quantitation of substances such as impurities challenging to separate by HPLC in a scientifically valid manner, which is impossible for conventional HPLC-UV detection, our method can assign confidence intervals to quantitative data. Therefore, the adopted approach is expected to resolve the issues associated with assessing impurities in the quality control of pharmaceuticals.

Keywords: Impurity, Coelution, Bayesian inference

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Nakai Y*, Noda A*, Yamamoto E. Algorithm for the early prediction of drug stability using bayesian inference and multiple measurements: Application for predicting the stability of silodosin tablets.
J Pharm Biomed Anal. 2023;233:115442. doi:10.1016/j.jpba.2023.115442.

The stability of active pharmaceutical ingredients (APIs) and formulations has become a major chemistry, manufacturing, and control (CMC) concern in the pharmaceutical industry because it can

determine the feasibility of research and development, the development period, and the development costs of a certain formulation. To streamline the research and development of pharmaceutical products and create useful pharmaceutical products at an early stage, a technology that predicts the stability of formulations at an early stage and with a high degree of accuracy is needed. When predicting the stability of a substance, highly reliable data are required; however, the stability data are affected by analytical variations that depend on the experimenter, measurement device, and conditions used. Although these variations greatly affect the prediction accuracy, a stability prediction method that considers these variations has not yet been developed. Here, short-term stability data under accelerated conditions were obtained at three institutions using silodosin tablets as a model sample. By combining Bayesian inference with the temporal change in the amount of the main degradation products obtained and the conventional humidity-corrected Arrhenius equation, we developed a new algorithm that provides a narrow confidence interval, even when using data with variations. By using this algorithm and setting an appropriate number of conditions, we were able to obtain a valid confidence intervals in a short period of time. Here, by performing more measurements than those suggested by the minimum measurement frequency indicated in the guideline specified in the International Council for Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use, we developed a method that can be used to reasonably predict the long-term stability of the drugs, even if the data measurement interval is short. Our results will help solve various problems in today's pharmaceutical product development scenario and contribute to worldwide health and welfare.

Keywords: Stability, Prediction, Impurity

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Yamamoto E, Kan-no H, Ando D, Miyazaki T, Koide T, Izutsu K, Sato Y. Formic acid-aided sample preparation method for sensitive and simultaneous analysis of eight nitrosamines in poorly-water soluble pharmaceutical drugs using liquid chromatography–ultraviolet detection.

J Pharm Biomed Anal Open. 2023;2:100020. doi:10.1016/j.jpbao.2023.100020.

There has been a growing concern over the contamination of pharmaceutical products with nitrosamines (NAs) such as N-nitrosodimethylamine (NDMA). To quantify NA levels in drugs using reversed-phase liquid chromatography (LC), the sample solution should achieve a high drug concentration to detect trace NAs, and an appropriate amount of hydrophilic NAs should be retained. However, these are difficult to achieve, and no suitable method has yet been developed. The present study was the first to develop a sample preparation method to achieve this by combining drugs with formic acid (FA), followed by the removal of active pharmaceutical ingredients (APIs) from samples via crystallization. This method was successfully applied for the sensitive quantification of eight NAs in poorly water-soluble acidic atorvastatin (ATS) and basic itraconazole (ITC) via LC–ultraviolet (LC-UV) detection. The removal rate of ITC via recrystallization exceeded 99.96%, whereas most NAs remained as solutes. Assuming that the enhancement in ITC solubility directly translates to heightened analytical sensitivity, a > 100-fold increase in sensitivity was attained compared to conventional methodologies. This sample preparation method would be applicable to other poorly water-soluble drugs, contributing to the control of NA content in various formulations to realize the safe delivery of pharmaceuticals to patients.

Keywords: Sample preparation, Solubilization, Sensitive analysis

Takada J*, Hamada K*, Zhu X*, Tsuboko Y, Iwasaki K*: Biaxial tensile testing system for measuring mechanical properties of both sides of biological tissues.

J Mech Behav Biomed Mater. 2023;146:106028. doi:10.1016/j.jmbbm.2023.106028.

The aortic wall exhibits a unique elastic behavior, periodically expanding in aortic diameter by approximately 10% during heartbeats. This elastic behavior of the aortic wall relies on the distinct yet interacting mechanical properties of its three layers: intima, media, and adventitia. Aortic aneurysms develop as a result of multifactorial remodeling influenced by mechanical vulnerability of the aortic

wall. Therefore, investigating the mechanical response of the aneurysmal wall, in conjunction with changes in microstructural parameters on both the intimal and adventitial sides, may offer valuable insights into the mechanisms of aortic aneurysm development or rupture. This study aimed to develop a biaxial tensile testing system to measure the mechanical properties of both sides of the tissue to gain insights concerning the interactions in anisotropic layered tissue. The biaxial tensile test set-up consisted of four motors, four cameras, four load cells, and a toggle switch. Porcine ascending aortas were chosen as the test subject. Graphite particles with diameters of approximately 5–11 [μm] were randomly applied to both sides of the aorta. Strain measurements were obtained using the stereo digital-image correlation method. Because stretching a rectangular specimen with a thread inevitably concentrates and localizes stress, to reduce this effect the specimen's shape was investigated using finite element analysis.

The finite element analysis showed that a cross-shaped specimen with diagonally cut edges would be suitable. Therefore, we prepared specimens with this novel shape. This test system showed that mechanical response of the aortic tissue was significantly different between the intimal and adventitial side in the high-strain range, due to the disruption of collagen fibers. The adventitia side exhibited a smaller elastic modulus than the intimal side, accompanied by disruption of collagen fibers in the adventitia, which were more pronounced in the longitudinal direction. In contrast, in the mid-strain range, the elastic modulus did not differ between the intimal and adventitial sides, irrespective of longitudinal or circumferential direction, and collagen fibers were not disrupted but elongated.

A biaxial tensile test system, which measures the mechanical properties of both sides of biological tissues and the shape of the specimen for reducing the concentration of stress at the chuck region, was developed in this study. The biaxial tensile testing system developed here is useful for better understanding the influences of mechanical loads and tissue degeneration on anisotropic, layered biological tissues.

Keywords: biaxual tensile testing system, biological tissue, incremental elastic modulus, finite element analysis, porcine ascending thoracic aorta

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Okamura T[*1,2], Iwasaki K[*1], Lu H[*1], Zhu X[*1], Fujimura T[*2], Kitaba N[*1], Murakami K[*1], Nakamura R[*1], Mitsui H[*1], Tsuboko Y, Miyazaki Y[*2], Matsuyama T[*2]: Importance of optimal rewiring guided by 3-dimensional optical frequency domain imaging during double-kissing culotte stenting demonstrated through a novel bench model.

Sci Rep. 2023;13:13511. doi:10.1038/s41598-023-40606-7.

The usefulness of optical frequency domain imaging (OFDI) guidance on two-stenting at left main bifurcation has not been evaluated. Here, we used a novel bench model to investigate whether pre-defined optimal rewiring with OFDI-guidance decreases acute incomplete stent apposition (ISA) at the left main bifurcation segment. A novel bench simulation system was developed to simulate the foreshortening and overlapping of daughter vessels as well as left main bifurcation motion under fluoroscopy. Double-kissing (DK) culotte stenting was performed using the novel bench model under fluoroscopy with or without OFDI-guidance. In the OFDI-guidance group, if the guidewire did not pass through the pre-defined optimal cell according to the 3-dimensional OFDI, additional attempts of rewiring into the jailed side branch were performed. The success rate of optimal jailed side branch rewiring after implantation of the first and second stent under OFDI-guidance was significantly higher than that under only angio-guidance. After completion of the DK-culotte stenting, the incidence and volume of ISA at the bifurcation segment in the OFDI-guidance group was significantly lower than that in the angio-guidance group. Online 3-dimensional OFDI-guided DK-culotte stenting according to a pre-defined optimal rewiring point might be superior to only angio-guided rewiring for reducing ISA at the bifurcation.

Keywords: biomedical engineering, interventional cardiology

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Toda S[*1], Hashimoto Y[*1], Nakamura N[*2], Yamada M[*3], Nakaoka R, Nomura W[*4], Yamamoto M[*5], Kimura T[*1], Kishida A[*1]: Effects of polymeric materials on activation of THP-1-derived macrophages during differentiation by PMA
Adv Biomed Eng. 2024; 13:1-10.

Evaluation of biomaterial properties using THP-1 cells require the establishment of standardized protocols. Two potential methods are available, which differ in the timing of cell contact with the material; i.e. cell differentiation occurring simultaneous with or prior to polarization. No reports have examined the activation state of macrophages during differentiation. The aim of this study was to clarify the effects of biomaterials on THP-1 cells during differentiation. THP-1 cells were seeded on polymeric materials in the absence and presence of phorbol-12-myristate-13-acetate, and M1/M2 polarization was induced. The differentiation from THP-1 cells into macrophages was evaluated by loss of proliferation and acquisition of adhesion. The activation levels and M1/M2 polarization of M0 were assessed by IL-1β and MRC1 mRNA expression. Undifferentiated THP-1 cells were not markedly stimulated by interaction with biomaterials. However, THP-1 cells seed- ed on all the test materials differentiated into macrophages, and the macrophages polarized into different activated states depending on the material. These findings revealed the effects of material stimulation on macrophage activation state during differentiation. These results suggest that the step of cell differentiation and the step of contact with the material should be separated during systematic evaluation of biomaterials.

Keywords: macrophage differentiation, polarization, material derived stimulation

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Toda S[*1], Hashimoto Y[*1], Nakamura N[*2], Yamada M[*3], Nakaoka R, Nomura W[*4], Yamamoto M[*5], Kimura T[*1], Kishida A[*1]: Characteristics of macrophage aggregates prepared by rotation culture and their response to polymeric material
J Artif Organs. 2024; 1-9.

Understanding the interaction between macrophages and biomaterials is important for the creation of new biomaterials and the development of technologies to control macrophage function. Since macrophages are strongly adhesive, caution is required when performing *in vitro* evaluations. Similarly, when THP-1 cells, macrophage precursor cells, are differentiated into macrophages using phorbol-12-myristate-13-acetate (PMA), it becomes difficult to detach them from the adherent substrate, which has been a problem on investigation of immunological responses to biomaterials. In this study, the interaction of THP-1 cell-differentiated macrophages with biomaterials was analyzed based on a new method of seeding THP-1 cells. THP-1 cells were cultured in static and rotation culture without and with PMA. In undifferentiated THP-1 cells, there was no change in cellular function between static and rotation cultures. In rotation culture with PMA, THP-1 cells differentiated and formed macrophage aggregates. IL-1β and MRC1 expression in macrophage aggregates was examined after differentiation and M1/M2 polarization. Macrophage aggregates in rotation culture tended to be polarized toward M2 macrophages com- pared with those in static culture. In the evaluation of the responses of macrophage aggregates to several kinds of polymeric materials, macrophage aggregates showed different changes in MRC1 expression over time at 30, 50, and 70 rpm. Rotation speed of 30 rpm was considered most appropriate condition in that it gave stable results with the same trend as obtained with static culture. The use of macrophage aggregates obtained by rotational culture is expected to provide new insights into the evaluation of inflammatory properties of biomaterials.

Keywords: rotation culture, macrophage aggregate, material property evaluation

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Takada J*, Morimura H*, Hamada K*, Okamoto Y*, Mineta S*, Tsuboko Y, Hattori K*, Iwasaki K*: A tissue-silicone integrated simulator for right ventricular pulsatile circulation with severe functional tricuspid regurgitation.

Sci Rep. 2024;14:5120. doi:10.1038/s41598-024-55058-w.

There is a great demand for development of a functional tricuspid regurgitation (FTR) model for accelerating development and preclinical study of tricuspid interventional repair devices. This study aimed to develop a severe FTR model by creating a tissue-silicone integrated right ventricular pulsatile circulatory simulator. The simulator incorporates the porcine tricuspid annulus, valve leafets, chordae tendineae, papillary muscles, and right ventricular wall as one continuous piece of tissue, thereby preserving essential anatomical relationships of the tricuspid valve (TV) complex. We dilated the TV annulus with collagenolytic enzymes under applying stepwise dilation, and successfully achieved a severe FTR model with a regurgitant volume of 45 ± 9 mL/beat and a flow jet area of 15.8 ± 2.3 cm^2 (n= 6). Compared to a normal model, the severe FTR model exhibited a larger annular circumference (133.1 ± 8.2 mm vs. 115.7 ± 5.5 mm; p = 0.009) and lower coaptation height (6.6 ± 1.0 mm vs. 17.7 ± 1.3 mm; p = 0.003). Following the De-Vega annular augmentation procedure to the severe FTR model, a significant reduction in regurgitant volume and flow jet area were observed. This severe FTR model may open new avenues for the development and evaluation of transcatheter TV devices.

Keywords: biomedical engineering, experimental models of disease, valvular disease

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Sakoda, H., Tsuboko, Y., Okamoto, Y., Yamamoto, E., Imagama, T.*1, Sakai, T.*1, Hamada, H.*2, Sugano, N.*2 : Lipid index to quantify the absorbed lipids in retrieved UHMWPE components of joint arthroplasty.

Biomed Mater Eng. 2024;35:293-302. PMID: 38277279. doi: 10.3233/BME-230183.

Background: The ultra-high molecular weight polyethylene (UHMWPE) component of artificial joints is one of the most important factors affecting the clinical outcomes of joint arthroplasty. Although the possibility of *in vivo* UHMWPE degradation caused by absorbed lipids has been reported, a quantitative evaluation of this phenomenon has not yet been performed.

Objective: This study aimed to establish the lipid index (LI) as a quantitative indicator of the amount of absorbed lipids and the first step to quantify their effects on UHMWPE.

Methods: The LI was defined using the infrared spectrum obtained with a Fourier-transform infrared spectrophotometer and verified using the retrieved UHMWPE components.

Results: The LI was consistent with the amount of extract recovered in reflux extraction with hexane. In addition, the LI could replace lipid extraction for calculating the oxidation index (OI) because the value obtained by subtracting the LI from the OI showed good agreement with the OI obtained after lipid extraction.

Conclusions: The LI represents the amount of lipids absorbed by UHMWPE and is useful for quantitatively evaluating the effects of lipids on UHMWPE. In addition, the LI enables OI measurements that are unaffected by absorbed lipids without requiring troublesome lipid-extraction procedures.

Keywords: Peak separation, FTIR, retrieval study

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臨床バイオメカニクス 2023;44:117-120.

人工関節に使用される超高分子量ポリエチレン（UHMWPE）の生体脂質に起因した劣化が懸念されて

いるが，その直接的な証拠はまだない．脂質劣化は表面近傍に局所的である可能性があるため，我々は，位置分解能の高いマイクロスラリーエロージョン法を用いた強度測定による劣化評価を試みてきたが，浅い測定範囲のため，表面下で生じるUHMWPEのラジカルに起因した酸化劣化を検出できていなかった．そこで本研究では，機械加工による表面除去と組み合わせた高深度測定法を開発し，再置換のため抜去された２例を用いてラジカルによる劣化が検出可能か検証した．

酸化度の上昇が認められなかった試料では測定された強度も一様であったのに対し，酸化度の上昇が認められた試料では表面下で強度低下が認められ，本法でラジカルによる劣化を検出可能であることがわかった．また，いずれの試料でも摺動面直下で強度低下が見られ，脂質劣化である可能性が考えられた．

Keywords: UHMWPE, Artificial joint, Degradation

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迫田秀行，岡本吉弘，山本栄一，今釜崇[*1]，坂井孝司[*1]，濱田英敏[*2]，菅野伸彦[*2]：抜去人工股関節ライナーの簡易的摩耗量評価法の開発．
臨床バイオメカニクス 2023;44:121-125.

人工股関節ライナーの摩耗は臨床成績に影響する重要な因子の一つである．しかし，多数の試料の多角的な解析を必要とする抜去インプラント解析では，手間と時間を要する既存法による運用が負担になる可能性がある．そこで，ライナーの厚さ測定により簡便に摩耗量を推定する方法について検討した．

本法では，摩耗は一方向に進行しているものと仮定し，ライナーの中心軸から等距離にある，低摩耗側と高摩耗側の2か所を測定し，その差を測定摩耗量とした．真の摩耗量と測定摩耗量の関係を幾何学的に求め，様々な条件における測定摩耗量の理論値を計算した．3例の抜去インプラントについて実測したところ，実測値は理論値とほぼ一致した．また，測定摩耗量は真の摩耗量のおよそ0.4～0.9倍となることがわかった．この誤差は，様々な要因に伴う摩耗量の増減に比べ小さいと考えられることから，本法により，臨床上有用な精度で簡便に摩耗量を推定できる可能性がある．

Keywords: UHMWPE, Artificial joint, Degradation

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Yoshitomi T[*1], Nishi I[*1], Onuki A[*2], Tsunoda T[*2], Chiba M[*3], Oizumi S[*3], Tanaka R[*4], Muraki S[*4], Oshima N, Uemura H[*1], Tahara M, Sakai S: Development of a standard test method for insecticides in indoor air by GC-MS with solid-phase adsorption/solvent extraction.
BPB Reports 2023;6:76-80. doi:10.1248/bpbreports.6.3_76

Semi-volatile organic compounds (SVOCs), which can cause indoor air pollution, include plasticizers, insecticides, and flame retardants. In Japan, the Ministry of Health, Labour and Welfare has set guidelines for indoor air concentrations of di-n-butyl phthalate and di-2-ethylhexyl phthalate in plasticizers and fenobucarb, diazinon, and chlorpyrifos in insecticides. However, this analytical method has only been tentatively proposed for more than 20 years. In this study, we attempted to construct an analytical method for insecticides for which guideline values have been established based on recently standardized sampling and extraction methods for phthalates in indoor air. The results of the recovery tests for the insecticides were excellent, with recovery rates and relative standard deviations in the ranges 88%–104% and 1.4%–7.5%, respectively. Furthermore, the limits of detection and quantification were less than 1/50 of the current guideline values. Additionally, inter-laboratory validation was conducted at five research institutions. By excluding outliers with the Grubbs test, the accuracies were in the ranges of 81.9%–126.3%, 76.8%–121.7%, and 76.7%–112.8% for chlorpyrifos, diazinon, and fenobucarb, respectively. The target ranges for repeatability (RSD_r) and reproducibility (RSD_R) were 30% and 35%, respectively, and the validation results met these criteria. Based on these results, we propose the developed method as the standard test method for insecticide-originated pollutants in indoor air in Japan.

Keywords: indoor air, semi-volatile organic compound, interlaboratory validation

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for Establishing a Standard Test Method for Volatile Organic Compounds in Indoor Air in Japan using Solvent Extraction.
BPB Reports 2024;7:39-43. doi:10.1248/bpbreports.7.2_39

The Ministry of Health, Labour and Welfare of Japan has set the guideline values for indoor air concentrations of 13 volatile organic compounds (VOCs) and semi-volatile organic compounds from 1997 to 2002. However, in 2019, the guideline values for three of these substances, including xylene, were revised and regulated more strictly. Additionally, the manual for analysis of VOCs in indoor air, established in 2001 by the Committee on Sick House Syndrome: Indoor Air Pollution, has not been updated for over 20 years. In this study, we confirmed that the current analytical method for VOCs in indoor air using solvent extraction which was established in 2001, is applicable to VOCs that have been revised or added since then. We proposed it as a standard test method and performed an inter-laboratory validation study in five laboratories to prove this. This validation study included nine substances: six VOCs with current guideline values and three VOCs as candidates for newly setting guideline values. Additional amount in this study was set as 1 μg, less than one-tenth of the guideline value for xylene. The results showed that the average recovery, repeatability, and reproducibility for the nine substances in the five laboratories were 75.4%–115%, 0.78%–9.6%, and 3.6%–21%, respectively. These values satisfied the determined criteria ranges, suggesting that our proposed analytical method can be used as a standard test method.
Keywords: indoor air, volatile organic compound, inter-laboratory validation

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小林憲弘，土屋裕子，木下輝昭[*1]，高木総吉[*2]，中嶋京介[*3]，広木孝行[*4]，平林達也[*5]，藤井裕美[*6]，栗原正憲[*7]，関川慎也[*8]，奥村学[*9]，古口健太郎[*10]，樋口雄一[*11]，大瀧翔吾[*12]，代龍之介[*13]，古川浩司[*14]，松巾宗平[*15]，松澤悠[*16]，髙原玲華[*17]，五十嵐良明：液体クロマトグラフ質量分析計による水道水中のメチダチオンオキソンの分析法の検討と妥当性評価.
水道協会雑誌 2023;92(7):5–17.

2022年4月にメチダチオンオキソンが新たに水道水質検査の対象農薬リストに追加されたことから，本研究では液体クロマトグラフタンデム質量分析計（LC-MS/MS）を用いた水道水中のメチダチオンオキソンの分析条件の検討と，18機関のバリデーション試験による分析法の妥当性評価を行なった．アスコルビン酸ナトリウムまたはチオ硫酸ナトリウムで残留塩素を除去した水道水を用いた添加回収試験の結果，いずれの機関もメチダチオン（DMTP）の目標値の1/100よりも低濃度の0.03 μg/Lの添加試料の真度および併行精度が良好であったことから，本分析法はメチダチオンオキソンの水質検査に適用できると考えられた.
Keywords: water analysis, pesticides, LC/MS

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小林憲弘：水質事故迅速モニタリング手法の開発と普及に関する研究.
地球環境 2023;28(2):171–178.

水質汚染事故発生時には即座に濃度把握が必要となることから，事故発生時に採水現場において測定が可能な簡易分析法と，標準物質を必要とせず広範囲の物質を分析可能なGC/MSスクリーニング分析法について検討した．簡易分析法に関しては，ホルムアルデヒドを対象に定量精度を検討した結果，市販の測定キットを用いた場合は濃度が過大評価となる可能性も示唆されたが，迅速・簡便な方法として有用であることが示された．スクリーニング分析法に関しては複数の装置により作成した

検量線および定量値の誤差について評価した結果，異なる装置で作成した検量線を用いた場合も，対象物質の多くを5倍以内の誤差で定量できることが示された．さらに，実試料を用いた複数機関でのバリデーション試験により，機関による定性・定量結果の違いを検証した結果，解析者のトレーニングを行うことにより，各農薬の定量下限の3倍以上であれば解析者の判断はほぼ一致することが示された．

Keywords: screening analysis, formaldehyde, pesticides

小林憲弘，土屋裕子，石井一行*，馬場紀幸*，林田寛司*：パージ・トラップ-ガスクロマトグラフィー質量分析による水道水中の揮発性有機化合物の分析精度に影響を与える要因の解析．

環境化学 2024;34:1–8. doi: 10.5985/jec.34.1

パージ・トラップ-ガスクロマトグラフィー質量分析（PT-GC/MS）による水道水中の25種の揮発性有機化合物（VOC）の分析において，試験操作時の器具・溶媒等の冷却と，検水の希釈が分析精度に与える影響を評価するため，様々な試験条件下で水道水への添加回収試験を行ない，試験結果を比較した．ヘンリー定数が小さく揮発性の高い化合物ほど，冷却を行なわなかった場合に真度および併行精度が大きく影響を受けた．また，検水を希釈して測定した場合は，器具・溶媒等の冷却を行なっても真度が低下し，併行精度も悪化したことから，PT-GC/MSによる水道水中のVOC分析では器具・溶媒等を冷却して試験操作を行なうとともに，検水を希釈せずに分析を行なった方がよいと考えられる．

Keywords: volatile organic compounds（VOCs）, PT-GC/MS, drinking water

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環境科学会誌 2024;37(2):53–63. doi: 10.11353/sesj.37.53

ガスクロマトグラフィー質量分析（GC/MS）による水道水中農薬168種のスクリーニング分析データベースをアジレント・テクノロジー社製の装置で作成し，標準液および河川水試料を用いて定性・定量精度の検証を行った．データベース作成から約1ヶ月後に再度，各農薬0.05 mg/Lの標準液をデータベース作成時と同一条件でスクリーニング分析を行ったところ，データベースに登録した168農薬のうち162農薬を検出できた．検出できなかった6農薬は定量下限が0.05～0.1 mg/Lの範囲にあったが，水道水中の農薬検査で要求される定量下限は十分に満たした．一方，誤同定に注意を要する農薬も幾つか存在することが明らかになった．実試料へのスクリーニング分析の適用時には，これらの農薬の定性には特に注意する必要がある．多摩川中流域5地点の河川水にスクリーニング分析を適用した結果，異なる検査員が実施した通常分析と検出農薬は全て一致した．また，スクリーニング分析による定量値は通常分析の0.63～0.98倍の範囲にあり，河川水試料から検出された農薬については，スクリーニング分析法の定量誤差は通常分析と比べて1/2～2倍以内に収まることが示された．スクリーニング分析法は標準品を用いずにデータベースに登録した農薬を迅速・簡便に，なおかつ通常分析と比べて一定の誤差範囲内の定量精度で分析可能であることから，水道水中農薬の検査においてスクリーニング分析法の有用性は高いと考えられる．

Keywords: screening analysis, pesticides, GC/MS

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Nishi I*, Yoshitomi T*, Nakano F*, Uemura H*, Tahara M, Kawakami T: Development of safer and improved analytical method for polycyclic aromatic hydrocarbons in creosote products.

J Chromatogr A 2023;1698:464007. doi:10.1016/j.chroma.2023.464007

Polycyclic aromatic hydrocarbons (PAHs) in creosote products used for wood preservation are regulated in Japan. Although the analytical method for this regulation has been stipulated by law, two main problems have been highlighted, namely the use of dichloromethane, a potential carcinogen, as a solvent and inadequate purification. Therefore, an analytical method to solve these problems was developed in this study. Actual creosote-treated wood samples were examined, and it was found that acetone could be used as an alternative solvent. Purification methods using centrifugation, silica gel cartridges, and strong anion exchange (SAX) cartridges were also developed. It was found that the SAX cartridges strongly retained PAHs, and an effective purification method was developed using this phenomenon, in which contaminants were removed by washing with diethyl ether/hexane (1/9 v/v), which could not be achieved

with a silica gel cartridge. This strong retention was attributed to cation-π interactions. The analytical method developed in this study yielded good recoveries (81.4-113.0%) with low relative standard deviations (<6.8%), and the limit of quantification (0.02-0.29 μg/g) was significantly lower than the current creosote product regulation. Therefore, this method can safely and effectively extract and purify PAHs from creosote products.
Keywords: creosote, PAHs, strong anion exchange

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Tahara M, Kawakami T, Ikarashi Y: GC-MS analysis of primary aromatic amines originated from azo dyes in commercial textile or leather products using helium alternative carrier gas.
J AOAC Int 2024;107:61-8. doi:10.1093/jaoacint/qsad116

In recent years, due to the global shortage of helium gas, the development of gas chromatography (GC) analytical methods using alternatives to helium carrier gases is necessary. The objective of this study was to examine the applicability of hydrogen and nitrogen as alternative carrier gases using the test method for azo compounds in the Act on Control of Household Products Containing Harmful Substances of Japan. The gas chromatograph mass spectrometer (GC-MS) analytical method using hydrogen and nitrogen as alternative carrier gases was compared with a method using helium for 26 primary aromatic amines (PAAs) originated from azo dyes. When hydrogen and nitrogen were used as carrier gases under the same conditions used during analysis using helium (same column, gas flow rate, oven temperature conditions, etc.), sufficient peak separation of 26 PAAs was obtained. The sensitivities of the methods using helium and hydrogen were comparable, whereas the sensitivity was lower when nitrogen was used, with the detection limits ranging from 1/220 to 1/25. However, all carrier gases achieved quantification at concentrations below the standard value (30 μg/g) of the Act on Control of Household Products Containing Harmful Substances, and the results were in agreement with the standard value for the target product. Our results indicated that hydrogen or nitrogen can be used as alternative carrier gases to helium for GC-MS analysis of azo compounds producing specific aromatic amines.
Keywords: primary aromatic amines, helium alternative carrier gas, GC-MS

大嶋智子*, 河上強志: 繊維製品に含まれる防炎加工剤トリス（1-アジリジニル）ホスフィンオキシドのGC-MS分析法.
薬学雑誌 2024;144:119-27. doi:10.1248/yakushi.23-00156

Tris(1-aziridinyl)phosphine oxide (APO) used as flame retardant in textile products, such as curtains, carpets, and sleeping clothes, is prohibited in Japan under the "Act on the Control of Household Products Containing Harmful Substances." This study developed a GC-MS-based method to quantify APO more accurately and safely than the current official method. The APO in textile products was extracted with methanol, the extract was replaced with acetone instead of hexane as previously reported, and purified by florisil cartridge column. This cleanup method was instead of the harmful and carcinogenic dichloromethane used for open column to purify the sample in the official method, giving consideration to health of analysts. For accurate and sensitive quantification, deuterated compound, APO-d_{12}, was used as a surrogate standard. The calibration curve displayed linearity within the 0.01–2.0 μg/mL range for APO. The detection limit for APO was 0.008 μg/g with S/N=5, which was 50 times more sensitive than the current detection limit of 0.4 μg/g, enabling the analysis of sufficiently low concentrations. The recoveries in non-treatment cloth and flame-retardant textiles were 73.5–126.6% and relative standard deviations were 3.3–24.6% when 2 μg APO was added to 0.5 g of samples, confirming that it can be analyzed satisfactorily. Thus, the developed method is applicable to textile products of various materials.
Keywords: tris(1-aziridinyl)phosphine oxide, frame retardants, GC-MS

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臨床皮膚科 2024;78:106-11. doi:10.11477/mf.1412207199

30歳台，女性．術創にダーマボンド®アドバンスド（DA）を使用した約１か月後より，使用部位に一致して米粒大の丘疹が集簇し，使用部位以外の軀幹四肢にも丘疹や浮腫性紅斑が出現した．製品（as is）と主成分である２-オクチルシアノアクリレートのオープンテストが陽性を示し，DAに含まれる２-オクチルシアノアクリレートによる接触皮膚炎症候群と診断した．アクリレート・メタクリレート関連化学物質のパッチテストはすべて陰性であった．ダーマボンド®シリーズによる接触皮膚炎は，特に初回使用例では発症時期が約１か月後と比較的遅く，また，接触皮膚炎症候群への進展例が多いのが特徴である．DAの術創への長期接触による感作や症状悪化の可能性から，可能な限り早期に除去することが重要と考えた．自験例では，DA以外に交差感作の可能性のある他のシアノアクリレート製品やアクリレート・メタクリレートの使用を避けるよう指導した．

Keywords: ダーマボンド®アドバンスド, 2-オクチルシアノアクリレート, 接触皮膚炎症候群

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内山奈穂子，細江潤子，石附京子，新井玲子，杉本直樹，鈴木梓*1，浅野龍二*1，五十嵐靖*1，三浦亨*2，武藤康弘*2，末松孝子*3，小松功典*3，日向野太郎*4，古川茶勲*4，嶋田典基*5，合田幸広：ブシモノエステルアルカロイドの相対モル感度（RMS）を用いた日本薬局方定量法の検討．

YAKUGAKU ZASSHI 2023;143(11): 951–62. doi: 10.1248/yakushi.23-00122

Recently, a novel quantitative method using relative molar sensitivity (RMS) was applied to quantify the ingredients of drugs and foods. An important development in this regard can be observed in the Japanese Pharmacopoeia (JP) 18, where the quantification of perillaldehyde, an unstable compound, in crude drug "Perilla Herb," was revised to incorporate the RMS method. In this study, the primary objective was to improve the tester safety and reduce the amount of reagents used in the JP test. To achieve this, the quantification of three toxic Aconitum monoester alkaloids (AMAs) was explored using the RMS method, employing a single reference compound for all three targets. These AMAs, namely benzoylmesaconine hydrochloride, benzoylhypaconine hydrochloride, and 14-anisoylaconine hydrochloride, which are the quantitative compounds of Kampo extracts containing Aconite Root (AR), were quantified using the reference compound benzoic acid (BA). Reliable RMS values were obtained using both 1H-quantitative NMR and HPLC/UV. Using the RMS of three AMAs relative to the BA, the AMA content (%) in commercial AMAs quantitative reagents were determined without analytical standards. Moreover, the quantitative values of AMAs using the RMS method and the calibration curve method using the three analytical standards were similar. Additionally, similar values were achieved for the three AMAs in the Kampo extracts containing AR using the RMS and the modified JP18 calibration curve methods. These results suggest that the RMS method is suitable for quantitative assays of the Kampo extracts containing AR and can serve as an alternative to the current method specified in the JP18.

Keywords: relative molar sensitivity, 1H-quantitative NMR, Aconitum monoester alkaloids, benzoic acid

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

Chem. Pharm. Bull. 2024;72(1):36-40. doi: 10.1248/cpb.c23-00635

The spectrum of ^{31}P-NMR is fundamentally simpler than that of ^{1}H-NMR; consequently identifying the target signal(s) for quantitation is simpler using quantitative ^{31}P-NMR (^{31}P-qNMR) than using quantitative ^{1}H-NMR (^{1}H-qNMR), which has been

already established as an absolute determination method. We have previously reported a ^{31}P-qNMR method for the absolute determination of cyclophosphamide hydrate and sofosbuvir as water-soluble and water-insoluble organophosphorus compounds, respectively. This study introduces the purity determination of brigatinib (BR), an organophosphorus compound with limited water solubility, using ^{31}P-qNMR at multiple laboratories. Phosphonoacetic acid (PAA) and 1,4-BTMSB-d_4 were selected as the reference standards (RSs) for ^{31}P-qNMR and ^{1}H-qNMR, respectively. The qNMR solvents were chosen based on the solubilities of BR and the RSs for qNMR. CD_3OH was selected as the solvent for ^{31}P-qNMR measurements to prevent the influence of deuterium exchange caused by the presence of exchangeable intramolecular protons of BR and PAA on the quantitative values, while CD_3OD was the solvent of choice for the ^{1}H-qNMR measurements to prevent the influence of water signals and the exchangeable intramolecular protons of BR and PAA. The mean purity of BR determined by ^{31}P-qNMR was 97.94 ± 0.69%, which was in agreement with that determined by ^{1}H-qNMR (97.26 ± 0.71%), thus indicating the feasibility of purity determination of BR by ^{31}P-qNMR. Therefore, the findings of this study may provide an effective method that is simpler than conventional ^{1}H-qNMR for the determination of organophosphorus compounds.

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

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Hirasawa Y*, Kase A*, Okamoto A*, Suzuki K*, Hiroki M*, Kaneda T*, Uchiyama N, Morita H*: Vincazalidine A, an Unique Bisindole Alkaloid from *Catharanthus roseus*.

J. Nat. Med. 2024;78:382–92. doi:10.1007/s11418-023-01775-x

A new dimeric indole alkaloid, vincazalidine A consisting of an aspidosperma type and a modified iboga type with 1-azatricyclo ring system consisting of one azepane and two piperidine rings coupled with an oxazolidine ring was isolated from *Catharanthus roseus*, and the structure including absolute stereochemistry was elucidated on the basis of spectroscopic data as well as DP4 statistical analysis. Vincazalidine A induced G2 arrest and subsequent apoptosis in human lung carcinoma cell line, A549 cells.

Keywords: Vincazalidine A, Bisindole alkaloid, *Catharanthus roseus*

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Akiyama H*, Ishibashi A*, Kai T*, Kikuchi A*, Taguchi T, Fukiwake T*, Tsutsumi T, Asakura H, Ito R*: Determination of Cyanide and Cyanoglycosides in Sweetened Bean Paste by HPLC with Fluorescence Detection.

Biol. Pharm. Bull. 2023;46:1024-1026. doi: https://doi.org/10.1248/bpb.b23-00118

It is necessary to evaluate the efficiency of reduction for cyanide and cyanoglycosides during the manufacturing process from raw material beans to sweetened bean paste in a food hygiene control system from the viewpoint of food safety. Analytical methods for cyanide and cyanoglycoside determination in sweetened bean paste by HPLC with fluorescence detection were developed. In analysis of collection time of free cyanide in the free cyanide assay, the recovery was improved by extending the collection time, the recovery rate was >80% by 2h. The accuracy, repeatability and intra-laboratory precision of the free cyanide assay were 82.3, 2.0, and 2.4%, respectively. The method for cyanoglycoside analysis was evaluated by 5 repeated spiked recovery experiments at a concentration of 10ppm. The accuracy, repeatability and intra-laboratory precision of the cyanoglycoside method were 82.2, 1.9, and 3.4%, respectively. These analytical methods will enable the analysis of cyanide

and cyanoglycosides in sweetened bean paste without using steam distillation method in the pretreatment.
Keywords: hazard analysis and critical control point (HACCP), cyanide, sweetened bean paste

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坂井隆敏，菊地博之，根本了，穐山浩*，田口貴章，堤智昭：LC-MS/MSを用いた畜産物中の酢酸メレンゲステロールの分析法.
食品衛生学雑誌，2024;65(1):15-19. doi: https://doi.org/10.3358/shokueishi.65.15

The present study verified that it is possible to analyze melengesterol acetate using the existing multi-residue method. Melengestrol acetate was extracted from livestock products using acidic acetonitrile acidified with acetic acid in the presence of *n*-hexane and anhydrous sodium sulfate. The crude extracts were cleaned up using an octadecylsilanized silica gel cartridge column. Separation by HPLC was performed using an octadecylsilanized silica gel column with linear gradient elution of 0.1 vol% formic acid and acetonitrile containing 0.1 vol% formic acid. For the determination of the analyte, tandem mass spectrometry with positive ion electrospray ionization was used. In recovery tests using four livestock products fortified with maximum residue limits levels of melengestrol acetate (0.001–0.02 mg/kg), the truenesses ranged from 82% to 100%, and the repeatabilities for the entire procedure ranged from 0.5 RSD% to 5.6 RSD%. In recovery tests using 11 livestock products fortified with 0.0005 mg/kg of melengestrol acetate, the truenesses ranged from 88% to 99%, and the repeatabilities ranged from 1.3 RSD% to 5.4 RSD%. The limit of quantification for melengestrol acetate in livestock products was 0.0005 mg/kg.
Keywords: melengestrol acetate, LC-MS/MS, livestock products

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Zhang T, Takatsuki S, Sato T*, Tobiishi K*, Hori T*, Nabeshi H, Tsutsumi T: Polychlorinated Biphenyl Concentrations and Estimated Intakes in Fish Oil Supplements on the Japanese Market.
J. Food Prot., 2024; 87:100235. doi: 10.1016/j.jfp.2024.100235

Polychlorinated biphenyls (PCBs) are synthetic organic contaminants that are widespread in the environment. There are 209 PCB congeners. Fish oil produced from marine fish is widely used as a health supplement. PCB contamination of fish oil is of concern. We determined the concentrations of all 209 PCB congeners in commercially available fish oil supplements from Japan and estimated PCB intakes for humans consuming the supplements. We determined the concentrations of non–dioxin - like PCBs separately. The total PCB concentrations in 37 fish oil supplements purchased in Japan were 0.024–19 ng/g whole weight, and the non–dioxin - like PCB concentration range was also 0.024–19 ng/g whole weight. The total PCB intakes calculated for a 50 kg human consuming the supplements were 0.039–51 ng/day (0.00078–1.0 ng/(kg body weight per day)) and the non–dioxin - like PCB intake range was also 0.039–51 ng/day (0.00078–1.0 ng/(kg body weight per day)). The total PCB intakes were much lower than the tolerable daily intake of 20 ng/(kg body weight per day) recommended by the WHO. The results indicated that PCBs in the fish oil supplements pose acceptable risks to humans consuming the fish oil supplements daily.
Keywords: Fish oil supplements, Intake estimation, Non–dioxin - like PCBs, Polychlorinated biphenyls

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志田（齊藤）静夏，齋藤真希，堤智昭：クロレラ加工品中のフェオホルバイド等クロロフィル分解物試験法の改良.
食品衛生学雑誌，2023;64(6):191-199. doi: https://doi.org/10.3358/shokueishi.64.191

An official analytical method for chlorophyll degradation compounds, including pheophorbide, in chlorella products, is described in notification Kanshoku No. 99 (May 8, 1981). However, this method has several operational issues, such as the formation of emulsion during liquid-liquid partitioning. Additionally, impurities present in the reagents (sodium sulfate decahydrate or anhydrous sodium sulfate) used to prepare saturated sodium sulfate solution can degrade pheophorbide and other related compounds, resulting

in a significant decrease in analytical values. In this study, we thoroughly examined each step of the official method to enhance the operability and develop an alternative method that eliminates the need for saturated sodium sulfate solution. The developed method was evaluated for pheophorbide a and pyropheophorbide a at 100 mg%. Satisfactory analytical performance was achieved with trueness of 100% for pheophorbide a and 90% for pyropheophorbide a, and relative standard deviations of intra- and inter-day precision below 5% for both compounds. The proposed method is considered suitable for regulatory analysis of chlorophyll degradation compounds and would be useful for quality control of chlorella products.

Keywords: pheophorbide, chlorophyll, chlorella

Suzuki Y, Harimoto M, Takahashi M, Akiyama H[*1], Hirose A[*2], Tsutsumi T: Analysis of Silver-containing Nanoparticles in Oysters Using Single-particle ICP-MS.

Journal of Environmental Chemistry, 2024;34:9-20. doi: 10.5985/jec.34.9.

Silver-containing nanoparticles (Ag-NPs) are now used in a wide range of consumer products for their antibacterial properties. Human exposure to Ag-NPs can occur not only through contact with these products but also through foods that have become contaminated with Ag-NPs released from these products into the environment. However, the status of NPs in foods remains unknown due to the lack of an analytical method for the examination of NPs contained in solid samples such as foods. Recent evidence suggests that Ag-NPs released into the environment may accumulate in filter feeders such as oysters. Here, we developed a method for measuring the concentrations of Ag-NPs in oysters using single-particle inductively coupled mass spectrometry (spICP-MS). Ag-NPs were extracted from oysters using one of four extraction procedures (ultrasonic crushing, alkali-treated hydrolysis, solubilization with surfactant, or enzymatic digestion), and recoveries of spiked Ag-NP (diameter, 60 nm) in terms of particle size, particle number concentration, and particle mass concentration were evaluated. Among the four extraction procedures, enzymatic degradation afforded the highest recoveries for particle number concentration (85 ± 13%) and particle mass concentration (93 ± 14%) and had the smallest effect on particle size (110 ± 3%). Using our developed spICP-MS approach with enzymatic degradation, we examined 24 samples of rock and Pacific oysters purchased on the Japanese market between 2019 and 2020 and found that they contained Ag-NPs at 6.3×10^5 to 7.7×10^8 particles/g. The particle mass concentrations ranged from 0.13 to 98 ng/g, and an average of 1.2% of the total Ag was present as nanoparticles. The present data also suggest a difference in the size of the Ag-NPs accumulated in rock oysters (28 ± 2 nm) and Pacific oysters (25 ± 4 nm); further studies are needed to examine this finding in more detail.

Keywords: nanoparticle, silver, spICP-MS

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Suzuki Y, Kondo M, Harimoto M, Okamoto Y, Tanaka Y[*1], Ogra Y[*1], Akiyama H[*2]: Bayesian estimation to deconvolute single-particle ICP-MS data with a mixed Poisson distribution.

J. Anal. At. Spectrom., 2024;39(1):190-203. doi: 10.1039/D3JA00220A.

Single-particle ICP-MS (spICP-MS) is an established method for the determination of inorganic nanoparticle (NP) mass distributions and particle number concentrations. However, spICP-MS is not applicable to some cases, especially cases that require distinguishing signals from dissolved ions and signals from relatively small NPs. To deconvolute spICP-MS data, which is obtained by setting the dwell time similar to the particle event duration time, a Bayesian estimation method was developed for spICP-MS analysis using silver (Ag) and silica (SiO_2) NPs. The signal distributions of the spICP-MS data were parameterised using a Bayesian estimation method on the assumption that they could be described by mixed Poisson distributions. Analytical results were then compared to results obtained with conventional criteria. When the instrument parameters were set so that the particle-event duration was within 2 readings and hence did not deviate from the assumptions of the current Bayesian model, better estimation results could be obtained with the Bayesian estimation method than with methods based on conventional criteria, especially

for a sample with high particle number concentration. Furthermore, applying the specific informative prior distribution enabled us to obtain reasonable estimation results, even when the signal counts were 6 or less and the background counts were high. Because appropriate NP information was obtained for Ag-NP and SiO_2-NP, the Bayesian estimation method can be universally adopted with inorganic NPs detectable by ICP-MS.

Keywords: nanoparticle, spICP-MS, Bayesian estimation

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Wakui N[*1], Inoue K[*2], Nunome M[*2], Suzuki Y, Takagi A[*3], Ito R[*1], Iwasaki Y[*1], Sugiura J[*4], Akiyama H[*1]: Implementation and Evaluation of Risk Communication Regarding Residual Pesticides.
Food Hyg. Saf., 2024;65(1):20-23. doi: 10.3358/shokueishi.65.20.

In this study, a public seminar on risk communication methods was conducted to raise awareness and disseminate accurate knowledge about residual pesticides to consumers. Additionally, surveys on consumer awareness were conducted on the attendees before and after the seminar to evaluate its effectiveness. Responses were obtained from 84 participants. The paired t-test was used to analyze the changes in awareness before and after the seminar. The results showed significant improvements in "trust in the government" and "understanding of residual pesticides." Furthermore, step-wise multiple regression analysis was performed to explore the factors influencing satisfaction with the risk communication seminar, and the item "understanding of the safety of residual pesticides in food" was extracted. Understanding food safety is a crucial concern in daily life for consumers. To enable consumers to have an accurate understanding of food risks and make appropriate judgments, it is essential to continue implementing risk communication and conveying information about food safety and security in the future.

Keywords: risk communication, residual pesticides, food safety

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Torii A[*1], Seki Y[*1], Arimoto C[*1], Hojo N[*1], Iijima K[*1], Nakamura K, Ito R[*2], Yamakawa H[*1], Akiyama H[*2]: Development of a simple and reliable LC-MS/MS method to simultaneously detect walnut and almond as specified in food allergen labelling regulations in processed foods.
Current Research in Food Science, 6;100444 (2023). doi: 10.1016/j.crfs.2023.100444

We developed a simple and reliable analytical method using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to simultaneously detect walnut and almond as specified in regulations for food allergen labelling in processed foods. Five specific target peptides derived from walnut 2S albumin and 7S globulin and three target peptides from almond 11S globulin were selected by analysing several varieties of walnut and almond, eight kinds of other nuts, and ten kinds of major allergen ingredients or cereals. The limit of detection for the walnut 2S albumin peptide GEEMEEMVQSAR (m/z 698.3 [precursor] > 316.1 [product]) was 0.22 ± 0.02 μg/g, and that for almond 11S globulin peptide GNLDFVQPPR (m/z 571.8 [precursor] > 369.2 [product]) was 0.08 ± 0.02 μg/g when extracted walnut and almond protein were spiked into butter cookie chocolate ice cream. These peptides had good linearity (R^2 > 0.999) for each calibration curve with a range of 0.1–50 μg/mL protein concentration in the sample solutions, and sufficient recovery rates (90.4–101.5%) from the spiked samples. The developed analytical approach is applicable to a wide variety of processed foods for food allergen labelling.

Keywords: LC-MS/MS, detection, allergen

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Yamasaki Y, Suzuki Y, Kitayama I, Nunome M, Kondo M, Sakai T, Nemoto S, Akiyama H*,

Tsutsumi T: Validation of analytical method and surveillance for gibberellic acid in banana, cherry, and kiwi fruit.
Food Hyg Saf Sci, 2023;64(4):123–129. doi: 10.3358/shokueishi.64.123

Gibberellic acid (GA_3) is commonly used as a plant growth regulator in many food crops owing to its essential signaling functions during plant growth and development. In Japan, a threshold for administrative action for GA_3 content of 0.3 mg/kg applies in produce in which maximum residue limits have not been established. Although the threshold is based on previous studies, the GA_3 concentrations in individual foods are still unknown. Thus, we surveyed the concentrations of GA_3 in banana, cherry, and kiwi fruit on the Japanese market. We developed and validated a method for the analysis of GA_3 using solid-phase extraction and LC-MS/MS in accordance with accepted criteria of trueness, repeatability, and selectivity. The limits of detection and of quantification were determined as 0.005 and 0.05 mg/kg, respectively, in all fruits. Concentrations of GA_3 did not exceed 0.3 mg/kg regardless of ripeness, suggesting the reasonability of the current regulation of GA_3 in banana, cherry, and kiwi fruit. These findings can support prompt administrative action on these fruits, contributing to the regulation of GA_3 in Japan.
Keywords: gibberellic acid, analytical method, surveillance

* Hoshi University

岡部 亮*，根本了，青柳光敏*：LC-MS/MSを用いた畜産物中のフルベンダゾールおよび代謝物の分析法.
食品衛生学雑誌，2023;64(4):130-135. doi: https://doi.org/10.3358/shokueishi.64.130

This study proposes a method to determine flubendazole and metabolite R35475 in livestock products using tandem mass spectrometry coupled with positive ion electrospray ionization. Acetone is used to extract flubendazole and metabolite R35475 from the livestock samples. These extracts were purified using an SCX cartridge column (500 mg). Furthermore, high-performance liquid chromatography was performed on an Inertsil ODS-4 column with a gradient formed using methanol and water, both of which contain 5 mmol/L of ammonium acetate. The recovery tests using bovine muscle, fat, liver, milk, and egg fortified at the maximum residue limits of analytes or 0.005 mg/kg revealed that the trueness (n=5) of flubendazole and metabolite R35475 ranged from 89.4 to 106.4% with a repeatability rate of 1.7–7.8% .
Keywords: flubendazole, livestock products, LC-MS/MS

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Sato T*[1,2], Tobiishi K*[1], Hori T*[1], Tsutsumi T, Akiyama H*[3], Matsui T*[2]: Simultaneous determination of hexabromocyclododecanes, Polybrominated diphenyl ethers, and dechlorane-related compounds in boxed sushi meals using a developed analytical method.
Food Science and Technology Research, 2023;29(4):347-356. doi: https://doi.org/10.3136/fstr.FSTR-D-22-00204

We investigated the concentrations of halogenated flame retardants (HFRs), which include hexabromocyclododecanes (HBCDDs), polybrominated diphenyl ethers (PBDEs), and dechloranes and related compounds (DRCs), in 25 typical ready-made boxed sushi meals (each divided into seafood and non-seafood portions) using a developed simultaneous analytical method involving accelerated solvent extraction and gel permeation chromatographic separation. The developed method yielded good recoveries of surrogates (72–122%). HBCDDs, PBDEs, and DRCs were detected in all seafood portions. While DRCs were also frequently detected in non-seafood portions, HBCDDs and PBDEs were hardly detected. The estimated dietary intakes of HBCDDs, PBDEs, and DRCs from boxed sushi meals were well below the corresponding health-based guideline values. In conclusion, our study suggests that the intake of HFRs from boxed sushi meals poses low concern for consumer health and that the developed simultaneous analytical method is highly useful for determining HFRs in seafood-based meals.
Keywords: halogenated flame retardant, seafood, intake

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食品衛生学雑誌 2023;64:240–245. doi: 10.3358/shokueishi.64.240

「第２版　食品中の食品添加物分析法」に収載されている亜硝酸ナトリウム分析法（2 版法）では，試料によっては試験溶液が混濁し，ろ過が困難となる場合がある．近年，これらの問題点を解消した改良分析法が報告された．そこで，その改良分析法について，2 版法の改正を視野に，単一試験室による妥当性確認を行い，8 機関で共同実験を行った．亜硝酸ナトリウム表示のないたらこ，魚肉ソーセージ，ハムを用い，使用基準の上限量を添加濃度として単一試験室による妥当性確認を実施した結果，真度88～92%，併行精度2.0～3.0%，室内精度3.2～4.3%と推定され，目標値（真度：70～120%，室内精度：15%未満，併行精度：室内精度以下）を満たしていた．また．共同実験では，同じ3 試料を配布し，各機関において定量下限値の2 倍および使用基準の上限量の2 濃度を添加し併行分析（n=3）した．得られた分析値から推定された真度は82～95%，併行精度は2.3～5.8%，室間再現精度は3.5～11%であった．以上より，本研究で検証した分析法は，食品中の亜硝酸根の測定に有用と考えられ，2 版法の改正法としても妥当であることが示された．

Keywords: 食品添加物，発色剤，亜硝酸塩，妥当性確認

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*6（一財）東京顕微鏡院
*7（一財）日本食品分析センター

稲井隆之*，浮田英生*，大橋篤志*，樺沢正志*，児高由以子*，澤野友信*，関谷史子*，土屋一行*，寺川将樹*，長屋有紀子*，松井敏晃*，久保田浩樹，建部千絵，佐藤恭子，多田敦子：日本における食品香料化合物の使用量調査結果（2020年）．

日本食品化学学会誌 2023;30:158–164. doi: 10.18891/jjfcs.30.3_158

日本香料工業会は，国際香料工業会（IOFI）が５年ごとに実施している世界的な使用量調査に合わせ，2020年１月から12月にかけて日本で使用されている香料原料の調査を実施した．最新の食品香料の使用量調査の結果を報告する．日本における2020年の香料数は1,843品目，総使用量は1,272トンであった．今回の使用量調査では，「少量多品種の香料」が使用されていることが概ね確認された．また，新たに指定された香料については，推定摂取量が当該構造分類の許容摂取量以下であるか，または推定摂取量と無影響レベル（NOAEL）との間に十分な余裕があり，安全な使用が確認された．

Keywords: 使用量調査，食品香料化合物，推定摂取量

* 日本香料工業会

阿部 裕，山口未来，片岡洋平，六鹿元雄，佐藤恭子，杉本直樹：ポリ塩化ビニル製おもちゃの使用可塑剤調査（2019～2020）．

食品衛生学雑誌 2023;64:145-153. doi: 10.3358/shokueishi.64.145

2019～2020年に市販されていたポリ塩化ビニル（PVC）製おもちゃ220検体を対象に使用可塑剤の調査を行った．その結果，15種類の可塑剤が同定された．この内フタル酸エステル類（PAEs）は４種類であった．また，本研究では同定に至らなかったが，PAEsと推定されるこれまで検出したことのない３種類の可塑剤も検出された．軟質PVC製おもちゃ209検体ではテレフタル酸ジ（２-エチルヘキシル）（DEHTP）の検出率が最も高く，指定おもちゃでは71.2%，指定おもちゃ以外では88.9%であり，過去の調査から徐々に増加している傾向がみられた．その他の特徴として，アセチルクエン酸トリブチルやアジピン酸エステル類の使用量の減少が確認された．規制対象の６種のPAEsは指定おもちゃでは引き続き使用されていなかったが，フタル酸ジイソブチルの使用が増加した．一方指定おもちゃ以外ではPAEsのうち特にフタル酸ジ（２-エチルヘキシル）の検出率が約１/10に減少した．１検体あたりの使用量は５年前の調査から継続して低いレベルを維持していた．このように，現在国内で流通するPVC製おもちゃに主に使用されている可塑剤はDEHTPであり，その他の可塑剤の使用は減少していることが明らかとなった．

Keywords：ポリ塩化ビニル，可塑剤，乳幼児用おもちゃ

阿部裕，山口未来，片岡洋平，六鹿元雄，佐藤恭子，杉本直樹：ポリメタクリル酸メチル製食品用器具・容器包装のメタクリル酸メチル試験法へのHPLCの適用．

日本食品化学学会誌 2023;30:109-113. doi: 10.18891/jjfcs.30.2_109

ポリメタクリル酸メチル製食品用器具・容器包装のメタクリル酸メチル（MMA）試験法へのHPLCの適用性を検討した．HPLC条件は日本産業規格（JIS）を参考にして，カラムはODS系カラム，移動相はアセトニトリルおよび水，検出波長は205 nmとした．さらにMMAのピークが夾雑ピークと重複しないようにグラジエント条件を最適化した．MMA試験法では，MMAの標準溶液は20% エタノール（EtOH）で調製することとされているが，室温で保管するとMMAは不安定である可能性が疑われた．そこで標準溶液はEtOHで調製することとし，さらに試験溶液はEtOHで10倍以上希釈して分析したところ，MMAを安定して測定できることが明らかとなった．本法について限度試験および定量試験としての性能を評価した結果，いずれも真度，併行精度および再現精度（室内精度）のパラメーターは目標値を満たしており，本分析法が規格の適否判定を行う分析法として有用であると考えられた．

Keywords：メタクリル酸メチル，HPLC，食品用器具・容器包装

増本直子，杉本直樹，佐藤恭子：食品添加物公定書収載品目における基原生物に使用される学名の考え方とその表記法．

食品衛生学雑誌 2023;64:77-88. doi: 10.3358/shokueishi.64.78

天然物由来の食品添加物では，その基原生物が一義的に特定されるよう，成分規格に基原生物の学名および和名が明記されている．誤った基原生物の使用は想定外の健康被害を招く恐れがあり，学名や和名の正確性は添加物の有効かつ安全な使用に欠かせない．しかし，過去に公的な成分規格等で定義された学名が最新の分類学による学名と一致せず，詳しい調査が必要とされることが多々ある．そこで，食品添加物の原料の範囲を合理的かつ持続的に制御するために，トレーサビリティに重点をおいた方針を策定し，具体的な学名および和名の調査法および表記法を定めた．この方針に従い，既存添加物である「香辛料抽出物」，「アラビアガム」および「カラギナン」について，基原生物を精査したところ，学名や和名の決定はおおむね可能であったが，一部の生物で学名改変に伴い想定する種の範囲が広がるものもあった．トレーサビリティ確保の方針は有用だが，それに従った結果，成分規格等で定義された種と異なる種が含まれることにならないか等の確認も必要だと考える．

Keywords：天然添加物，学名，基原生物

Masumoto N, Ohno T*, Suzuki T*, Togawa T*, Sugimoto N: Application of the relative molar sensitivity method using GC-FID to quantify safranal in saffron (*Crocus sativus L.*).

J. Nat. Med. 2023;77:829-838. doi: 10.1007/s11418-023-01724-8

Safranal is one flavor component of saffron, which is used as a spice, food additive, and crude drug. In ISO3632, safranal is defined as the compound that contributes to the quality of saffron, and many quantitative determination methods for safranal have been reported. However, safranal is volatile and degrades easily during storage, and an analytical standard with an exact known purity is not commercially available, making it difficult to quantify accurately the content of safranal in saffron. Here, we developed a method for quantifying safranal using relative molar sensitivity (RMS), called the RMS method, using a GC-flame ionization detector (GC-FID). We determined the RMS of safranal to 1,4-bis(trimethylsilyl)benzene-d_4, a certified reference material commercially available, by a combination of quantitative NMR and chromatography. Using two GC-FID instruments made by different manufacturers to evaluate inter-instrument effect, the resultant RMS was 0.770, and the inter-instrument difference was 0.6%. The test solution, with a known safranal concentration, was measured by the RMS method, with an accuracy of 99.4-101%, repeatability of 0.81%, and reproducibility of 0.81-1.3%. Given the ease of degradation, high volatility, and uncertain purity of safranal reagents, the RMS method is a more accurate quantification approach compared to the calibration curve method and methods based on absorption spectrophotometry. Moreover, our findings revealed that the GC-FID makeup gas affected the RMS and quantitative values.

Keywords: 1H-quantitative NMR, GC-FID, Relative molar sensitivity

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Iwasaki D*, Kanazawa M*, Kawamoto F*, Araho D*, Murakami T*, Nishizaki Y, Masumoto N, Sugimoto N: A new single-reference quantitative method using liquid chromatography with relative molar sensitivity based on ^{1}H-qNMR for khellactone

esters from *Peucedanum japonicum* root extract.
Food Chem. 2023;427:136647. doi: 10.1016/j.foodchem.2023.136647

Khellactone ester (KLE) quantification using the absolute calibration method is difficult owing to the unavailability of standard reagents that can guarantee purity. Herein, a new method was developed to quantify KLEs from *Peucedanum japonicum* root extracts using liquid chromatography (LC) without utilizing standards. This method used relative molar sensitivity (RMS) and 7-ethoxy-4-methylcoumarin as a single-reference (SR) compound instead of KLE standards. RMS is the sensitivity ratio of SR to analytes, determined using an offline combination of quantitative NMR and LC. LC was performed using a triacontylsilyl silica gel column of superficially porous particles with a ternary mobile phase. The range of the method was 2.60–509 μmol/L. The accuracy and precision were reasonable. This is the first study to apply the RMS method to both conventional LC and ultra-high-performance liquid chromatography using the same mobile phase and column. This method may aid the quality assurance of foods containing KLEs.
Keywords: *Peucedanum japonicum*, Relative molar sensitivity, ^{1}H-qNMR

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Nishihara M*[1], Hirabuchi A*[1], Goto F*[1], Nishizaki Y, Uesugi S*[1], Watanabe A*[1], Tasaki K*[1,2], Washiashi R*[1], Sasaki N*[1,3]: Production of yellow-flowered gentian plants by genetic engineering of betaxanthin pigments.
New Phytologist, 2023;240:1177-1188. doi: 10.1111/nph.19218

Genetic engineering of flower color provides biotechnological products such as blue carnations or roses by accumulating delphinidin-based anthocyanins not naturally existing in these plant species. Betalains are another class of pigments that in plants are only synthesized in the order Caryophyllales. Although they have been engineered in several plant species, especially red-violet betacyanins, the yellow betaxanthins have yet to be engineered in ornamental plants. We attempted to produce yellow-flowered gentians by genetic engineering of betaxanthin pigments. First, white-flowered gentian lines were produced by knocking out the dihydroflavonol 4-reductase (DFR) gene using CRISPR/Cas9-mediated genome editing. Beta vulgaris BvCYP76AD6 and Mirabilis jalapa MjDOD, driven by gentian petal-specific promoters, flavonoid 3',5'-hydroxylase (F3'5'H) and anthocyanin 5,3'-aromatic acyltransferase (AT), respectively, were transformed into the above DFR-knockout white-flowered line; the resultant gentian plants had vivid yellow flowers. Expression analysis and pigment analysis revealed petal-specific expression and accumulation of seven known betaxanthins in their petals to c. 0.06-0.08 μmol g FW-1. Genetic engineering of vivid yellow-flowered plants can be achieved by combining genome editing and a suitable expression of betaxanthin-biosynthetic genes in ornamental plants.
Keywords: Japanese gentian, betaxanthin, genome editing

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Amakura Y*, Uchikura T*, Yoshimura M*, Masumoto N, Nishizaki Y, Sugimoto N: Chromatographic evaluation and characterization of constituents of sunflower seed extract used as food additives.
Chem. Pharm. Bull. 2024;72:93-97. doi 10.1248/cpb.c23-00670

Sunflower seed extract, an antioxidant agent registered on the List of Existing Food Additives in Japan, was evaluated using HPLC, and three common constituents were detected. These peaks were identified as monocaffeoylquinic acids (3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, and 5-*O*-caffeoylquinic acid [chlorogenic acid]). Upon scrutinizing other components, dicaffeoylquinic acids (isochlorogenic acids; 3,4-di-*O*-caffeoylquinic, 3,5-di-*O*-caffeoylquinic, and 4,5-di-*O*-caffeoylquinic acids) were also identified. Structures of two newly isolated compounds were determined to be 3-*O*-(3*S*-2-oxo-3-hydroxy-indole-3-acetyl)-5-*O*-caffeoylquinic and 4-*O*-(3*S*-2-oxo-3-hydroxy-indole-3-acetyl)-5-*O*-caffeoylquinic acids. To identify the components that contribute to the antioxidant activity of sunflower seed extract, we fractionated the food additive sample solution and

examined the active fractions for 2,2-diphenyl-1-picrylhydrazyl（DPPH）radical scavenging activity. Monocaffeoylquinic and dicaffeoylquinic acids showed high DPPH activity, including their contribution to the antioxidant activity of this food additive. DPPH radical scavenging activity of the new compounds showed almost the same value as that of the positive control, Trolox. Therefore, the contribution of these compounds was also considered.
Keywords: sunflower seed extract, existing food additive, antioxidant

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片岡洋平，六鹿元雄，阿部裕，近藤翠，四柳道代，佐藤恭子：ポリカーボネート製器具・容器包装の溶出試験におけるビスフェノールA分析法の改良，
食品衛生学雑誌 2023;64:111-115, doi:10.3358/shokueishi.64.111

ポリカーボネート製器具・容器包装の溶出試験の浸出用液がヘプタン，20%エタノール，４%酢酸における改良ビスフェノールA分析法を性能評価した．分析法の分析対象化合物はビスフェノールA，フェノールおよび*p-tert*-ブチルフェノールである．改良分析法の併行精度，室内精度，真度はそれぞれ0.2–1.8%，0.4–2.6%，95–102%の範囲にあった．これらの結果から，浸出用液がヘプタン，20%エタノール，４%酢酸における分析法として有用であると考えられた．さらに，蛍光検出器による測定法の適用性を検証した．分析法を性能評価した結果，本分析法の併行精度，室内精度，真度は，それぞれ0.1–2.9%，0.2–3.1%，94–101%の範囲にあった．したがって，蛍光検出器による測定も利用可能であることが確認された．
Keywords: ポリカーボネート，ビスフェノールA，溶出試験，食品用器具・容器包装

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食品衛生学雑誌 2023;64:154-160. doi:10.3358/shokueishi.64.154

ポリカーボネート製器具・容器包装の溶出試験におけるビスフェノールA分析法の浸出用液がヘプタンである場合の改良分析法について24試験所が参加する室間共同実験を行った．濃度非明示で２濃度の試料を配付し，計画書にしたがい試料中の分析対象化合物（ビスフェノールA，フェノールおよび*p-tert*-ブチルフェノール）濃度を定量した．得られた試験所の分析結果を基に，国際的なハーモナイズドガイドラインに沿って統計的に解析した．共同実験の結果として推定された室間再現相対標準偏差（RSD_R）とHorwitz/Thompson式により計算される予測室間相対標準偏差（$PRSD_R$）からHorRat値を算出した．その結果，２試料のHorRat値は３化合物をとおして0.15–0.37となり，Codex委員会が分析法承認のために設定している性能規準の指標である２未満を満たした．したがって，本分析法は規格の判定を行う分析法として有用であると考えられた．
Keywords: ポリカーボネート，ビスフェノール，室間共同試験

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Fujihara K, Yamaguchi M, Nishizaki Y, Abe Y, Mutsuga M, Sugimoto N: Identification of unknown plasticizers in polyvinyl chloride toys,
Jpn. J. Food Chem. Saf., 2023;30:149-157, doi:10.18891/

jjfcs.30.3_149

In our previous study, three unknown plasticizers were detected in five polyvinyl chloride (PVC) toys on the Japanese market. In this study, we isolated these three plasticizers (1–3), and their structures were determined using accurate mass and nuclear magnetic resonance (NMR) spectral data. One of the plasticizers was identified as bis(2-propylheptyl) phthalate (DPHP). The others were assumed to be (4-methyl-2-propylhexyl)(2-propylheptyl) phthalate and bis (4-methyl-2-propylhexyl) phthalate. This is the first study to detect these phthalic acid esters in PVC toys in Japan. Isolated DPHP was subjected to quantitative NMR (qNMR). Using the NMR solution with the absolute concentration as a calibrant, the DPHP concentration in five PVC toys was then determined by GC/MS. The amount of DPHP ranged from 14.5–21.9 wt%, whereas the DPHP level in one of the PVC toys was below the quantification limit (0.05 wt%).

Keywords: polyvinyl chloride (PVC), toy, plasticizer, phthalate, bis(2-propylheptyl) phthalate (DPHP)

岩越景子*，岩越一之*，長谷部恵美*，大須賀愛幸*，宮川弘之*，六鹿元雄，小林千種*：市販ポリエチレン製品から溶出される物質およびその溶出量に関する検討，

食品衛生学雑誌 2023;64:101-107, doi:10.3358/shokueishi.64.101

食品用として使用される可能性のある市販ポリ袋から，溶出が想定される物質についてLC-QTOFを用いたノンターゲット分析およびLC-MS/MSを用いた溶出量の把握を行った．LC-QTOFでの分析結果から推定された溶出物質を含む14物質について，移動相由来物質と試験溶液中のターゲット物質を分離するためのリテンションギャップ法を活用したLC-MS/MS分析法を構築した．本分析法を用いて市販のポリ袋9検体について溶出試験を実施したところ，最もIrganox 1076が溶出された試料では，EUのSMLの1/4の量が検出された．その他，Erucamide，Irganox 168-oxideの溶出が確認できた．

Keywords: 器具・容器包装，合成樹脂，ポリエチレン，溶出試験，リテンションギャップ法，液体クロマトグラフ-四重極飛行時間型質量分析装置，液体クロマトグラフ-四重極型質量分析装置

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Kishi E*, Ozaki A*, Ooshima T*, Abe Y, Mutsuga M, Yamaguchi Y, Yamano T*: Migration of catalyst elements from polyethylene terephthalate bottles into food simulants and mineral water under short- and long-term conditions,

Packaging Technology and Science, 2024;37:319-331, doi:10.1002/pts.2794

As polyethylene terephthalate (PET) bottles are common beverage containers, their safety is a matter of grave concern. In this study, migration behaviour of five elements (Ge, Ti, Sb, Co and P) used as catalysts, bluing agents and stabilizers during manufacturing from PET into beverages were assessed. Migration tests were performed on eight unused PET bottles under various conditions (food simulants: distilled water, 4% acetic acid and 50% ethanol; time: 10 days to 24 months; temperature: 25–60° C). The migration levels of two catalyst elements (Ge and Sb) under these conditions were 0.12–31.9 and <0.1–1.8, respectively, whereas Ti, Co and P were not detected (<2, <0.1 and <20 μg/L, respectively). The results showed storage temperature were positively correlated with migration of Sb and Ge in all food simulants. Different food simulants led to different levels of these elements migration at the same storage time and temperature, and the levels into 50% ethanol were higher than those into the other two food simulants. It was also shown that migration levels of Sb and Ge were below 2 and 20 μg/L even after 2 years of storage at room temperature, not exceeding the Japanese and EU regulation values. The Sb levels in distilled water were similar to those in commercially available bottled mineral waters, indicating that the migration tests provide a good reflection of the actual situation.

Keywords: antimony, food simulant, germanium, migration, mineral water, polyethylene terephthalate (PET) bottle

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Maronpot R*1, Ramot Y*2, Nyska A*3, Sproul C*4, Moore R*4, Bolon B*5, Hayashi SM: Oral chronic toxicity and carcinogenicity study of alpha-glycosyl isoquercitrin (AGIQ) in Sprague Dawley rats.

Regul. Toxicol. Pharmacol. 2023;140:105343. doi: 10.1016/j.yrtph.2023.105343

alpha-Glycosyl isoquercitrin (AGIQ) is a flavonoid

that possesses antioxidant and tumor suppressive capabilities and is marketed as a food additive in Japan. The aim of this study was to assess the potential for oral chronic toxicity and carcinogenicity of AGIQ in male and female Sprague Dawley rats following up to 5.0% dietary exposure. In the chronic toxicity study, rats were exposed to AGIQ or vehicle for one year with a 6-month interim termination point; for the carcinogenicity study, rats were treated for 24 months. No signs of AGIQ-related toxicity clinically or histologically were observed for up to one year except for yellow discoloration of bone. In the carcinogenicity study, a statistically significant increase in the incidence of malignant glioma of the brain or spinal cord was observed in female rats exposed to 5.0% AGIQ compared to those exposed to control feed. A Scientific Advisory Panel of experienced neuropathologists reviewed the gliomas (routine stains and glial cell markers) and concluded that the gliomas were a rare, spontaneous, rat-specific neoplasm: malignant microglial tumor. The lesions could not definitively be attributed to AGIQ exposure and have limited implications with respect to predicting human cancer risk.

Keywords: carcinogenicity, flavonoid, food additive safety

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Maronpot R*1, Ramot Y*2, Nyska A*3, Sproul C*4, Moore R*4, Koyanagi M*5, Chiba S*5, Nishino M*5, Hayashi SM: Chronic toxicity and carcinogenicity study of dietary gardenia blue in Sprague Dawley rats.
Food Chem. Toxicol. 2023;176:113734. doi: 10.1016/j.fct.2023.113734

In this combined chronic toxicity/carcinogenicity study of gardenia blue as a natural food color additive, Sprague Dawley rats were administered 0.5%, 2.5%, or 5.0% gardenia blue via the feed or carrier diet (0.0% gardenia blue) for 12 (chronic toxicity cohort) or 24 (carcinogenicity cohort) months. No abnormal clinical, ophthalmological, neurotoxicity or clinical pathology changes were attributed to treatment, and there was no increase in mortality due to gardenia blue exposure. The only treatment-related change was grossly observed blue discoloration of the stomach, intestines, and mesenteric lymph nodes as well as reversible dark discoloration of the kidneys all without associated histopathology. The no-observed-adverse-effect level (NOAEL) for gardenia blue exposure via the diet for one or two years was determined to be 5.0% (2175.3 mg/kg body weight/day in male rats and 3075.4 mg/kg body weight/day in female rats).

Keywords: carcinogenicity, gardenia blue, toxicity

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Maronpot RR*1, Streicker M*1, Mahapatra D*2, Moore R*2, Koyanagi M*3, Chiba S*3, Nishino M*3, Hayashi SM: Twelve-month in utero safety assessment of gardenia blue, a natural food colorant.
J. Toxicol. Pathol. 2023;36:171-179. doi: 10.1293/tox.2023-0030

Toxicity assessment of the food colorant Gardenia jasminoides Ellis at dietary exposures of 0.0%, 0.1%, 0.5%, 1.5%, 3.0% and 5.0% included measures of T-cell-dependent antibody response, neurotoxicity, and clinical and anatomic pathology in Sprague Dawley rats during mating, gestation, lactation, postnatal development, and following weaning for up to 12 months including 3- and 6-month interim evaluations. Blue coloration of the gastrointestinal tract, mesenteric lymph nodes and kidneys was present in treated rats only at necropsy with minimal blue coloration at the lowest dose and without histopathological correlates in any of the tissues. There was good survival with no consistent treatment-related changes in hematology, clinical chemistry, enhanced evaluation of lymphoid tissues, or tissue histopathology at interim and final time points. T-cell dependent antibody response and neurotoxicity screening were negative in treated rats. The no-observed-adverse-effect level (NOAEL) was determined to be 5.0% gardenia blue (2,854.5 and 3,465.4 mg/kg/day in parental males and females, respectively, prior to mating; 3,113.5 and 4,049.6 mg/

kg/day in male and female offspring, respectively, following up to 12 months of exposure.

Keywords: food colorant, gardenia blue, safety assessment

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Maronpot RR*1, Koyanagi M*2, Hayashi SM, Mahapatra D*3, Nishino M*2, Iniwa M*2: Gardenia blue is not carcinogenic in the rasH2 mouse.

Toxicol. Res. Appl. 2023;7:23978473231173093. doi: 10.1177/23978473231173093

Introduction: Gardenia blue is currently being considered as a naturally derived food colorant for use in the global marketplace. Methods: To assess its carcinogenic potential, 100 female and 100 male CByB6F1-Tg (HRAS)2Jic (rasH2) mice were allocated to four dose groups and exposed to gardenia blue in the diet for 26 weeks at dose levels of 0.0% (control), 0.5%, 2.5%, or 5.0% (corresponding to 0.0, 664.8, 3341.0, and 6623.2 mg/kg/day in male mice and 0.0, 1182.7, 5561.1, and 10,440.3 mg/kg/day in female mice, respectively). An additional group of 10 males and 10 females was administered intraperitoneal N-methyl-N-nitrosourea (MNU) as a positive control. Clinical observations, body and organ weights, clinical chemistry, hematology, and hormone analyses were performed in addition to urinalysis and histopathology. Results: The positive control elicited expected responses specific to rasH2 mice. There were sporadic background nondose-related findings in clinical pathology parameters and anatomic pathology common to rasH2 mice in the absence of any gardenia blue induced dose-related changes. Discussion: Under these study conditions, the no-observed-adverse-effect level was 5% gardenia blue (6623.2 mg/kg/day in male mice and 10,440.3 mg/kg/day in female mice). Conclusions: Based on this study a high dietary level of gardenia blue was negative for carcinogenicity in the rasH2 mouse test system.

Keywords: gardenia blue, carcinogenicity testing, rasH2 mouse

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Breslin WJ*1, Mesnard JL*2, Maronpot RR*3, Koyanagi M*4, Chiba S*4, Nishino M*4, Hayashi SM: Prenatal Developmental Toxicity of Gardenia Blue, a Natural Food Colorant, in Rats and Rabbits.

Toxicol. Res. Appl. 2023;7:23978473231169090. doi: 10.1177/23978473231169090

Introduction: Gardenia blue is a colorant widely used in Asia in food and beverages. The objectives of the present studies were to evaluate the maternal and prenatal embryo-fetal developmental toxicity of gardenia blue in rats and rabbits. Methods: Sprague Dawley rats and New Zealand White rabbits were administered gardenia blue daily by oral gavage at doses of 0 (deionized water vehicle), 500, 1000 or 2000 mg/kg/day on Gestation Days 6 through 20 (rats) and 7 through 28 (rabbits). Endpoints evaluated included clinical observations, body weight, food consumption, thyroid hormones (rats), thyroid weights and histopathology (rats), gross pathologic changes, ovarian and uterine observations, fetal weight and anogenital distance (rats) and fetal morphology (external, visceral and skeletal). Results: Treatment related maternal findings attributed to the blue/dark color of the test substance included body surface staining, and dark/blue discoloration of the kidneys, gastrointestinal track and mesenteric lymph nodes at all or most doses in the rat and/or rabbit. Slight reductions in food consumption without effects on body weight were also observed in rats at all doses and in rabbits at 2000 mg/kg/day. There were no treatment related effects on maintenance of pregnancy, postimplantation loss, litter size, fetal weight and anogenital distance, or fetal external, visceral, or skeletal malformations and variations. Conclusions: Based on these results, the maternal and developmental no-observed-adverse-effect level for gardenia blue in rats and rabbits was ≥2000 mg/kg/day.

Keywords: food additive, gardenia blue, embryo-fetal

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Foster ML[*1], Mahapatra D[*1], Maronpot RR[*2], Nishino M[*3], Chiba S[*3], Koyanagi M[*3], Burleson F[*4], Hayashi SM: Extended one-generation reproductive toxicity study evaluating gardenia blue in Sprague Dawley rats.

Regul. Toxicol. Pharmacol. 2023;144:105472. doi: 10.1016/j.yrtph.2023.105472.

Gardenia blue powder was administered at 0.5%, 2.5%, or 5.0% in feed to male and female Sprague Dawley rats in an Extended One-Generation Reproductive Toxicity Study (OECD Test Guideline 443). The dosed diet began 14 days before mating and was continued at the same concentration level for the entire study for all parental animals (P0) and offspring (F1). At weaning, offspring were allocated into one of 5 cohorts for different endpoints. P0 and F1 animals had blue urine, blue or black feces, and blue discolorations in gastrointestinal organs, mesenteric lymph nodes, and kidneys. This treatment-related finding was not considered adverse as there were no histopathologic correlates. There was a dose-related increase in sperm concentration in P0 and F1 males. There were dose-related increases in heart weights of F1 postnatal day (PND) 21 males, male and female thyroid weights, and female TSH levels of PND 91 F1 offspring, with no histopathological correlate. There were no consistent treatment-related adverse effects on any other parameters evaluated for general toxicity, reproductive toxicity, developmental neurotoxicity, or developmental immunotoxicity. The highest dietary concentration (5.0%) of gardenia blue powder was the no observed adverse effect level (NOAEL) for male and female rats at all life stages evaluated.

Keywords: extended one-generation, gardenia blue, neurotoxicity

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Liu H[*1], Inoue R[*2], Koyanagi M[*3], Hayashi SM, Nagaoka K[*1]: Potential Effects of Alpha-Glycosyl Isoquercitrin on Memory by Altering the Gut Microbiota-Blood-Brain Axis in Mice.

J. Agric. Food Chem. 2023;71:15991-16002. doi: 10.1021/acs.jafc.3c00897.

Alpha-glycosyl isoquercitrin (AGIQ), composed of isoquercitrin and glycosylated quercetin, has multiple biological effects. Here, we further examined the influence of AGIQ on brain function and provided its potential mechanism. Male C57BL/6 mice were treated with 0, 0.005, and 0.05% AGIQ in drinking water for 4 weeks prior to behavioral testing. Behavior tests showed that 0.05% AGIQ treatment significantly improved learning and memory function without affecting emotion. In the hippocampus, the gene expression of antioxidative defense enzymes was upregulated after 0.05% AGIQ treatment. In contrast, AGIQ caused significant alterations in the microbial abundance of genera Akkermansia, Bifidobacterium, and Alistipes associated with memory function. Metabolomics analysis identified that taurine concentration was significantly increased in serum and hippocampus from AGIQ-treated mice. The correlation analysis suggested that elevated serum taurine levels were closely related to the abundance of Akkermansia, indicating the underlying crosstalk of gut microbiota and serum metabolites. *In vitro* fecal culture further demonstrated that AGIQ could increase the level of Akkermansia. Taurine could exert antioxidant activity in SH-SY5Y neuroblastoma cell lines *in vitro*. Finally, vancomycin-induced alterations of gut microbiota attenuated the taurine increases in the serum and the antioxidant gene level in the hippocampus by AGIQ. Taken together, it is likely that AGIQ could increase genus Akkermansia abundance and ultimately increase taurine levels in serum and hippocampus to improve learning and memory function, relying on the gut microbiota-blood-brain axis. Our results supply a new view for understanding effects of AGIQ on brain function.

Keywords: gut microbiota, interaction, taurine

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Liu H[*1], Li J[*1], Takahashi S[*1], Toyoda A[*2], Inoue R[*3], Koyanagi M[*4], Hayashi SM, Xu M[*5], Yamamoto Y[*1], Nagaoka K[*1]: Alpha-glycosyl isoquercitrin alleviates subchronic social defeat stress-induced depression symptoms by modulating the microbiota-gut-brain axis in mice.

Life Sci. 2024;344:122561. doi: 10.1016/j.lfs.2024.122561

Aims: Increasing evidence suggests a link between gut microbial dysbiosis and the pathogenesis of depression. Alpha-glycosyl isoquercitrin (AGIQ), consisting of isoquercitrin and its glycosylated quercetin, has beneficial effects on the gut microbiome and brain function. Here, we detected the potential antidepressant impact of a four-week administration of AGIQ and its underlying mechanisms using a mouse model of depression. Main methods: Male C57BL/6 mice were orally administered AGIQ (0.05% or 0.5% in drinking water) for 28 days; subchronic social defeat stress was performed in the last 10 days. Behavior tests were conducted to assess anxiety and depressive-like behaviors. Additionally, evaluations encompassed 5-hydroxytryptamine (5-HT) levels, the gut microbiota composition, lipopolysaccharide (LPS) concentrations, short-chain fatty acids levels, and intestinal barrier integrity changes. Key findings: AGIQ significantly alleviated depression-like behaviors and increased hippocampal 5-HT levels. Further, AGIQ mitigated stress-induced gut microbial abnormalities and reduced the levels of LPS in the serum, which affected the relative gene expression levels of 5-HT biosynthesis enzymes *in vitro*. Furthermore, AGIQ reversed the reduced butyrate levels in cecal contents and improved the impaired intestinal barrier by increasing the expression of colonic zonula occluden-1 (ZO-1) and occludin, thereby decreasing LPS leakage. Significance: Our results suggest that AGIQ could improve stress-induced depression by regulating the gut microbiome, which inhibits LPS production and maintains the gut barrier. This is the first report on the potential effect of AGIQ on depression via the gut microbiota-brain axis, shedding new light on treatment options.

Keywords: butyrate, depression, gut microbiota

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Shimojima Y[*1], Kanai Y [*2], Moriyama T [*2], Arakawa S [*3], Tamura Y [*4], Okada Y, Morita Y [*5]: Environmental Monitoring of Food Manufacturing Facilities for *Listeria*: A Case Study.

Journal of Food Protection. 2023;86:100149. doi: 10.1016/j.jfp.2023.100149

Environmental monitoring programs (EMPs) for food production facilities are useful for verifying general sanitation controls and are recommended as verification measures to ensure that the Hazard Analysis Critical Control Point plan is working effectively. In this study, EMPs for *Listeria* were conducted at three food production facilities to assess the efficacy of sanitation control and establish effective sanitation control methods. In Facility A, *L. monocytogenes* was detected in the clean area although on non-food-contact surfaces. Normal cleaning combined with disinfection with carbonated hypochlorite water proved effective. At Facility B, a salad product and its ingredients were positive for *L. monocytogenes* serotype 3b. The bacterial count was <10/g in all samples. The ingredients were commercially purchased blocks that were sliced in a slicer at Facility B and used as salad toppings. Because both unopened blocks were negative for *L. monocytogenes*, contamination of the slicer was suspected. Sampling of the slicer revealed that contamination by *L. monocytogenes* serotype 3b was more extensive after use than before use. Therefore, the slicer was disassembled, cleaned, and disinfected thoroughly. Therefore, efforts were made to frequently clean and disinfect the cart. EMPs revealed the presence of *Listeria* in each facility and allowed remedial measures to be undertaken. Continued monitoring and Plan-Do-Check-Act cycles were considered desirable.

Keywords: environmental monitoring program, food safety, *Listeria monocytogenes*

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岡田由美子，鈴木穂高[*1]，筒浦さとみ[*2]，西海理之[*2]，百瀬愛佳，野田衛：加熱不十分な鶏肉製品に対する調理前の高圧処理による食中毒菌低減効果の検討.
日本食品微生物学会雑誌. 2024;41:30-36. doi: 10.5803/jsfm.41.30

Raw and/or insufficient heat-cooked chicken meats are the major vehicles of human campylobacteriosis in Japan. These often cause human foodborne salmonellosis as well. Hence, the establishment of effective treatments for reduction of these pathogens from chicken meats in combination with heat processing is an important issue for food safety. In this study, the effects of high hydrostatic pressure (HHP) treatment prior to heat cooking for reduction of foodborne pathogens in cooked chicken meat was examined. Raw yakitori samples which were treated with HHP at 500 MPa for 10 min showed negative results of *Campylobacter and Salmonella* by detection methods after cooking at 200℃ for 5 min. On the other hand, HHP treatments with 300 MPa and 400 MPa for 10 min were not sufficient for inactivation of these pathogens under condition used in this study. Color and hardness of the HHP-treated yakitori samples were almost comparable to the samples without HHP treatment after cooking. These results suggest the effectiveness of HHP treatment prior to heat cooking to reduce the foodborne cases caused by insufficient cooked meats.
Keywords: high hydrostatic pressure, chicken meat, foodborne bacteria

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佐々木貴正*1，米満研三*2，百瀬愛佳，上間匡：成鶏肉のカンピロバクターおよびサルモネラ汚染状況と株性状.
食品衛生学雑誌 2023;64(4):117-122. doi: 10.3358/shokueishi.64.117

成鶏肉も人の食用に供されるが，カンピロバクターおよびサルモネラ汚染率や分離株の薬剤耐性に関する報告はほとんどない．そこで，成鶏胸肉における両菌の汚染状況と薬剤耐性状況を調査した．51鶏群に由来する胸肉を調査したところ，カンピロバクターおよびサルモネラの汚染率は，それぞれ92.2％および35.5％であった．*Campylobacter jejuni*はカンピロバクター株の87.5％を占め，薬剤耐性率はアンピシリンが最も高く（45.3％），次いでテトラサイクリン（14.3％），シプロフロキサシン（14.3％）の順であった．カンピロバクター腸炎が疑われる場合に第一次選択薬として推奨されるエリスロマイシンに耐性を示す株はなかった．サルモネラでは，*Salmonella Corvallis*（30.4％）が最も多く，次いで*S.* Braenderup（21.7％）で，サルモネラ株の30.4％がストレプトマイシン耐性であった．サルモネラ腸炎の第一次選択薬の１つであるシプロフロキサシンに耐性を示す株はなかった．成鶏肉は両菌に汚染されていたが，エリスロマイシン耐性カンピロバクターおよびシプロフロキサシン耐性サルモネラは分離されず，これら抗菌薬の有効性は維持されていた.
Keywords: カンピロバクター，鶏肉，サルモネラ

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佐々木貴正*1，古谷陽子*2，上間匡，百瀬愛佳，山﨑栄樹*1，岡村雅史*1，浅井鉄夫*3：ブロイラー群のカンピロバクターおよびサルモネラの保菌と鶏肉汚染との関連性.
鶏病研究会報 2023;59(2):61-68.

カンピロバクター属菌（カンピロバクター）およびサルモネラ属菌（サルモネラ）はブロイラーの消化管内容物中に生息していることがあり，食鳥処理場における食鳥処理工程で鶏肉がこれらの菌により汚染される．我々は，鶏肉の主産地である九州地方の食鳥処理場６施設において，ブロイラー群およびその食鳥処理後の鶏肉における両菌の保有および汚染状況を調査した．2022年６～11月の間に35ブロイラー群の盲腸内容物（各群５羽）とその胸肉（各群１製品）を採取した．カンピロバクターは25群（71.4%），サルモネラは27群（77.1%）の盲腸内容物から分離された．保菌群内における５羽の保菌率については，カンピロバクターは保菌25群中23群（92.0%）が100%（５/５）であった一方で，サルモネラは27群中22群（81.5%）が60%（３/５）以下であった．カンピロバクター保菌鶏における盲腸内容物中のカンピロバクター平均菌数は7.31log10CFU/gであった．*Campylobacter jejuni*の59.1%（13/22）がシプロフロキサシンに耐性を示した．サルモネラ保菌27群中26群から*Salmonella* Schwarzengrundが分離され，このうち22群（84.6%）から分離された*S.* Schwarzengrundは，ストレプトマイシン，カナマイシンおよびテトラサイクリンの３抗菌薬に耐性を示した．７群に対して抗菌薬が投与されていたが，分離株の薬剤耐性との関連性は認められなかった．カンピロバクターは胸肉の62.9%（22/35）から分離され，遺伝学的解析の結果，いずれも食鳥処理前の保菌群に由来することが示唆された．盲腸内容物中のカンピロバクター菌数が6.51log10 CFU/g以下であった保菌３群の胸肉からカンピロバクターは分離されなかった．サルモネラは胸肉の85.7%（30/35）から分離され，25検体から分離された菌株の血清型は食鳥処理前の保菌

群における血清型と一致し，残りの５検体の鶏肉はサルモネラ非保菌群に由来するものであった．本研究により，九州地方から出荷されたブロイラー群は，高率にカンピロバクターまたはサルモネラを保菌し，これらブロイラー群から生産された鶏肉は高率に両菌に汚染されている実態が明らかとなった．

Keywords: カンピロバクター，鶏肉，サルモネラ

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Sasaki Y*1, Ikeda T*2, Yonemitsu K*3, Kuroda M*3, Ogawa M*4, Sakata R*4, Uema M, Momose Y, Ohya K, Watanabe M, Hara-Kudo Y, Okamura M*1, Asai T*5: Antimicrobial resistance profiles of *Campylobacter jejuni* and *Salmonella spp.* isolated from enteritis patients in Japan.

J Vet Med Sci. 2023;85(4):463-470. doi: 10.1292/jvms.22-0424

Understanding the antimicrobial resistance of *Campylobacter jejuni* and *Salmonella spp.* isolated from patients with enteritis will aid in therapeutic decision-making. This study aimed to characterize *C. jejuni* and *Salmonella spp.* isolates from patients with enteritis. For *C. jejuni*, the resistance rates against ampicillin, tetracycline, and ciprofloxacin were 17.2%, 23.8%, and 46.4%, respectively. All the *C. jejuni* isolates were susceptible to erythromycin, which is recommended as a first-choice antimicrobial if Campylobacter enteritis is strongly suspected. *C. jejuni* was classified into 64 sequence types (STs), and the five major STs were ST22, ST354, ST21, ST918, and ST50. The ciprofloxacin-resistance rate of ST22 was 85.7%. For Salmonella, the resistance rates against ampicillin, cefotaxime, streptomycin, kanamycin, tetracycline, and nalidixic acid were 14.7%, 2.0%, 57.8%, 10.8%, 16.7%, and 11.8%, respectively. All the *Salmonella spp.* isolates were susceptible to ciprofloxacin. Therefore, fluoroquinolones are the recommended antimicrobials against *Salmonella* enteritis. *S.* Thompson, *S.* Enteritidis, and *S.* Schwarzengrund were the three most prevalent serotypes. The two cefotaxime-resistant isolates were serotyped as S. Typhimurium and were found to harbor $bla_{\text{CMY-2}}$. The results of this study would help select antimicrobials for treating patients with Campylobacter and *Salmonella* enteritis.

Keywords: antimicrobial resistance, *Campylobacter, Salmonella*

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Sakuma M*1, Hashimoto M*2, Nishi K*2, Tohya M, Hishinuma T*1, Shimojima M*3, Tada T*1, Kirikae T*1: Emergence of colistin-resistant *Acinetobacter modestus* harbouring the intrinsic phosphoethanolamine transferase EptA.

J Glob Antimicrob Resist. 2023;33:101-108. doi: 10.1016/j.jgar.2023.02.023.

In this study, we determined the effect of endogenous phosphoethanolamine transferase from *Acinetobacter modestus*. Acinetobacter spp. strain JUAB9 was isolated from a sample of nasal secretions taken in 2019 from a hospitalised pet cat in Japan. The whole genome was sequenced by next-generation sequencers, and transformants of *Escherichia coli*, Klebsiella pneumoniae, and *Enterobacter cloacae* carrying the gene encoding phosphoethanolamine transferase from *A. modestus* were constructed. Electrospray ionization mass spectrometry was used to analyze the lipid A modification of the *E. coli* transformants. Sequencing of the whole genome showed that the isolate harbored the gene encoding phosphoethanolamine transferase, *eptA_AM*, on its chromosome. Transformants of *E. coli*, *K. pneumoniae*, and *E. cloacae* harboring both the promoter and the *eptA_AM* from *A. modestus* had 32-, 8-, and 4-fold higher minimum inhibitory concentrations (MICs) for colistin, respectively, than transformants harboring control vectors. The genetic environment surrounding the *eptA_AM* of *A. modestus* was similar to that surrounding the *eptA_AM* of *Acinetobacter junii* and *Acinetobacter* venetianus. Analysis using electrospray ionization mass spectrometry showed that *eptA_AM* modifies lipid A in Enterobacterales.

Keywords: *Acinetobacter modestus*, colistin resistance

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Sci Rep. 2024;14(1):2749. doi: 10.1038/s41598-024-52538-x.

The emergence and spread of carbapenem-resistant species of *Acinetobacter* spp. and *Pseudomonas* spp. is a serious health concern. Routine antimicrobial disk susceptibility testing performed in clinical laboratories cannot distinguish between isolates that are highly carbapenem-resistant and those that are moderately carbapenem-resistant. This study describes antimicrobial susceptibility tests using disks containing high doses (1000 μg) of meropenem. The diameters of the zone of inhibition were significantly negatively correlated with the MICs of *Pseudomonas* and *Acinetobacter* species for meropenem (R^2: 0.93 and 0.91, respectively) and imipenem (R^2: 0.75 and 0.84, respectively). Double disk synergy tests with clavulanic acid or sodium mercaptoacetate can detect ESBL or MBL producers. Susceptibility testing with disks containing high doses of meropenem can easily detect carbapenem-resistant isolates quantitatively. These disks may be useful in clinical laboratories because of their technical ease, stability, and relatively low cost.

Keywords: carbapenem-resistant, antimicrobial susceptibility tests, disk

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Kobayashi K*1, Kubota H*1, Tohya M, Ushikubo M*2, Yamamoto M*2, Ariyoshi T*1, Uchitani Y*1, Mitobe M*1, Okuno R*1, Nakagawa I*3, Sekizaki T*3,4, Suzuki J*1, Sadamasu K*1: Characterization of pig tonsils as niches for the generation of *Streptococcus suis* diversity.

Vet Res. 2024;55(1):17. doi: 10.1186/s13567-024-01270-5.

Streptococcus suis causes meningitis, septicemia, endocarditis, and other diseases in pigs and humans. We obtained 42 and 50 *S. suis* isolates from porcine endocarditis and palatine tonsils in clinically healthy pigs in Japan, respectively. In this study, we determined their sequence type (ST), *cps* genotype, serotype, and the presence of classical major toxicity-related marker genes (*mrp*, *epf,* and *sly*). Forty-two isolates from endocarditis lesions were assigned to a limited number of ST and clonal complexes (CC). In contrast, the 50 isolates from tonsils were diverse in their traits and degree of virulence, suggesting that tonsils can accommodate a variety of *S. suis* isolates. The goeBURST full algorithm, using tonsil isolates from this study and those obtained from the database, showed that the major CC and many other clusters were composed of isolates originating from different countries. The results of this study showed that the STs are very similar to each other despite the differences in the country of origin. These findings indicate that *S. suis*, with similar as well as different genomic variants, survives independently within the tonsils across different geographic locations. Thus, unlike endocarditis lesions, the pig tonsils appear to correspond to various *S. suis* lineages. This study suggests that *S. suis* acquired its diversity through spontaneous mutations during establishment and persistence within the pig tonsils.

Keywords: *Streptococcus suis*, goeBURST

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Takei S*1,2, Teramoto K*2,3, Sekiguchi Y*4, Ihara H*2,5, Tohya M, Iwamoto S*6, Tanaka K*6, Khasawneh A*1, Horiuchi Y*1, Misawa S*2,7, Naito T*2,8, Kirikae T*2,9, Tada T*10, Tabe Y*1,2.

Identification of Mycobacterium abscessus using the peaks of ribosomal protein L29, L30 and hemophore-related protein by MALDI-MS proteotyping.

Sci Rep. 202;14(1):11187. doi: 10.1038/s41598-024-61549-7.

Mycobacteroides (*Mycobacterium*) *abscessus* causes a variety of infections in humans. Recently, *M. asscessus* is increasingly being detected in clinical specimens. Taxonomically, *M. abscessus* consists of three subspecies of *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*, which differ in their susceptibility to macrolides. In order to rapidly identify these three subspecies, we determined biomarker proteins using matrix-assisted laser desorption/ionization mass spectrometry (MALDI- MS). Thirty-three clinical strains of *M. abscessus* were accurately identified at the subspecies level by three biomarker protein peaks. This study ultimately demonstrates the potential of routine MALDI-MS-based testing methods for the early identification and treatment of *M. abscessus* infections.

Keywords: *Mycobacteroides* (*Mycobacterium*) *abscessus*, matrix-assisted laser desorption/ionization mass spectrometry (MALDI- MS)

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Ukai R*1, Uchida H*2, Sugaya K*1, Onose J*1, Oshiro N, Yasumoto T*3, Abe N*1: Structural assignment of the product ion generated from a natural ciguatoxin-3C congener, 51-hydroxyciguatoxin-3C, and discovery of distinguishable signals in congeners bearing the 51-hydroxy group.

Toxins 2024;16:89. doi: 10.3390/toxins16020089

Ciguatoxins (CTXs) stand as the primary toxins causing ciguatera fish poisoning (CFP) and are essential compounds distinguished by their characteristic polycyclic ether structure. In a previous report, we identified the structures of product ions generated via homolytic fragmentation by assuming three charge sites in the mass spectrometry (MS)/MS spectrum of ciguatoxin-3C (CTX3C) using LC-MS. This study aims to elucidate the homolytic fragmentation of a ciguatoxin-3C congener. We assigned detailed structures of the product ions in the MS/MS spectrum of a naturally occurring ciguatoxin-3C congener, 51-hydroxyciguatoxin-3C (51-hydoxyCTX3C), employing liquid chromatography/quadrupole time-of-flight mass spectrometry with an atmospheric pressure chemical ionization (APCI) source. The introduction of a hydroxy substituent on C51 induced different fragmentation pathways, including a novel cleavage mechanism of the M ring involving the elimination of 51-OH and the formation of enol ether. Consequently, new cleavage patterns generated product ions at m/z 979 ($C_{55}H_{79}O_{15}$), 439 ($C_{24}H_{39}O_7$), 149 ($C_{10}H_{13}O$), 135 ($C_9H_{11}O$), and 115 ($C_6H_{11}O_2$). Additionally, characteristic product ions were observed at m/z 509 ($C_{28}H_{45}O_8$), 491 ($C_{28}H_{43}O_7$), 481 ($C_{26}H_{41}O_8$), 463 ($C_{26}H_{39}O_7$), 439 ($C_{24}H_{39}O_7$), 421 ($C_{24}H_{37}O_6$), 171 ($C_9H_{15}O_3$), 153 ($C_9H_{13}O_2$), 141 ($C_8H_{13}O_2$), and 123 ($C_8H_{11}O$).

Keywords: ciguatoxins (CTXs), 51-hydroxyciguatoxin-3C (51-hydroxyCTX3C), LC-APCI-QTOFMS

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Kobayashi M*, Masuda J*, Oshiro N: Detection of extremely low level ciguatoxins through monitoring of lithium adduct ions by liquid chromatography-

triple quadrupole tandem mass spectrometry.

Toxins 2024;16:170. doi:10.3390/toxins16040170.

Ciguatera poisoning (CP) is the most common type of marine biotoxin food poisoning worldwide, and it is caused by ciguatoxins (CTXs), thermostable polyether toxins produced by dinoflagellate Gambierdiscus and Fukuyoa spp. It is typically caused by the consumption of large fish high on the food chain that have accumulated CTXs in their flesh. CTXs in trace amounts are found in natural samples, and they mainly induce neurotoxic effects in consumers at concentrations as low as 0.2 μg/kg. The U.S. Food and Drug Administration has established CTX maximum permitted levels of 0.01 μg/kg for CTX1B and 0.1 μg/kg for C-CTX1 based on toxicological data. More than 20 variants of the CTX1B and CTX3C series have been identified, and the simultaneous detection of trace amounts of CTX analogs has recently been required. Previously published works using LC-MS/MS achieved the safety levels by monitoring the sodium adduct ions of CTXs ($[M+Na]^+ > [M+Na]^+$). In this study, we optimized a highly sensitive method for the detection of CTXs using the sodium or lithium adducts, $[M+Na]^+$ or $[M+Li]^+$, by adding alkali metals such as Na^+ or Li^+ to the mobile phase. This work demonstrates that CTXs can be successfully detected at the low concentrations recommended by the FDA with good chromatographic separation using LC-MS/MS. It also reports on the method's new analytical conditions and accuracy using $[M+Li]^+$.

Keywords: ciguatera poisoning, ciguatoxin, liquid chromatography-triple quadrupole tandem mass spectrometer (LC-MS/MS)

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Ando M*1, Yamaguchi H*1, Morimoto A*1, Iwashita N*1,2, Takagi Y*1,3, Nagane M*1, Yoshinari T, Fukuyama T*1: Chronic oral exposure to low-concentration fumonisin B2 significantly exacerbates the inflammatory responses of allergies in mice via inhibition of IL-10 release by regulatory T cells in gut-associated lymphoid tissue.

Arch Toxicol. 2023;97:2707-2719. doi: 10.1007/s00204-023-03579-0.

Contamination with fumonisins produced by *Fusarium* spp. is rapidly growing in both developing and developed countries. The purpose of this study was to determine whether oral exposure to fumonisin contributed to the development of allergic diseases. We initially examined the immunotoxic potential of short-term, oral administration of fumonisin B_1 (FB1, 1 mg/kg) and fumonisin B_2 (FB2, 1 mg/kg), both naturally occurring fumonisins, using a BALB/c mouse model of allergic contact dermatitis and Dermatophagoides farina-induced asthma. Using an NC/nga mouse model of atopic dermatitis (AD), we evaluated the adverse effects of subchronic oral exposure to low concentrations of FB2 (2 or 200 μg/kg). Finally, we explored the influence of FB2 on regulatory T cell proliferation and function in mesenteric lymph nodes after 1-week oral exposure to FB2 in BALB/c mice. Oral exposure to FB2 markedly exacerbated the symptoms of allergy, including skin thickness, histological evaluation, immunocyte proliferation, and proinflammatory cytokine production, although no change was observed following exposure to FB1. Furthermore, oral exposure to low concentrations of FB2 considerably exacerbated the AD scores, skin thickness, transepidermal water loss, histological features, and proinflammatory cytokine production. The aggravated allergic symptoms induced by oral exposure to FB2 could be attributed to the direct inhibition of IL-10 production by regulatory T cells in mesenteric lymph nodes. Our findings indicate that the recommended maximum fumonisin level should be reconsidered based on the potential for allergy development.

Keywords: atopic dermatitis, fumonisin B_2, IL-10

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Arai S, Hirose S, Yanagimoto K*1, Kojima Y*2, Yamaya S*3, Yamanaka T*4, Matsunaga N*5, Kobayashi A*6, Takahashi N*7, Konno T*8, Tokoi Y*9, Sakakida N*10, Konishi N*11, Hara-Kudo Y: An interlaboratory study on the detection method for *Escherichia albertii* in food using real time PCR assay and selective agars.

Int J Food Microbiol. 2024;414:110616. doi: 10.1016/j.ijfoodmicro.2024.110616.

Escherichia albertii is an emerging enteropathogen.

Although *E. albertii*-specific detection and isolation methods have been developed, their efficiency on food samples have not yet been systematically studied. To establish a series of effective methods for detecting *E. albertii* in food, an interlaboratory study was conducted in 11 laboratories using enrichment with modified *E. coli* broth supplemented with cefixime and tellurite (CT-mEC), real-time PCR assay, and plating on four kinds of selective agars. This study focused on the detection efficiency of an *E. albertii*-specific real-time PCR assay (EA-rtPCR) and plating on deoxycholate hydrogen sulfide lactose agar (DHL), MacConkey agar (MAC), DHL supplemented with rhamnose and xylose (RX-DHL), and MAC supplemented with rhamnose and xylose (RX-MAC). Chicken and bean sprout samples were inoculated with *E. albertii* either at 17.7 CFU/25 g (low inoculation level) or 88.5 CFU/25 g (high inoculation level), and uninoculated samples were used as controls. The sensitivity of EA-rtPCR was 1.000 for chicken and bean sprout samples inoculated with *E. albertii* at low and high inoculation levels. The Ct values of bean sprout samples were higher than those of the chicken samples. Analysis of microbial distribution by 16S rRNA gene amplicon sequencing in enriched cultures of bean sprout samples showed that approximately >96% of the population comprised unidentified genus of family Enterobacteriaceae and genus Acinetobacter in samples which *E. albertii* was not isolated. The sensitivity of the plating methods for chicken and bean sprout samples inoculated with a high inoculation level of *E. albertii* was 1.000 and 0.848-0.970, respectively. The sensitivity of the plating methods for chicken and bean sprout samples inoculated with a low inoculation level of *E. albertii* was 0.939-1.000 and 0.515-0.727, respectively. The *E. albertii*-positive rate in all colonies isolated in this study was 89-90% in RX-DHL and RX-MAC, and 64 and 44% in DHL and MAC, respectively. Therefore, the sensitivity of RX-supplemented agar was higher than that of the agars without these sugars. Using a combination of enrichment in CT-mEC and *E. albertii* isolation on selective agars supplemented with RX, *E. albertii* at an inoculation level of over 17.5 CFU/25 g of food was detected with a sensitivity of 1.000 and 0.667-0.727 in chicken and bean sprouts, respectively. Therefore, screening for *E. albertii*-specific genes using EA-rtPCR followed by isolation with RX-DHL or RX-MAC is an efficient method for *E. albertii* detection in food.

Keywords: bean sprouts, chicken, *E. albertii*

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Daud N*1, Currie V*1, Duncan G*1, Filipe JAN*1,2, Yoshinari T, Stoddart G*3, Roberts D*3, Gratz SW*1: Free and Modified Mycotoxins in Organic and Conventional Oats (*Avena sativa* L.) Grown in Scotland.

Toxins (Basel). 2023;15:247. doi: 10.3390/toxins15040247.

Small grain cereals are frequently infected with mycotoxigenic *Fusarium* fungi. Oats have a particularly high risk of contamination with type A trichothecene mycotoxins; their glucoside conjugates have also been reported. Agronomy practices, cereal variety and weather conditions have been suggested to play a role in *Fusarium* infection in oats. The current study investigates concentrations of free and conjugated *Fusarium* mycotoxins in organic and conventional oats grown in Scotland. In 2019, 33 milling oat samples (12 organic, 21 conventional) were collected from farmers across Scotland, together with sample questionnaires. Samples were analysed for 12 mycotoxins (type A trichothecenes T-2-toxin, HT-2-toxin, diacetoxyscirpenol; type B trichothecenes deoxynivalenol, nivalenol; zearalenone and their respective glucosides) using LC-MS/MS. The

prevalence of type A trichothecenes T-2/HT-2 was very high (100% of conventional oats, 83% of organic oats), whereas type B trichothecenes were less prevalent, and zearalenone was rarely found. T-2-glucoside and deoxynivalenol-glucoside were the most prevalent conjugated mycotoxins (36 and 33%), and co-occurrence between type A and B trichothecenes were frequently observed (66% of samples). Organic oats were contaminated at significantly lower average concentrations than conventional oats, whereas the effect of weather parameters were not statistically significant. Our results clearly indicate that free and conjugated T-2- and HT-2-toxins pose a major risk to Scottish oat production and that organic production and crop rotation offer potential mitigation strategies.

Keywords: *Fusarium* mycotoxins, masked mycotoxins, oats

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Hirose S, Konishi N*1, Sato M*2, Suzumura K*3, Obata H*1, Ohtsuka K*2, Doi R*2, Goto K*3, Kai A*4, Arai S, Hara-Kudo Y: Growth and survival of *Escherichia albertii* in food and environmental water at various temperatures.
J Food Prot. 2024; 87:100249. doi: 10.1016/j.jfp.2024.100249.

Escherichia albertii is an emerging foodborne pathogen that causes diarrhea. *E. albertii* has been isolated from various foods, including pork and chicken meat, and environmental waters, such as river water. Although many food poisoning cases have been reported, there have been insufficient analyses of bacterial population behaviors in food and environmental water. In this study, we inoculated 2-5 log CFU of *E. albertii* into 25 g of pork, chicken meat, Japanese rock oyster, Pacific oyster, and 300 mL of well water and seawater at 4° C, 10° C, 20° C, and 30° C, and analyzed the bacterial population behavior in food and environmental water. After 3 days at 4° C, the population of *E. albertii* strain EA21 and EA24 in foods maintained approximately 4 log CFU/25 g. After 3 days at 10° C, the population of *E. albertii* strains in pork and oysters maintained approximately 4 log CFU/25 g, and that in chicken meat increased to approximately 5-6 log CFU/25 g. After 2 days at 20° C, *E. albertii* strains grew to approximately 6-7 log CFU/25 g in pork and chicken meat, and *E. albertii* strain EA21 but not EA24 grew to 4.5 log CFU/25 g in Japanese rock oyster, *E. albertii* strain EA21 but not EA24 slightly grew to 3.1 log CFU/25 g in Pacific oyster. After 1 day at 30° C, *E. albertii* strains grew to approximately 7-8 log CFU/25 g in chicken meat and pork, grew to approximately 4-6 log CFU/25 g in Japanese rock oyster, and 6-7 log CFU/25 g in Pacific oyster. These results suggest that *E. albertii* survives without growth below 4° C and grew rapidly at 20° C and 30° C in foods, especially in meat. *E. albertii* strains did not grow in well water and seawater at 4° C, 10° C, 20° C, and 30° C. The population of *E. albertii* strains in well water and seawater decreased faster at 30° C than at 4° C, 10° C, and 20° C, suggesting that *E. albertii* has low viability at 30° C in environmental water.

Keywords: environmental water, *Escherichia albertii,* food

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Hirose S, Ohya K, Yoshinari T, Ohnishi T, Mizukami K*1, Suzuki T*1, Takinami K*1, Suzuki T, Lee K*2, Iyoda S*2, Akeda Y*2, Yahata Y*2, Tsuchihashi Y*2, Sunagawa T*2, Hara-Kudo Y: Atypical diarrhoeagenic *Escherichia coli* in milk related to a large foodborne outbreak.
Epidemiol Infect. 2023;151:e150. doi: 10.1017/S0950268823001395.

A foodborne outbreak related to milk cartons served in school lunches occurred in June 2021, which involved more than 1,800 cases from 25 schools. The major symptoms were abdominal pain, diarrhoea, vomiting, and fever. Although major foodborne toxins and pathogens were not detected, a specific *Escherichia coli* strain, serotype OUT (OgGp9):H18, was predominantly isolated from milk samples related to the outbreak and most patients tested. The strains from milk and patient stool samples were identified as the same clone by core genome multilocus sequence typing and single-nucleotide polymorphism analysis.

The strain was detected in milk samples served for two days related to the foodborne outbreak at a rate of 69.6% and levels of less than ten most probable number/100 mL but not on days unrelated to the outbreak. The acid tolerance of the strain for survival in the stomach was similar to that of enterohaemorrhagic *E. coli* O157:H7, and the same inserts in the chu gene cluster in the acid fitness island were genetically revealed. The pathogenicity of the strain was not clear; however, it was indicated that the causative pathogen was atypical diarrhoeagenic *E. coli* OUT (OgGp9):H18.

Keywords: *Escherichia coli* O157:H7, diarrhea, food poisoning

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Hirose S, Watanabe M, Tada A, Sugimoto N, Sato K, Hara-Kudo Y.: Suitability of Culture Broth and Conditions for *Escherichia coli* Growth and Gas Production as a Test for Food Additives in EC Broth.
Shokuhin Eiseigaku Zasshi. 2023;64:69-77. doi: 10.3358/shokueishi.64.69.

The growth and gas production test for *Escherichia coli* in the microbiological examination of food additives is stipulated in the ninth edition of Japan's Specifications and Standards for Food Additives (JSFA) and described as a part of the "Confirmation Test for *Escherichia coli*" in "Microbial Limit Tests" in the same manuscript. The growth and gas production test for *E. coli* indicated that the positive or negative of "gas production and/or turbidity" in EC broth should be confirmed after incubating at 45.5±0.2° C for 24±2 h. If both gas production and turbidity are negative, the culture is additionally incubated up to 48±2 h to determine *E. coli* contamination. The internationally referenced Bacteriological Analytical Manual of the U.S. FDA had revised the incubation temperature in tests for coliforms and *E. coli* from 45.5±0.2° C to 44.5±0.2° C in 2017. Therefore, we conducted research in anticipation of this temperature change being reflected in the microbiological examination of the JSFA. We used seven EC broth products and six food additives across eight products that are available in Japan in order to compare the growth and gas production at temperatures of 45.5±0.2° C and 44.5±0.2° C of *E. coli* NBRC 3972, which is designated as the test strain in JSFA. Both with/without food additives, the number of EC broth products in which medium turbidity and gas production by the strain were positive in three out of three tubes at all test times was greater at 44.5±0.2° C than at 45.5±0.2° C. These results suggest that the growth and gas production test for *E. coli* could be more appropriately conducted by incubation at 44.5±0.2° C in the "Confirmation Test for *Escherichia coli*" for *E. coli* in the JSFA in comparison to 45.5±0.2° C. Furthermore, there were differences in the growth and gas production of *E. coli* NBRC 3972 depending on the EC broth product used. Therefore, the importance of "Media growth promotion test" and "Method suitability test" in the ninth edition of the JSFA should be emphasized.

Keywords: EC broth, *Escherichia coli,* culture condition

Ojiro R*1, Okano H*1, Ozawa S*1, Yamagata H*2, Zou X*1, Tang Q*1, Jin M*3, Sasaki K*1, Yoshida T*1, Yoshinari T, Shibutani M*1: Pharmacokinetics and 28-day repeated-dose toxicity of enniatin B after oral administration in mice.
Food Chem Toxicol. 2023;177:113814. doi: 10.1016/j.fct.2023.113814.

Enniatins are emerging mycotoxins that contaminate foods. The present study investigated the oral pharmacokinetics and 28-day repeated-dose oral toxicity of enniatin B (ENNB) in CD1 (ICR) mice. In the pharmacokinetic study, male mice received a single oral or intravenous dose of ENNB [30 mg/kg body weight (BW) and 1 mg/kg BW, respectively]. After oral dosing, ENNB exhibited 139.9% bioavailability, a 5.1-h elimination half-life, 5.26% fecal excretion from 4 to 24 h post-dose, and upregulation of Cyp7a1, Cyp2a12, Cyp2b10, and Cyp26a1 in the liver 2 h post-dosing. In the 28-day toxicity study, ENNB was administered to male and female mice by oral gavage at 0, 7.5, 15, and 30 mg/kg BW/day. Females (7.5 and 30 mg/kg) showed dose-unrelated decreased food consumption without accompanying changes in clinical parameters. Males (30 mg/kg) showed low red blood cell counts and high blood urea nitrogen levels and absolute kidney weights; however, other related

parameters including the histopathology of systemic organs/tissues were unchanged. These results suggest that ENNB may not induce toxicity after 28 days of oral administration in mice, despite high absorption. The no-observed-adverse-effect level of ENNB after 28 days of repeated oral doses was 30 mg/kg BW/day for both sexes of mice.

Keywords: enniatin B, general toxicity, mouse

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Sasaki Y*1,2, Ikeda T*3, Yonemitsu K*4, Kuroda M*5, Ogawa M*6, Sakata R*6, Uema M, Momose Y, Ohya K, Watanabe M, Hara-Kudo Y, Okamura M*2, Asai T*1: Antimicrobial resistance profiles of *Campylobacter jejuni* and *Salmonella* spp. isolated from enteritis patients in Japan.

J Vet Med Sci. 2023; 85:463-470. doi: 10.1292/jvms.22-0424.

Understanding the antimicrobial resistance of *Campylobacter jejuni* and *Salmonella* spp. isolated from patients with enteritis will aid in therapeutic decision-making. This study aimed to characterize *C. jejuni* and *Salmonella* spp. isolates from patients with enteritis. For *C. jejuni*, the resistance rates against ampicillin, tetracycline, and ciprofloxacin were 17.2%, 23.8%, and 46.4%, respectively. All the *C. jejuni* isolates were susceptible to erythromycin, which is recommended as a first-choice antimicrobial if *Campylobacter* enteritis is strongly suspected. *C. jejuni* was classified into 64 sequence types (STs), and the five major STs were ST22, ST354, ST21, ST918, and ST50. The ciprofloxacin-resistance rate of ST22 was 85.7%. For *Salmonella*, the resistance rates against ampicillin, cefotaxime, streptomycin, kanamycin, tetracycline, and nalidixic acid were 14.7%, 2.0%, 57.8%, 10.8%, 16.7%, and 11.8%, respectively. All the *Salmonella* spp. isolates were susceptible to ciprofloxacin. Therefore, fluoroquinolones are the recommended antimicrobials against *Salmonella* enteritis. *S.* Thompson, *S.* Enteritidis, and *S.* Schwarzengrund were the three most prevalent serotypes. The two cefotaxime-resistant isolates were serotyped as *S.* Typhimurium and were found to harbor blaCMY-2. The results of this study would help select antimicrobials for treating patients with *Campylobacter* and *Salmonella* enteritis.

Keywords: antimicrobial resistance, *Campylobacter, Salmonella*

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Watanabe M, Konuma R*1, Hasegawa K*2, Kimura N*3, Kobayashi N*4, Kamata Y*5, Yoshino H*6, Takatori K*7, Hara-Kudo Y: An experimental verification of fungal overgrowth in temporary houses at the site of the Great East Japan Earthquake.

J Microoorganism Control. 2024;29:45-48. doi: 10.4265/jmc.29.1_45.

Fungal contamination in the indoor air of prefabricated temporary houses at the site of the Great East Japan Earthquake revealed extremely high levels compared to those found in conventional residences. We experimentally investigated fungal growth levels on different interior materials to support fungal overgrowth in prefabricated temporary houses. Three species each of allergenic fungi and invasive fungi observed in temporary housing were selected for inoculation tests with various interior materials. The experiments with fungal inoculation were conducted in conformance with standards for industrial products described in the Japanese" JIS Z 2911:2018 Methods of test for fungus resistance" with small modifications. After incubation, visual and stereomicroscopic assessments were performed to determine fungal growth levels. The viability of the fungi varied according to the interior material type. Our findings demonstrate the importance of antifungal measures in indoor environments and the need for additional research on the growth levels of fungal species on various interior materials.

Keywords: fungal overgrowth, gypsum board, indoor environmen

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Yoshinari T, Sekine A*, Kobayashi N*, Nishizaki Y, Sugimoto N, Hara-Kudo Y, Watanabe M. Determination of the biological origin of enzyme preparations using SDS-PAGE and peptide mass fingerprinting.
Food Addit Contam Part A. 2023;40(6):711-722. doi: 10.1080/19440049.2023.2211678.

Enzymes are mainly extracted from the culture broth of microorganisms. Various commercially available enzyme preparations (EPs) are derived from different microorganisms, and the source of the EP should be the same as that mentioned in the manufacture's information. The development of analytical methods that can determine the origin of the final products is important for ensuring that the EPs are nontoxic, especially when used as food additives. In this study, various EPs were subjected to SDS-PAGE, and the main protein bands were excised. After in-gel digestion, the generated peptides were analysed using MALDI-TOF MS, and protein identification was performed by searching the set of peptide masses against protein databases. In total, 36 EPs including amylase, β-galactosidase, cellulase, hemicellulase and protease were analysed, and the information about the enzyme sources was obtained for 30 EPs. Among these, the biological sources determined for 25 EPs were consistent with the manufacturer's information; for the remaining five, enzymes produced by closely-related species were shown as matching proteins due to high sequence similarity. Six enzymes derived from four microorganisms could not be identified because their protein sequences were not registered in the database. As these databases are expanded, this approach of using SDS-PAGE and peptide mass fingerprinting (PMF) can determine the biological origin of enzymes rapidly and contribute to ensuring the safety of EPs.

Keywords: Enzyme preparation, MALDI-TOF MS, biological origin

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Yoshinari T, Sugita-Konishi Y*1, Sato E*2, Takeuchi H*3, Taniguchi M*4, Fukumitsu T*5, Shimoyama A*6, Nakamura A*7, Murayama S*8, Owaki S*9, Miyake S*10, Hara-Kudo Y: Survey and risk assessment of aflatoxins and sterigmatocystin in Japanese staple food items and the evaluation of an in-house ELISA technique for rapid screening.
Food Control. 2024;157:110154. doi: 10.1016/j.foodcont.2023.110154.

Aflatoxins and sterigmatocystin (STC) are carcinogenic mycotoxins frequently detected in many kinds of agricultural products. An analytical method for the simultaneous detection of these mycotoxins was developed using immunoaffinity chromatography clean-up and LC–MS/MS techniques to assess the health risks associated with the exposure to aflatoxins and STC present in staple Japanese food items. A survey targeting Japanese staple food items, including rice and wheat, was conducted after validating the analytical method by conducting a single-laboratory test. A total of 550 samples were analyzed. Aflatoxins were not detected in all samples, whereas STC was detected in 22.4% of the tested samples. The dietary exposure to STC was estimated based on the results of the survey, and the amount of food intake ranged from 0.11 to 0.67 ng/kg body weight/day. The results obtained following the margin of exposure (MOE) approach revealed that STC in staple food items posed little risk to the general Japanese population. In addition, an ELISA technique for detecting STC in brown rice and wheat samples was developed, as high levels of contamination were observed in these food items. The performance of the developed ELISA technique was evaluated using STC-spiked samples, and the average recoveries were in the ranges of 94.6–121.2% for brown rice and 91.5–117.9% for wheat. The developed two analytical methods based on LC-MS/MS and ELISA techniques were efficiently utilized for assessing the health risks associated with exposure to these carcinogenic mycotoxins.

Keywords: aflatoxin, sterigmatocystin, occurrence

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Nakamura M, Ohoka N, Shibata N, Inoue T, Tsuji G, Demizu Y: Development of STING degrader with double covalent ligands.
Bioorg. Med. Chem. Lett., 2024;102:129677. doi:10.1016/j.bmcl.2024.129677

Stimulator of interferon genes (STING), a homodimeric membrane receptor localized in the endoplasmic reticulum, plays a pivotal role in signaling innate immune responses. Inhibitors and proteolysis-targeting chimeras (PROTACs) targeting STING are promising compounds for addressing autoinflammatory and autoimmune disorders. In this study, we used a minimal covalent handle recently developed as the ligand portion of an E3 ligase. The engineered STING degrader with a low molecular weight compound covalently binds to STING and E3 ligase. Degrader 2 showed sustained STING degradation activity at lower concentrations (3 μM, 48 h, about 75% degradation) compared to a reported STING PROTAC, SP23. This discovery holds significance for its potential in treating autoinflammatory and autoimmune diseases, offering promising avenues for developing more efficacious STING-targeted therapies.

Keywords: STING, covalent ligand, protein degradation

Yamamoto K*, Torigoe K*, Kuriyama M*, Demizu Y, Onomura O*: [3+2] Cycloaddition of heteroaromatic N-ylides with sulfenes.
Org. Lett., 2024;26:798-803. doi:10.1021/acs.orglett.3c03878

A (3+2) cycloaddition of heteroaromatic N-ylides with sulfenes, which are generated in situ from sulfonyl chlorides, has been developed. A variety of ylides were transformed into the corresponding sulfone-embedded N-fused heterocycles in high yields. Hexafluoroiso- propyl mesylate was demonstrated to be a suitable reactant for quinolinium ylides. Furthermore, this cycloaddition could be performed with an ylide prepared by a Cu-catalyzed ylide transfer reaction in a one- pot manner, extending the substrate scope to an unisolable ylide.

Keywords: (3+2) cycloaddition, heteroaromatic N-ylide

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Tsuji G, Kurohara T, Shoda T, Yokoo H, Ito T, Masada S, Uchiyama N, Yamamoto E, Demizu Y: *In silico* prediction of N-nitrosamine formation pathways in pharmaceutical products.
Chem. Pharm. Bull., 2024;72:166-172. doi:10.1248/cpb.c23-00550

The recent discovery of N-nitrosodimethylamine (NDMA), a mutagenic N-nitrosamine, in pharmaceuticals has adversely impacted the global supply of relevant pharmaceutical products. Contamination by N-nitrosamines diverts resources and time from research and development or pharmaceutical production, representing a bottleneck in drug development. Therefore, predicting the risk of N-nitrosamine contamination is an important step in preventing pharmaceutical contamination by DNA-reactive impurities for the production of high-quality pharmaceuticals. In this study, we first predicted the degradation pathways and impurities of model pharmaceuticals, namely gliclazide and indapamide, *in silico* using an expert-knowledge software. Second, we verified the prediction results with a demonstration test, which confirmed that N-nitrosamines formed from the degradation of gliclazide and indapamide in the presence of hydrogen peroxide, especially under alkaline conditions. Furthermore, the pathways by which degradation products formed were determined using ranitidine, a compound previously demonstrated to generate NDMA. The prediction indicated that a ranitidine-related compound served as a potential source of nitroso groups for NDMA formation. *In silico* software is expected to be useful for developing methods to assess the risk of N-nitrosamine formation from pharmaceuticals.

Keywords: N-nitrosamine, degradation, *in silico* prediction

Hirano M, Yokoo H, Ohoka N, Ito T, Misawa T, Oba M*, Inoue T, Demizu Y: Rational design of amphipathic antimicrobial peptides with alteration of L-/D-amino acids that form helical structures.
Chem. Pharm. Bull., 2024;72:149-155. doi:10.1248/cpb.c23-00465

Antimicrobial peptides (AMPs) are promising therapeutic agents against bacteria. We have previously reported an amphipathic AMP Stripe composed of cationic L-Lys and hydrophobic L-Leu/L-Ala residues, and Stripe exhibited potent antimicrobial activity against Gram-positive and Gram-negative bacteria. Gramicidin A (GA), composed of repeating sequences of L- and D-amino acids, has a unique β6.3-helix structure and exhibits broad antimicrobial activity. Inspired by the structural properties and antimicrobial activities of LD-alternating peptides such as GA, in this study, we designed Stripe derivatives with LD-alternating sequences. We found that simply alternating L- and D-amino acids in the Stripe sequence to give StripeLD caused a reduction in antimicrobial activity. In contrast, AltStripeLD, with cationic and hydrophobic amino acids rearranged to yield an amphipathic distribution when the peptide adopts a β6.3-helix, displayed higher antimicrobial activity than AltStripe. These results suggest that alternating L-/D-cationic and L-/D-hydrophobic amino acids in accordance with the helical structure of an AMP may be a useful way to improve antimicrobial activity and develop new AMP drugs.
Keywords: antimicrobial peptide, α-helix

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Po-C. Shih*, Naganuma M, Tsuji G, Demizu Y, Naito M*: Development of decoy oligonucleotide-warheaded chimeric molecules targeting STAT3.
Bioorg. Med. Chem., 2023;95:117507. doi:10.1016/j.bmc.2023.117507

Proteolysis-targeting chimera (PROTAC) technology is a disruptive innovation in the drug development community, and over 20 PROTAC molecules are currently under clinical evaluation. These PROTAC molecules contain small-molecule warheads that bind to target proteins. Recently, oligonucleotide-warheaded PROTACs have emerged as a promising new tool to degrade DNA-binding proteins such as transcription factors. In this study, we applied an oligonucleotide-warheaded PROTAC technology to induce the degradation of signal transducer and activator of transcription 3 (STAT3), which is a hard-to-target protein. A double-stranded decoy oligonucleotide specific to STAT3 was conjugated to E3 binders (pomalidomide, VH032, and LCL161) to generate PROTAC molecules that recruited different E3 ubiquitin ligases cereblon (CRBN), von Hippel-Lindau (VHL), and inhibitor of apoptosis protein (IAP), respectively. One of the resulting PROTAC molecules, POM-STAT3, which recruits CRBN, potently induces STAT3 degradation. STAT3 degradation by POM-STAT3 was abolished by scrambling the oligonucleotide sequences of POM-STAT3 and by adding a double-stranded decoy oligonucleotide against STAT3 in a competitive manner, suggesting the significance of oligonucleotide sequences in STAT3 degradation. Moreover, POM-STAT3-induced STAT3 degradation was suppressed by the CRBN binder thalidomide, proteasome inhibitor bortezomib, E1 inhibitor MLN7243, and siRNA-mediated depletion of CRBN, indicating that STAT3 degradation is mediated by the ubiquitin-proteasome system, which involves CRBN as the responsible E3 ubiquitin ligase. Consistent with STAT3 degradation, NCI-H2087 cell viability was severely reduced following POM-STAT3 treatment. Thus, POM-STAT3 is a STAT3 degrader that potentially has cytocidal activity against cancer cells that are highly dependent on STAT3 signaling, which implies that inducing protein degradation by decoy oligonucleotide-warheaded PROTAC molecules could be harnessed to be therapeutic against oncogenic transcription factors.
Keywords: Nucleic acids, STAT3, Targeted protein degradation

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Ito T, Yokoo H, Kato T*, Doi M*, Demizu Y: Sculpting secondary structure of a cyclic peptide: Conformational analysis of a cyclic hexapeptide

containing a combination of L-Leu, D-Leu and Aib residues.

ACS Omega, 2023;8:44106-44111. doi:10.1021/acsomega.3c06397

We have previously reported that cyclo(L-Leu-D-Leu-Aib-L-Leu-D-Leu-Aib) (2), a cyclic hexapeptide consisting of heterochiral L-Leu and D-Leu (L-Leu-D-Leu) residues with achiral 2-aminoisobutyric acid (Aib) residues, forms a figure-8 conformation. In this study, we newly designed cyclo(L-Leu-D-Leu-Aib-D-Leu-L-Leu-Aib)+ (4), an epimer of 2, and examined the conformational differences between 2 and 4 by X-ray crystallographic analysis. Peptide 4 formed a planar cyclic conformation with an antiparallel β-sheet hydrogen-bonding pattern. This investigation demonstrates the potential to manipulate the molecular conformation of cyclic peptides by simply arranging the L- and D-amino acids and emphasizes that diverse conformations can be obtained by using cyclic peptides. Harnessing cyclic peptides as platforms for distinct molecular structures is a promising approach to expanding the chemical space for various applications.

Keywords: peptide, secondary structure, cyclic peptide

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Naganuma M, Ohoka N, Tsuji G, Inoue T, Naito M*, Demizu Y: Structural optimization of decoy oligonucleotide-based PROTAC that degrades the estrogen receptor.

Bioconjug. Chem., 2023;34:1780-1788. doi:10.1021/acs.bioconjchem.3c00332

Proteolysis-targeting chimera (PROTAC) technology is a disruptive innovation in the drug development community, and over 20 PROTAC molecules are currently under clinical evaluation. These PROTAC molecules contain small-molecule warheads that bind to target proteins. Recently, oligonucleotide-warheaded PROTACs have emerged as a promising new tool to degrade DNA-binding proteins such as transcription factors. In this study, we applied an oligonucleotide-warheaded PROTAC technology to induce the degradation of signal transducer and activator of transcription 3 (STAT3), which is a hard-to-target protein. A double-stranded decoy oligonucleotide specific to STAT3 was conjugated to E3 binders (pomalidomide, VH032, and LCL161) to generate PROTAC molecules that recruited different E3 ubiquitin ligases cereblon (CRBN), von Hippel-Lindau (VHL), and inhibitor of apoptosis protein (IAP), respectively. One of the resulting PROTAC molecules, POM-STAT3, which recruits CRBN, potently induces STAT3 degradation. STAT3 degradation by POM-STAT3 was abolished by scrambling the oligonucleotide sequences of POM-STAT3 and by adding a double-stranded decoy oligonucleotide against STAT3 in a competitive manner, suggesting the significance of oligonucleotide sequences in STAT3 degradation. Moreover, POM-STAT3-induced STAT3 degradation was suppressed by the CRBN binder thalidomide, proteasome inhibitor bortezomib, E1 inhibitor MLN7243, and siRNA-mediated depletion of CRBN, indicating that STAT3 degradation is mediated by the ubiquitin-proteasome system, which involves CRBN as the responsible E3 ubiquitin ligase. Consistent with STAT3 degradation, NCI-H2087 cell viability was severely reduced following POM-STAT3 treatment. Thus, POM-STAT3 is a STAT3 degrader that potentially has cytocidal activity against cancer cells that are highly dependent on STAT3 signaling, which implies that inducing protein degradation by decoy oligonucleotide-warheaded PROTAC molecules could be harnessed to be therapeutic against oncogenic transcription factors.

Keywords: Biopolymers, Degradation, Ligands

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* Graduate School of Pharmaceutical Sciences, The University of Tokyo

Hirano M, Yokoo H, Goto C, Oba M *, Misawa T, Demizu Y: Magainin 2-derived stapled peptides derived with the ability to deliver pDNA, mRNA, and siRNA into cells.

Chem. Sci., 2023;14:10403-10410. doi:10.1039/D3SC04124G

We have developed cell-penetrating stapled peptides based on the amphipathic antimicrobial peptide magainin 2 for intracellular delivery of nucleic acids such as pDNA, mRNA, and siRNA. Various types of stapled peptides with a cross-linked structure were synthesised in the hydrophobic region of the amphipathic structure, and their efficacy in

intracellular delivery of pDNA was evaluated. The results showed that the stapled peptide st7-5 could deliver pDNA into cells. To improve the deliverability of st7-5, we further designed st7-5_R, in which the Lys residues were replaced by Arg residues. The peptide st7-5_R formed compact and stable complexes with pDNA and was able to efficiently transfer pDNA into the cell. In addition to pDNA, st7-5_R was also able to deliver mRNA and siRNA into the cell. Thus, st7-5_R is a novel peptide that can achieve efficient intracellular delivery of three different nucleic acids.
Keywords: Magainin 2, peptide, delivery

* Medical Chemistry, Graduate School of Medical Science, Kyoto Prefectural University of Medicine

Ito T, Matsunaga N, Kurashima M, Demizu Y, Misawa T: Enhancing chemical stability through structural modification of antimicrobial peptides with non-proteinogenic amino acids.
Antibiotics, 2023;12:1326. doi:10.3390/antibiotics12081326

We have developed cell-penetrating stapled peptides based on the amphipathic antimicrobial peptide magainin 2 for intracellular delivery of nucleic acids such as pDNA, mRNA, and siRNA. Various types of stapled peptides with a cross-linked structure were synthesised in the hydrophobic region of the amphipathic structure, and their efficacy in intracellular delivery of pDNA was evaluated. The results showed that the stapled peptide st7-5 could deliver pDNA into cells. To improve the deliverability of st7-5, we further designed st7-5_R, in which the Lys residues were replaced by Arg residues. The peptide st7-5_R formed compact and stable complexes with pDNA and was able to efficiently transfer pDNA into the cell. In addition to pDNA, st7-5_R was also able to deliver mRNA and siRNA into the cell. Thus, st7-5_R is a novel peptide that can achieve efficient intracellular delivery of three different nucleic acids.
Keywords: antimicrobial peptide, secondary structure, digestion tolerance

Murakami Y*, Ishida S*, Demizu Y, Terayama K*: Design of antimicrobial peptides containing non-proteinogenic amino acids using multi-objective Bayesian optimization.
Digital Discovery, 2023;2:1347-1353. doi: 10.1039/d3dd00090 g

Antimicrobial peptides (AMPs) have attracted attention as next-generation antimicrobial drugs. Designing AMPs while considering multiple properties, such as antimicrobial activities and toxicity, requires numerous trials and errors by chemists. In this study, we propose MODAN, a machine learning-assisted AMP design framework based on multi-objective Bayesian optimisation. The primary advantage of MODAN is its ability to handle various non-proteinogenic amino acids, which have recently shown the potential of activity enhancement, and this flexibility has not been achieved by previous studies. In addition, multi-objective Bayesian optimisation enables simultaneous improvement of antimicrobial activity and toxicity. We have succeeded in designing peptides that have potent antimicrobial and low haemolytic activities within two rounds of MODAN recommendation and experimentation, based on a strategy that chemists do not usually consider.
Keywords: antimicrobial peptide, Bayesian optimization, non-proteinogenic amino acid

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Ito T, Hashimoto W, Ohoka N, Misawa T, Inoue T, Kawano R*, Demizu Y: Structure-activity relationship study of helix-stabilized antimicrobial peptides containing non-proteinogenic amino acids.
ACS Biomater. Sci. Eng., 2023;9:4654-4661. doi:10.1021/acsbiomaterials.3c00759

Helical amphipathic peptides containing cationic and hydrophobic amino acid residues can possess potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. In this study, several amphipathic peptides with enhanced helical structures containing nonproteinogenic amino acids were designed, and the relationships between the antimicrobial activity, hemolytic activity, and cytotoxicity were evaluated. In particular, the effect on the antimicrobial activity and cytotoxicity of the number and position of stapling structures introduced into the sequence was investigated. Peptide stp1 containing α,α-disubstituted amino acids showed potent antimicrobial activity against multidrug-resistant

bacteria (MDRP, SP45, and Staphylococcus aureus) without causing appreciable hemolytic activity or cytotoxicity. The cytotoxicity was found to be somewhat correlated to the hydrophobicity of the peptides.

Keywords: Antimicrobial peptide, Amphipathic peptide, Helical structure

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Tsuchiya K*, Horikoshi K, Fujita M, Hirano M, Miyamoto M, Yokoo H, Demizu Y: Development of hydrophobic cell-penetrating stapled peptides as drug carriers.

Int. J. Mol. Sci., 2023;24:11768. doi:10.3390/ijms241411768

Cell-penetrating peptides (CPPs) are widely used for the intracellular delivery of a variety of cargo molecules, including small molecules, peptides, nucleic acids, and proteins. Many cationic and amphiphilic CPPs have been developed; however, there have been few reports regarding hydrophobic CPPs. Herein, we have developed stapled hydrophobic CPPs based on the hydrophobic CPP, TP10, by introducing an aliphatic carbon side chain on the hydrophobic face of TP10. This side chain maintained the hydrophobicity of TP10 and enhanced the helicity and cell penetrating efficiency. We evaluated the preferred secondary structures, and the ability to deliver 5(6)-carboxyfluorescein (CF) as a model small molecule and plasmid DNA (pDNA) as a model nucleotide. The stapled peptide F-3 with CF, in which the stapling structure was introduced at Gly residues, formed a stable α-helical structure and the highest cell-membrane permeability via an endocytosis process. Meanwhile, peptide F-4 demonstrated remarkable stability when forming a complex with pDNA, making it the optimal choice for the efficient intracellular delivery of pDNA. The results showed that stapled hydrophobic CPPs were able to deliver small molecules and pDNA into cells, and that different stapling positions in hydrophobic CPPs can control the efficiency of the cargo delivery.

Keywords: cell-penetrating peptide, stapled peptide, hydrophobic peptide

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Tsujimura H, Naganuma M, Ohoka N, Inoue T, Naito M *, Tsuji G, Demizu Y: Development of DNA aptamer-based PROTACs that degrade the estrogen receptor.

ACS Med. Chem. Lett., 2023;14:27-832. doi:10.1021/acsmedchemlett.3c00126

Targeted protein degradation (TPD), using chimeric molecules such as proteolysis-targeting chimeras (PROTACs), has attracted attention as a strategy for selective degradation of intracellular proteins by hijacking the ubiquitin-proteasome system (UPS). However, it is often difficult to develop such degraders due to the absence of appropriate ligands for target proteins. In targeting proteins for degradation, the application of nucleic acid aptamers is considered to be effective because these can be explored using systematic evolution of ligand by exponential enrichment (SELEX) methods. In this study, we constructed chimeric molecules in which nucleic acid aptamers capable of binding to the estrogen receptor α (ERα) and E3 ubiquitin ligase ligands were linked via a linker. ERα aptamer-based PROTACs were found to degrade ERα via the UPS. These findings represent the development of novel aptamer-based PROTACs that target intracellular proteins and are potentially applicable to other proteins.

Keywords: ubiquitin-proteasome system, PROTAC, aptamer

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辻厳一郎，内山奈穂子，出水庸介: 日本薬局方の国際化の一層の推進を目指した定量法改正に関する研究

医薬品医療機器レギュラトリーサイエンス, 2023;52:257-265. doi:10.51018/pmdrs.54.3_257

日本薬局方（JP）は本邦の医薬品の性状及び品質の適正な確保に必要な規格・基準及び標準的試験法等のための公的な規範書である．本研究においては，第十九改正JPの作成基本方針の５本柱のうち，「最新の学問・技術の積極的導入による質的向上」及び「医薬品のグローバル化に対応した国際化の一層の推進」の達成によるJPの品質向上を目的として取り組んだ．具体的には，JPの定量法として電位差滴定法が設定されているダントロ

レンナトリウム水和物について，海外薬局方で設定されているHPLC法を取り入れるための検討として，類縁物質の合成，それらのqNMRによる純度決定及びHPLCクロマトグラム上における完全分離条件の設定を行った．
Keywords: 薬局方の国際調和，ダントロレンナトリウム水和物，HPLC

Moriya S[*1], Funaki K[*1], Demizu Y, Kurihara M[*2], Kittaka A[*1], Sugiyama T[*1]: Design and properties of PNA containing a dicationic nucleobase based on N4-benzoylated cytosine.
Bioorg. Med. Chem. Lett., 2023;88:129287. doi:10.1016/j.bmcl.2023.129287

We report the synthesis of a peptide nucleic acid (PNA) monomer containing N4-bis(aminomethyl) benzoylated cytosine (BzC2+ base). The BzC2+ monomer was incorporated into PNA oligomers using Fmoc-based solid-phase synthesis. The BzC2+ base in PNA had two positive charges and exhibited greater affinity for DNA G base than the natural C base. The BzC2+ base stabilized PNA-DNA heteroduplexes through electrostatic attractions, even in high salt conditions. The two positive charges on the BzC2+ residue did not compromise the sequence specificity of PNA oligomers. These insights will aid the future design of cationic nucleobases.
Keywords: antigene, peptide nucleic acid, positive charge

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H. Xu, Kurohara T, Ohoka N, Tsuji G, Inoue T, Naito M[*], Demizu Y: Development of a versatile solid-phase synthesis of PROTAC with diverse E3 ligands
Bioorg. Med. Chem., 2023;86:117293. doi:10.1016/j.bmc.2023.117293

Developing highly active proteolysis-targeting chimeras (PROTACs) requires investigating a variety of ubiquitin ligase (E3 ligase) ligands and linker structures as well as their lengths. In this study, we developed a solid-phase synthesis method that affords PROTAC design diversity. We expanded the E3 ligand range to include Von Hippel-Lindau (VHL) and inhibitor of apoptosis protein (IAP) ligands because only the cereblon (CRBN) ligand thalidomide and its derivatives have been investigated for solid-phase synthesis of PROTACs. Moreover, we examined the suitability of a polyethylene glycol (PEG) rather than an alkyl linker used in our previous study for synthesizing PROTACs. Facile and rapid solid-phase synthesis methods using the above E3 ligands for developing PROTACs targeting bromodomain-containing protein 4 (BRD4) were accomplished. Western blotting analysis revealed that minor differences in the E3 ligand and linker type significantly affected the activity of the synthesized PROTACs. Our solid-phase PROTAC synthesis methods enable rapid synthesis of multiple PROTACs with various combinations of ligands for the protein-of-interest and E3 ligands and linkers that connect these ligands.
Keywords: Solid-phase synthesis, PROTAC, E3 ligands

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Soga K, Taguchi C, Sugino M, Egi T[*1], Narushima J, Yoshiba S, Takabatake R[*2], Kondo K[*3], Shibata N: Investigation of genetically modified maize imported into Japan in 2021/2022 and the applicability of Japanese official methods.
Food Hyg. Saf. Sci. 2023;64:218-225. doi: 10.3358/shokueishi.64.218

Given that the number of genetically modified (GM) maize events that have been announced as having undergone safety assessment procedures in Japan is increasing yearly, more information is needed about their actual recent domestic distribution in Japan. In this study, we investigated whether current Japanese official qualitative and quantitative methods (the current official methods) for GM maize can comprehensively target events in domestically distributed maize. For samples with the identity-preserved (IP) handling system and non-IP samples from the United States (US) and non-IP samples from Brazil, we performed event-specific real-time PCR targeting 25 authorized single GM maize events in addition to the current official methods. According to our results, 15 events targeted by the current official methods were detected, but insect-resistance (IR) Event5307 and herbicide-tolerant (HT) DAS40278, not

targeted by the current official methods, were detected in the US (one out of 5 lots) and Brazilian (four out of 5 lots) non-IP samples, respectively. Nevertheless, a survey of recent GM maize acreage in recent years has revealed that more than 95% of the acreage in US maize is occupied by HT or IR/HT stacked events, and that more than 95% of the acreage in Brazilian maize is occupied by IR or IR/HT stacked events. Because the current official methods can target all stacked events related to Event5307 and DAS40278, the only undetectable events are the single Event5307 and DAS40278, whose production is estimated to be less than 5% of the total production in the producing country. Therefore, we conclude that the current official methods for the labelling of GM maize should be maintained in view of practicability.

Keywords: genetically modified maize, japanese official method, real-time PCR

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Taguchi C, Shibata N, Soga K, Yoshiba S, Narushima J, Sugino M, Kondo K*: Providing appropriate information to consumers boosts the acceptability 1 of genome-edited foods in Japan.
GM Crops and Food. 2023;14:1-14. doi: 10.1080/21645698.2023.2239539

The Japanese Health Ministry recently granted permission for the market distribution of genome-edited (GE) foods, yet there remains a lack of full understanding among consumers regarding this technology. In this study, we conducted a survey to assess the acceptability of GE foods among Japanese consumers and examined the impact of providing information about GE foods on their acceptability. We conducted a web-based survey among 3,408 consumers aged 20-69 years, focusing on three aspects: (1) the commercial availability of GE foods, (2) the consumption of GE foods by others, and (3) your own consumption of GE foods. The survey findings revealed that participants were most accepting of the consumption of GE foods by others, followed by their acceptance of GE foods being commercially available. Notably, participants' acceptance of GE foods increased in all three aspects after they viewed an informative video. The video had a particularly strong impact on participants who fully or partially understood its content, compared to those who did not. Furthermore, regression analyses showed that participants' understanding of two key areas, namely "Why are GE foods important" and "What procedures are in place to ensure the safety of GE foods," played a crucial role in increasing acceptability. Overall, these results indicate that providing information about GE foods to Japanese consumers can effectively enhance their acceptance of such foods. The findings highlight the importance of understanding the benefits and safety measures associated with GE foods in influencing consumer attitudes.

Keywords: acceptability, genome-edited food, perception

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Takabatake R*1, Kagiya Y*2, Futo S*2, Minegishi Y*3, Soga K, Shibata N, Kondo K: Rapid Screening Detection of Genetically Modified Papaya by Loop-Mediated Isothermal Amplification.
Biol Pharm Bull. 2023;46:713-717. doi: 10.1248/bpb.b22-00874

A loop-mediated isothermal amplification (LAMP)-mediated screening detection method for genetically modified (GM) papaya was developed targeting the 35S promoter (P35S) of the cauliflower mosaic virus. LAMP products were detected using a Genie II real-time fluorometer. The limit of detection (LOD) was evaluated and found to be ≤0.05% for papaya seeds. We also designed a primer set for the detection of the papaya endogenous reference sequence, chymopapain, and the species-specificity was confirmed. To improve cost-effectiveness, single-stranded tag hybridization (STH) on a chromatography printed-array strip (C-PAS) system, which is a lateral flow DNA chromatography technology, was applied. LAMP amplification was clearly detected by the system at the LOD level, and a duplex detection of P35S and chymopapain was successfully applied. This simple and quick method for the screening of GM papaya will be useful for the prevention of environmental contamination of unauthorized GM crops.

Keywords: genetically modified, loop-mediated

isothermal amplification, papaya

*1 National Agriculture and Food Research Organization
*2 Fasmac Co., Ltd.
*3 NIPPON GENE Co., Ltd.

Goto*1 K, Tamehiro N, Yoshida T*1, Hanada H*2, Sakuma T*1, Adachi R, Kondo K*3, Takeuchi I*4: Novel machine learning method allerStat identifies statistically significant allergen-specific patterns in protein sequences

J Biol Chem. 2023;299(6):104733. doi:10.1016/j.jbc.2023.104733.

Cutting-edge technologies such as genome editing and synthetic biology allow us to produce novel foods and functional proteins. However, their toxicity and allergenicity must be accurately evaluated. It is known that specific amino acid sequences in proteins make some proteins allergic, but many of these sequences remain uncharacterized. In this study, we introduce a data-driven approach and a machine-learning method to find undiscovered allergen-specific patterns (ASPs) among amino acid sequences. The proposed method enables an exhaustive search for amino acid subsequences whose frequencies are statistically significantly higher in allergenic proteins. As a proof-of-concept, we created a database containing 21,154 proteins of which the presence or absence of allergic reactions are already known and applied the proposed method to the database. The detected ASPs in this proof-of-concept study were consistent with known biological findings, and the allergenicity prediction performance using the detected ASPs was higher than extant approaches, indicating this method may be useful in evaluating the utility of synthetic foods and proteins.

Keywords: allergen, amino acid, computational biology

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Murai U*1, Tajima R*1, Matsumoto M*1, Sato Y*2, Horie S*3, Fujiwara A, Koshida E*1, Okada E*1, Sumikura T*1, Yokoyama T*4, Ishikawa M*4, Kurotani K*5, Takimoto H*1: Validation of Dietary Intake Estimated by Web-Based Dietary Assessment Methods and Usability Using Dietary Records or 24-h Dietary Recalls: A Scoping Review.

Nutrients. 2023;15:1618. doi: 10.3390/nu15081816

The goal was to summarize studies comparing the accuracy of web-based dietary assessments with those of conventional face-to-face or paper-based assessments using 24-h dietary recall or dietary record methods in the general population. Using two databases, mean differences and correlation coefficients (CCs) for intakes of energy, macronutrients, sodium, vegetables, and fruits were extracted from each study independently by the authors. We also collected information regarding usability from articles reporting this. From 17 articles included in this review, the mean dietary intake differences in the web-based dietary assessment compared to conventional methods, were -11.5-16.1% for energy, -12.1-14.9% for protein, -16.7-17.6% for fat, -10.8-8.0% for carbohydrates, -11.2-9.6% for sodium, -27.4-3.9% for vegetables, and -5.1-47.6% for fruits. The CC was 0.17-0.88 for energy, protein, fat, carbohydrates, and sodium, and 0.23-0.85 for vegetables and fruits. In three out of four studies reporting usability, more than half of the participants preferred the web-based dietary assessment. In conclusion, % difference and CC of dietary intake were acceptable in both web-based dietary records and 24-h dietary recalls. The findings from this review highlight the possibility of wide-spread application of the web-based dietary assessment in the future.

Keywords: dietary assessment, scoping review, validity

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Fujiwara A, Fukunaga A*1,2, Murakami K*3, Inoue Y*1, Nakagawa T*4, Yamamoto S*4, Konishi M*1,

Mizoue T[*1]: Cross-Sectional Association between Dietary Hardness and Cognitive Dysfunction among Japanese Men in Their 60s: A Cross-Sectional Study. *Nutrients*. 2023;15:2485. doi: 10.3390/nu15112485

We aimed to examine the cross-sectional association between dietary hardness and cognitive dysfunction among Japanese men in their 60s. Participants were 1494 men aged 60-69 years from the baseline survey of Hitachi Health Study II (2017-2020). Dietary hardness was defined as an estimate of masticatory muscle activity involved in consuming solid foods. Habitual intake of these foods was assessed using a brief-type, self-administered diet history questionnaire. Cognitive dysfunction was defined as a score ≤ 13 points on the test battery for screening for Alzheimer's disease (MSP-1100). The mean (SD) age of participants was 63.5 (3.5) years. The prevalence of cognitive dysfunction was 7.5%. The ORs (95% CIs) for cognitive dysfunction in the second and third tertiles were: 0.77 (0.47, 1.26) and 0.87 (0.54, 1.41), respectively, after adjustment for socio-demographic factors (p for trend = 0.73). After further adjustment for protective nutrient intake against cognitive dysfunction, the corresponding figures were 0.72 (0.43, 1.21) and 0.79 (0.43, 1.46), respectively (p for trend = 0.57). Dietary hardness was not associated with the prevalence of cognitive dysfunction among Japanese men in their 60s. Future prospective studies are necessary to investigate the association between dietary hardness estimated by a validated questionnaire and cognitive dysfunctions.

Keywords: Japan, cognitive impairment, hardness, mastication, older adults

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Fujiwara A, Fukunaga A[*1,2], Murakami K[*3], Inoue Y[*1], Nakagawa T[*4], Yamamoto S[*4], Konishi M[*1], Mizoue T[*1]: Cross-Sectional Association between Estimated Hardness of the Habitual Diet and Depressive Symptoms in Older Japanese Men. *Nutrients*. 2023;15:3034. doi: 10.3390/nu15133034

This cross-sectional study aimed to investigate the association between dietary hardness and depressive symptoms in older Japanese men. Participants were 1487 men aged 60-69 years enrolled in the baseline survey of the Hitachi Health Study II (2017-2020). Habitual dietary intake was estimated by a brief-type, self-administered diet history questionnaire. Dietary hardness was defined as the magnitude of masticatory muscle activity necessary to consume solid foods. The participants who scored ≥ 9 points on a short version of the Center for Epidemiologic Studies Depression Scale were considered to have depressive symptoms. The prevalence of depressive symptoms was 12.7%. The ORs (95% CIs) for depressive symptoms in the third tertile of dietary hardness were significantly lower after adjustment for sociodemographic and lifestyle-related variables and mood-modulating nutrients (ORs [95% CIs]: 0.93 [0.63, 1.36] and 0.58 [0.35, 0.97] for the second and third tertile, respectively [p-value for trend = 0.04]). Dietary hardness was inversely associated with the prevalence of depressive symptoms in older Japanese men. Future studies should confirm these findings and clarify the role of consuming a hard diet in preventing depressive disorders.

Keywords: Japan, chewing, depressive disorder, dietary hardness, mastication

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Yuan X[*], Tajima R[*], Matsumoto M[*], Fujiwara A, Aoyama T[*], Okada C[*], Okada E[*], Takimoto H[*]: Analysing food groups and nutrient intake in adults who met and did not meet the daily recommended

vegetable intake of 350 g: the 2016 National Health and Nutrition Survey in Japan.

Journal of Nutritional Science. 2024;13:e12. doi: 10.1017/jns.2024.5

This study aimed to compare the differences in the intake of food groups and nutrients between Japanese adults who consumed the recommended daily vegetable intake (350 g/day) and those who did not. Dietary information was obtained from one-day dietary records collected from the 2016 National Health and Nutrition Survey, which was conducted in 46 prefectures in Japan. The participants aged ≥20 years (n = 21,606; 53.8% women) were classified into the < and ≥350 g/day groups. Inter-group differences for 17 food groups and 27 nutrients were assessed as percentages of consumers (food groups only) and energy-adjusted intake (units/MJ/d or % of total energy intake). Overall, 29% of participants consumed ≥350 g/day of vegetables. The ≥350 g/day group had a higher percentage of consumers and energy-adjusted intakes for all vegetable subgroups than the <350 g/day group. For other food groups, the ≥350 g/day group had higher percentages of consumers for all food groups, except for cereals, eggs, and condiments and seasonings, which showed no significant differences. However, the ≥350 g/day group had a significantly higher energy-adjusted intake for potatoes and other tubers, mushrooms, meats, and condiments and seasonings but a significantly lower value for cereals, eggs, savoury snacks and confectionaries, and beverages. The ≥350 g/day group had a significantly higher intake of almost all (25/27) nutrients, including sodium, than the <350 g/day group. Participants with vegetable intake ≥350 g/day might have a more favourable intake of food groups and nutrients; however, watching for salt intake is necessary when promoting vegetable intake.

Keywords: Japanese adults, dietary intake, national survey, vegetables

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Masuoka S*, Nishio J*, Yamada S*, Saito K, Kaneko K*, Kaburaki M*, Tanaka N*, Sato H*, Muraoka S*, Kawazoe M*, Mizutani S*, Furukawa K*, Ishii-Watabe A, Kawai S*, Saito Y, Nanki T*. Relationship Between the Lipidome Profile and Disease Activity in Patients with Rheumatoid Arthritis.

Inflammation. 2024;Feb 24. doi:10.1007/s10753-024-01986-8

The Lipid mediators have been suggested to play important roles in the pathogenesis of rheumatoid arthritis (RA). Lipidomics has recently allowed for the comprehensive analysis of lipids and has revealed the potential of lipids as biomarkers for the early diagnosis of RA and prediction of therapeutic responses. However, the relationship between disease activity and the lipid profile in RA remains unclear. In the present study, we performed a plasma lipidomic analysis of 278 patients with RA during treatment and examined relationships with disease activity using the Disease Activity Score in 28 joints (DAS28)-erythrocyte sedimentation rate (ESR). In all patients, five lipids positively correlated and seven lipids negatively correlated with DAS28-ESR. Stearic acid [FA(18:0)] (r = -0.45) and palmitic acid [FA(16:0)] (r = -0.38) showed strong negative correlations. After adjustments for age, body mass index (BMI), and medications, stearic acid, palmitic acid, bilirubin, and lysophosphatidylcholines negatively correlated with disease activity. Stearic acid inhibited osteoclast differentiation from peripheral blood monocytes in *in vitro* experiments, suggesting its contribution to RA disease activity by affecting bone metabolism. These results indicate that the lipid profile correlates with the disease activity of RA and also that some lipids may be involved in the pathogenesis of RA.

Keywords: Lipidomics, Lysophosphatidylcholine, Palmitic acid

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Ishikawa R, Saito K, Misawa T, Demizu Y, Saito Y. Identification of the Stapled α-Helical Peptide ATSP-7041 as a Substrate and Strong Inhibitor of OATP1B1 *In Vitro*.

Biomolecules. 2023;13:1002. doi:10.3390/biom13061002

ATSP-7041, a stapled α-helical peptide that inhibits murine double minute-2 (MDM2) and MDMX activities, is a promising modality targeting protein-protein interactions. As peptides of molecular weights over 1000 Da are not usually evaluated, data on the

drug-drug interaction (DDI) potential of stapled α-helical peptides remain scarce. Here, we evaluate the interaction of ATSP-7041 with hepatic cytochrome P450s (CYPs;CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP2D6) and transporters (organic anion transporting polypeptides (OATPs;OATP1B1 and OATP1B3), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP)). ATSP-7041 demonstrated negligible metabolism in human liver S9 fraction and a limited inhibition of CYP activities in yeast microsomes or S9 fractions. On the contrary, a substantial uptake by OATPs in HEK 293 cells, a strong inhibition of OATP activities in the cells, and an inhibition of P-gp and BCRP activities in reversed membrane vesicles were observed for ATSP-7041. A recent report describes that ALRN-6924, an ATSP-7041 analog, inhibited OATP activities *in vivo*;therefore, we focused on the interaction between ATSP-7041 and OATP1B1 to demonstrate that ATSP-7041, as a higher molecular weight stapled peptide, is a substrate and strong inhibitor of OATP1B1 activity. Our findings demonstrated the possibility of transporter-mediated DDI potential by high molecular weight stapled peptides and the necessity of their evaluation for drug development.

Keywords: ATSP-7041, MDM2/MDMX inhibitor, drug–drug interaction, organic anion transporting polypeptide 1B1, α-helical peptide

Sun Y, Saito K, Ushiki A[*1], Abe M[*2], Saito Y[*3], Kashiwada T[*3], Horimasu Y[*4], Gemma A[*3], Tatsumi K[*2], Hattori N[*4], Tsushima K[*5], Takemoto K, Ishikawa R, Momiyama T, Matsuyama SI, Arakawa N, Akane H, Toyoda T, Ogawa K, Sato M[*6], Takamatsu K[*6], Mori K[*7], Nishiya T[*7], Izumi T[*8], Ohno Y[*8], Saito Y, Hanaoka M[*1]. Identification of kynurenine and quinolinic acid as promising serum biomarkers for drug-induced interstitial lung diseases.

Respir Res. 2024;25:31. doi:10.1186/s12931-023-02653-6

Background:Drug-induced interstitial lung disease (DILD) is a lung injury caused by various types of drugs and is a serious problem in both clinical practice and drug development. Clinical management of the condition would be improved if there were DILD-specific biomarkers available;this study aimed to meet that need.

Methods:Biomarker candidates were identified by non-targeted metabolomics focusing on hydrophilic molecules, and further validated by targeted approaches using the serum of acute DILD patients, DILD recovery patients, DILD-tolerant patients, patients with other related lung diseases, and healthy controls.

Results:Serum levels of kynurenine and quinolinic acid (and kynurenine/tryptophan ratio) were elevated significantly and specifically in acute DILD patients. The diagnostic potentials of these biomarkers were superior to those of conventional lung injury biomarkers, Krebs von den Lungen-6 and surfactant protein-D, in discriminating between acute DILD patients and patients with other lung diseases, including idiopathic interstitial pneumonia and lung diseases associated with connective tissue diseases. In addition to identifying and evaluating the biomarkers, our data showed that kynurenine/tryptophan ratios (an indicator of kynurenine pathway activation) were positively correlated with serum C-reactive protein concentrations in patients with DILD, suggesting the potential association between the generation of these biomarkers and inflammation. Our *in vitro* experiments demonstrated that macrophage differentiation and inflammatory stimulations typified by interferon gamma could activate the kynurenine pathway, resulting in enhanced kynurenine levels in the extracellular space in macrophage-like cell lines or lung endothelial cells. Extracellular quinolinic acid levels were elevated only in macrophage-like cells but not endothelial cells owing to the lower expression levels of metabolic enzymes converting kynurenine to quinolinic acid. These findings provide clues about the molecular mechanisms behind their specific elevation in the serum of acute DILD patients.

Conclusions:The serum concentrations of kynurenine and quinolinic acid as well as kynurenine/tryptophan ratios are promising and specific biomarkers for detecting and monitoring DILD and its recovery, which could facilitate accurate decisions for appropriate clinical management of patients with DILD.

Keywords: Biomarker, Drug-induced interstitial lung diseases, Kynurenine

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Pineda Garcia JC*, Li RS*, Kikura-Hanajiri R, Tanaka Y*, Ishii Y*:Timeframe Analysis of Novel Synthetic Cannabinoids Effects:A Study on Behavioral Response and Endogenous Cannabinoids Disruption. Int.
J. Mol. Sci. 2024;25:3083. https://doi.org/10.3390/ijms25063083

This study investigates the impact of SCs consumption by assessing the effects of three novel synthetic cannabinoids (SCs);MDMB-CHMINACA, 5F-ADB-PINACA, and APICA post-drug treatment. SCs are known for their rapid onset (<1 min) and prolonged duration (≥5 h). Therefore, this research aimed to assess behavioral responses and their correlation with endocannabinoids (ECs) accumulation in the hippocampus, and EC's metabolic enzymes alteration at different timeframes (1-3-5-h) following drug administration. Different extents of locomotive disruption and sustained anxiety-like symptoms were observed throughout all-encompassing timeframes of drug administration. Notably, MDMB-CHMINACA induced significant memory impairment at 1 and 3 h. Elevated levels of anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) were detected 1 h post-MDMB-CHMINACA and 5F-ADB-PINACA administration. Reduced mRNA expression levels of fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL) (AEA and 2-AG degrading enzymes, respectively), and brain-derived neurotrophic factor (BDNF) occurred at 1 h, with FAAH levels remaining reduced at 3 h. These findings suggest a connection between increased EC content and decreased BDNF expression following SC exposure. Cognitive disruption, particularly motor coordination decline and progressive loss manifested in a time-dependent manner across all the analyzed SCs. Our study highlights the importance of adopting a temporal framework when assessing the effects of SCs.

Keywords: locomotive, behavior, indazole/indole–carboxamide

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Nakamura R, Arakawa N, Tanaka Y, Uchiyama N, Sekine A*1, Mashimo Y*1, Tsuji K*2, Kagawa T*3, Sato K*4, Watanabe M*5, Aiso M*6, Hiasa Y*7, Takei Y*8, Ohira H*9, Ayada M*10, Tsukagoshi E, Maekawa K*11, Tohkin M*12, Saito Y, Takikawa H*13. Significant association between HLA-B*35:01 and onset of drug-induced liver injury caused by Kampo medicines in Japanese patients.
Hepatol Res. 2023;53:440-449. doi:10.1111/hepr.13874.

Aim:Drug-induced liver injury (DILI) is a severe and life-threatening immune-mediated adverse effect, occurring rarely among treated patients. We examined genomic biomarkers in the Japanese population that predict the onset of DILI after using a certain class of drugs, such as Kampo products (Japanese traditional medicines).

Methods:A total of 287 patients diagnosed as DILI by hepatology specialists were recruited after written informed consent was obtained. A genome-wide association analysis and human leukocyte antigen (HLA) typing in four digits were performed.

Results:We found a significant association ($p=9.41\times10\text{-}10$) of rs146644517 (G>A) with Kampo product-related DILI. As this polymorphism is located in the HLA region, we evaluated the association of HLA types and found that 12 (63.2%) of 19 Kampo-DILI patients contained HLA-B*35:01, whereas only 15.2% were positive for this HLA among healthy volunteers. The odds ratio was 9.56 (95% confidence interval 3.75-24.46;$p=2.98\times10\text{-}6$, corrected $p=4.17\times10\text{-}5$), and it increased to 13.55 compared with the DILI patients not exposed to Kampo products. The individual crude drug components in the Kampo products, including Scutellaria root (ougon in Japanese), rhubarb (daiou), Gardenia fruit (sanshishi), and Glycyrrhiza (kanzou), were significantly associated with HLA-B*35:01.

Conclusions:HLA-B*35:01 is a genetic risk factor and a potential predictive biomarker for Kampo-induced DILI in the Japanese population.

Keywords: Kampo medicines, crude drug, drug-induced liver injury

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Adachi K*1, Ohyama K*2, Tanaka Y, Saito Y, Shimizu M*1, Yamazaki H*1. Modeled Hepatic/Plasma Exposures of Fluvastatin Prescribed Alone in Subjects with Impaired Cytochrome P450 2C9*3 as One of Possible Determinant Factors Likely Associated with Hepatic Toxicity Reported in a Japanese Adverse Event Database.
Biol Pharm Bull. 2024;47(3):635-640.

Fluvastatin is a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor that competitively inhibits human cytochrome P450 (P450) 2C9 *in vitro*. Drug interactions between a variety of P450 2C9 substrates/inhibitors and fluvastatin can increase the incidence of fluvastatin-related hepatic or skeletal muscle toxicity *in vivo*. In this survey, the prescribed dosage of fluvastatin was reduced or discontinued in 133 of 164 patients receiving fluvastatin alone, as recorded in the Japanese Adverse Drug Event Report database of spontaneously reported events. The median days to onset of fluvastatin-related disorders were in the range 30-35 d in the 87 patients. Therefore, we aimed to focus on fluvastatin and, using the pharmacokinetic modeling technique, estimated the virtual plasma and hepatic exposures in subjects harboring the impaired CYP2C9*3 allele. The plasma concentrations of fluvastatin modeled after a virtual oral 20-mg dose increased in homozygotes with CYP2C9*3;the area under the plasma concentration curve was 4.9-fold higher than that in Japanese homozygotes for wild-type CYP2C9*1. The modeled hepatic concentrations of fluvastatin in patients with CYP2C9*3/*3 after virtual daily 20-mg doses for 7 d were 31-fold higher than those in subjects with CYP2C9*1/*1. However, heterozygous Chinese patients with CYP2C9*1/*3 reportedly have a limited elevation (1.2-fold) in plasma maximum concentrations. Virtual hepatic/plasma exposures in subjects harboring the impaired CYP2C9*3 allele estimated using pharmacokinetic modeling indicate that such exposure could be a causal factor for hepatic disorders induced by fluvastatin prescribed alone in a manner similar to that for interactions with a variety of co-administered drugs.
Keywords: CYP2C9.3, pharmacokinetic modeling, statin tolerance

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Adachi K*1, Ohyama K*2, Tanaka Y, Sato T*1, Murayama N*1, Shimizu M*1, Saito Y, Yamazaki H*1. High hepatic and plasma exposures of atorvastatin in subjects harboring impaired cytochrome P450 3A4*16 modeled after virtual administrations and possibly associated with statin intolerance found in the Japanese adverse drug event report database.
*Drug Metab Pharmacokine*t. 2023;49:100486.

Drug interactions between atorvastatin and cytochrome P450 (P450) 3A substrates/inhibitors lead to an increased incidence of skeletal muscle or hepatic toxicity. However, in this survey, among 483 Japanese subjects administered atorvastatin alone, more than half (258) experienced statin intolerance and were unable to continue using the drug. Although many factors underly atorvastatin toxicity, the intrinsic clearance rate might be a contributing causal factor. The impaired P450 3A4 p.Thr185Ser variant, CYP3A4*16 (rs12721627), has been identified in East Asians with an allele frequency of 2.2%. Pharmacokinetically modeled plasma concentrations of atorvastatin increased after a virtual oral dose of 40 mg in CYP3A4*16 homozygotes;the maximum concentration and area under the concentration curve, respectively, were 3.3-fold and 4.2-fold those in subjects homozygous for CYP3A4*1. In subjects with CYP3A4*16/*16, the virtual hepatic concentrations of atorvastatin after daily doses of 10 mg for a week were similar to or higher than the plasma concentrations. These results suggest that the estimated high virtual plasma and hepatic exposures obtained by

pharmacokinetic modeling in subjects harboring impaired allele CYP3A4*16 may be one of the causal factors for statin intolerance in a manner similar to the well-known drug interactions caused by co-administrations of CYP3A inhibitors.

Keywords: CYP3A4.1, CYP3A4.16, CYP3A5

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Nguyen TTT*, Tanaka Y, Sanada M*, Hosaka M*, Tamai M*, Kagami K*, Komatsu C*, Somazu S*, Harama D*, Kasai S*, Watanabe A*, Akahane K*, Goi K*, Inukai T*. CRISPR/Cas9-Mediated Induction of Relapse-Specific NT5C2 and PRPS1 Mutations Confers Thiopurine Resistance as a Relapsed Lymphoid Leukemia Model.

Mol Pharmacol. 103(4);199-210. 2023.

6-Mercaptopurine (6-MP) is a key component in maintenance therapy for childhood acute lymphoblastic leukemia (ALL). Recent next-generation sequencing analysis of childhood ALL clarified the emergence of the relapse-specific mutations of the NT5C2 and PRPS1 genes, which are involved in thiopurine metabolism. In this scenario, minor clones of leukemia cells could acquire the 6-MP-resistant phenotype as a result of the NT5C2 or PRPS1 mutation during chemotherapy (including 6-MP treatment) and confer disease relapse after selective expansion. Thus, to establish new therapeutic modalities overcoming 6-MP resistance in relapsed ALL, human leukemia models with NT5C2 and PRPS1 mutations in the intrinsic genes are urgently required. Here, mimicking the initiation process of the above clinical course, we sought to induce two relapse-specific hotspot mutations (R39Q mutation of the NT5C2 gene and S103N mutation of the PRPS1 gene) into a human lymphoid leukemia cell line by homologous recombination (HR) using the CRISPR/Cas9 system. After 6-MP selection of the cells transfected with Cas9 combined with single-guide RNA and donor DNA templates specific for either of those two mutations, we obtained the sublines with the intended NT5C2-R39Q and PRPS1-S103N mutation as a result of HR. Moreover, diverse in-frame small insertion/deletions were also confirmed in the 6-MP-resistant sublines at the target sites of the NT5C2 and PRPS1 genes as a result of nonhomologous end joining. These sublines are useful for molecular pharmacological evaluation of the NT5C2 and PRPS1 gene mutations in the 6-MP sensitivity and development of therapy overcoming the thiopurine resistance of leukemia cells. SIGNIFICANCE STATEMENT:Mimicking the initiation process of relapse-specific mutations of the NT5C2 and PRPS1 genes in childhood acute lymphoblastic leukemia treated with 6-mercaptopurine (6-MP), this study sought to introduce NT5C2-R39Q and PRPS1-S103N mutations into a human lymphoid leukemia cell line by homologous recombination using the CRISPR/Cas9 system. In the resultant 6-MP-resistant sublines, the intended mutations and diverse in-frame small insertions/deletions were confirmed, indicating that the obtained sublines are useful for molecular pharmacological evaluation of the NT5C2 and PRPS1 gene mutations.

Keywords: mercaptopurine, NT5C2, PRPS1

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Tsukagoshi E, Nakamura R, Tanaka Y, Maekawa K*1, Hiratsuka M*2, Asada H*3, Saito S, Kikura-Hanajiri R. Validation of a genotyping technique for a surrogate marker of HLA-B*58:01 for allopurinol-induced Stevens–Johnson syndrome and toxic epidermal necrolysis in the Japanese population.

Drug Metab Pharmacokinet. 2023;49:100495. doi:10.1016/j.dmpk.2023.100495

Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are rare but severe cutaneous adverse drug reactions. Certain human leukocyte antigen (HLA) types have been associated with SJS/TEN onset, e.g., HLA-B*58:01 with allopurinol-induced SJS/TEN, but HLA typing is time-consuming and expensive;thus, it is not commonly used in clinical situations. In the previous work, we demonstrated that the single-nucleotide polymorphisms (SNP) rs9263726 was in absolute linkage disequilibrium with HLA-B*58:01 in the Japanese population, and can be used as a surrogate marker for the HLA. Here, we developed a new genotyping method for the surrogate SNP using the single-stranded tag hybridization chromatographic printed-array strip (STH-PAS) technique and performed an analytical validation. The results of genotyping rs9263726 using STH-PAS

correlated well with those obtained using the TaqMan SNP Genotyping Assay for 15 HLA-B*58:01-positive and 13 HLA-B*58:01-negative patients (analytical sensitivity and specificity were both 100%). Additionally, at least 1.11 ng of genomic DNA was sufficient to digitally and manually detect positive signals on the strip. Robustness studies showed that the annealing temperature (66℃) was the most important condition related to reliable results. Collectively, we developed an STH-PAS method that can rapidly and easily detect rs9263726 for predicting SJS/TEN onset.

Keywords: Stevens–Johnson syndrome, toxic epidermal necrolysis, human leukocyte antigen

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Tsukagoshi E, Nakamura R, Kaniwa N, Sai K, Kikura-Hanajiri R, Matsunaga K*1, Abe R*2, Asada H*3, Saito Y. Clinical profiles of Japanese patients with Stevens–Johnson syndrome/toxic epidermal necrolysis collected by a nationwide system from 2006 to 2023.
Biol Pharm Bull. 2024;47(1):88-97. doi:10.1248/bpb.b23-00595

Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) are potentially life-threatening severe cutaneous adverse drug reactions. These diseases are rare, and their onset is difficult to predict because of their idiosyncratic reactivity. The Japan Severe Adverse Reactions Research Group, led by the National Institute of Health Sciences, has operated a nationwide to collect clinical information and genomic samples from patients with SJS/TEN since 2006. This study evaluated the associations of clinical symptoms with sequelae and specific causative drugs/drug groups in Japanese patients with SJS/TEN to identify clinical clues for SJS/TEN treatment and prognosis. Acetaminophen, antibiotics, and carbocisteine were linked to high frequencies of severe ocular symptoms and ocular sequelae ($P < 0.05$). For erythema and erosion areas, antipyretic analgesics had higher rates of skin symptom affecting <10% of the skin than the other drugs, suggesting narrower lesions ($P < 0.004$). Hepatic dysfunction, was common in both SJS and TEN, and antiepileptic drugs carried higher risks of hepatic dysfunction than the other drug groups ($P = 0.0032$). This study revealed that the clinical manifestations of SJS/TEN vary according to the causative drugs.

Keywords: Clinical information, Drug, Japanese

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Fukunaga K*1, Tsukagoshi E, Kurata M*2, Mizukawa Y*2, Niihara H*3, Morita E*3, Watanabe Y*4, Yamaguchi Y*4, Watanabe H*5, Nakajima S*6, Nomura T*6, Kabashima K*6, Tohyama M*7, Azukizawa H*8, Asada H*8, Hasegawa A*9, Hama N*9, Ozeki T*1, Mashimo Y*10, Sekine A*10, Matsunaga K*11, Tanaka Y, Nakamura R, Abe R*9, Mushiroda Taisei*1, Saito Y. Differential effects of HLA-B*15:11 and HLA-A*31:01 on carbamazepine-induced cutaneous adverse reactions.
J Invest Dermatol. 2024;144(4):908-911.e7. doi:10.1016/j.jid.2023.09.282

Introduction:Carbamazepine (CBZ)-induced cutaneous adverse drug reactions (cADRs) are significant public health concerns. Previous studies have identified associations between human leukocyte antigen (HLA)-A*31:01 or HLA-B*15:11 and CBZ-induced cADRs. However, the effects of these HLA-alleles on different cADRs, such as Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN), drug-induced hypersensitivity syndrome or drug reaction with eosinophilia and systemic symptoms (DIHS/DRESS), maculopapular exanthema (MPE), and erythema multiforme (EM), remain unclear. We aimed to identify genetic variants associated with the risk of different cADRs.

Methods:Genome-wide association studies of 131 cases and 2,823 controls in two independent Japanese populations were performed. Meta-analysis of HLA-B*15:11 was conducted across Japan, Korea, Southern China, and Thailand, and the effects of HLA-A*31:01 and HLA-B*15:11 on the risk of different cADRs were assessed.

Results:HLA-B*15:11 was associated with the risk of SJS/TEN (odds ratio [OR] = 18.2, $p = 3.02 \times 10 - 12$). This association was replicated in different populations

(Koreans, Southern Chinese, and Thais). Furthermore, strong associations were observed between HLA-A*31:01 and cADRs other than SJS/TEN (DIHS/DRESS, OR=10.1, p=8.64 × 10−20;MPE, OR=9.1, p=1.51 × 10−5;EM, OR=7.4, p=0.0019).

Discussion:In conclusion, in populations where HLA-B*15:02 is rare, HLA-B*15:11 and HLA-A*31:01 could be used as biomarkers in predicting the risk of CBZ-induced cADRs.

Keywords: Cutaneous adverse drug reaction, Genome-wide association study

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Saito H, Yokota S, Kitajima S:Immunohistochemical analysis of the vimentin filaments in Sertoli cells is a powerful tool for the prediction of spermatogenic dysfunction.

Acta Histochemica. 2023;125(5):152046. doi:10.1016/j.acthis.2023.152046

The close interaction between male germ cells and Sertoli cells, a type of somatic cell found in the seminiferous tubules of mammalian testis, is essential for the normal progression of spermatogenesis in mammals. Vimentin is an intermediate filament protein that primarily provides mechanical support, preserves cell shape, and maintains the nuclear position, and it is often used as a marker to identify Sertoli cells. Vimentin is known to be involved in many diseases and aging processes;however, how vimentin is related to spermatogenic dysfunction and the associated functional changes is still unclear. In a previous study, we reported that vitamin E deficiency affected the testes, epididymis, and spermatozoa of mice, accelerating the progression of senescence. In this study, we focused on the Sertoli cell marker vimentin and explored the relationship between the cytoskeletal system of Sertoli cells and spermatogenic dysfunction using testis tissue sections that caused male reproductive dysfunction with vitamin E deficiency. The immunohistochemical analysis showed that the proportion of the vimentin-positive area in seminiferous tubule cross-sections was significantly increased in testis tissue sections of the vitamin E-deficient group compared with the proportion in the control group. The histological analysis of testis tissue sections from the vitamin E-deficient group showed that vimentin-positive Sertoli cells were greatly extended from the basement membrane, along with an increased abundance of vimentin. These findings suggest that vimentin may be a potential indicator for detecting spermatogenic dysfunction.

Keywords: Sertoli cell, testicular toxicity, vimentin

Miyauchi A*1, Akashi T*1, Yokota S, Taquahashi Y, Hirose A*2, Hojo M*3, Yoshida H*1, Kurokawa M*1, Watanabe W*1:Effects of inhalation of multi-walled carbon nanotube (MWCNT) on respiratory syncytial virus (RSV) infection in mice.

J Toxicol Sci. 2023;48(7):411-420. doi:10.2131/jts.48.411.

Multi-walled carbon nanotubes (MWCNTs), a kind of nanomaterial, are widely used in battery electrodes and composite materials, but the adverse effects associated with their accumulation in the living body have not been sufficiently investigated. MWCNTs are a fibrous material with molecules similar to asbestos fibers, and there are concerns about its effects on the respiratory system. In this study, we conducted a risk assessment by exposing mice using a previously developed nanomaterial inhalation exposure method. We quantified the exposure in the lungs by a lung burden test, evaluated the deterioration due to pneumonia using respiratory syncytial virus (RSV) infection, and measured inflammatory cytokines in bronchoalveolar lavage fluid (BALF). As a result, in the lung burden test, the amount of MWCNT in the lung increased according to the inhalation dose. In the RSV infection experiment, CCL3, CCL5, and TGF-β, which are indicators of inflammation and lung fibrosis, were elevated in the MWCNT-exposed group.

Histological examination revealed cells phagocytosing MWCNT fibers. These phagocytic cells were also seen during the recovery period from RSV infection. The present study found that MWCNT remained in the lungs for about a month or more, suggesting that the fibers may continue to exert immunological effects on the respiratory system. Furthermore, the inhalation exposure method enabled the exposure of nanomaterials to the entire lung lobe, allowing a more detailed evaluation of the effects on the respiratory system.

Keywords: MWCNT, pneumonia, RSV

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Hojo M*1, Maeno A*1, Sakamoto Y*1, Yamamoto Y*1, Taquahashi Y, Hirose A*2, Suzuki J*1, Inomata A*1, Nakae D*3:Time-Course of Transcriptomic Change in the Lungs of F344 Rats Repeatedly Exposed to a Multiwalled Carbon Nanotube in a 2-Year Test.
Nanomaterials (Basel). 2023 19;13(14):2105. doi:10.3390/nano13142105.

Despite intensive toxicological studies of carbon nanotubes (CNTs) over the last two decades, only a few studies have demonstrated their pulmonary carcinogenicities in chronic animal experiments, and the underlying molecular mechanisms are still unclear. To obtain molecular insights into CNT-induced lung carcinogenicity, we performed a transcriptomic analysis using a set of lung tissues collected from rats in a 2-year study, in which lung tumors were induced by repeated intratracheal instillations of a multiwalled carbon nanotube, MWNT-7. The RNA-seq-based transcriptome identified a large number of significantly differentially expressed genes at Year 0.5, Year 1, and Year 2. Ingenuity Pathway Analysis revealed that macrophage-elicited signaling pathways such as phagocytosis, acute phase response, and Toll-like receptor signaling were activated throughout the experimental period. At Year 2, cancer-related pathways including ERBB signaling and some axonal guidance signaling pathways such as EphB4 signaling were perturbed. qRT-PCR and immunohistochemistry indicated that several key molecules such as Osteopontin/Spp1, Hmox1, Mmp12, and ERBB2 were markedly altered and/or localized in the preneoplastic lesions, suggesting their participation in the induction of lung cancer. Our findings support a scenario of inflammation-induced carcinogenesis and contribute to a better understanding of the molecular mechanism of MWCNT carcinogenicity.

Keywords: MWNT-7, lung cancer, transcriptomics

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Yoshioka H*1, Wu S*2, Moriishi T*2, Tsukiboshi Y*1, Yokota S, Miura N*3, Yoshikawa M*4, Matsui N*1, Inagaki N*1, Matsushita Y*2, Nakao M*4:*Sasa veitchii* extract alleviates non-alcoholic steatohepatitis in methionine-choline deficient diet-induced mice by regulating peroxisome proliferator-activated receptor alpha.
Traditional and kanpo. 2023;10(3):259-268. doi:10.1002/tkm2.1385

Aim:Nonalcoholic steatohepatitis (NASH) is a pandemic liver disease. This study aimed to explore the protective effects of *Sasa veitchii* extract (SE) in a mouse model of methionine–choline-deficient (MCD) diet-induced NASH.

Methods:Eight-week-old male C57BL/6J mice were fed a normal diet or an MCD diet for 8 weeks (0–8 weeks). SE (0.1 mL) was administered orally once daily for the latter period (4 weeks;5–8 weeks). Body weight was measured weekly. The mice were euthanized and plasma samples were collected. Livers were collected and weighed.

Results:The MCD diet decreased the liver/body weight ratio, elevated plasma alanine aminotransferase and aspartate aminotransferase levels, and increased liver oxidative stress, fibrosis, and inflammation. These changes were alleviated by SE administration. We also found that the MCD-induced increase in triglycerides and lipid droplets in the liver was attenuated by SE. Furthermore, MCD-induced glutathione peroxidase-4 and peroxisome proliferator-activated receptor-alpha downregulation was sustained by SE administration.

Conclusion:Our results showed that SE protected mice against MCD-induced hepatic injury, including oxidative stress and inflammation, by modulating peroxisome proliferator-activated receptor alpha

activation.

Keywords: nonalcoholic steatohepatitis, *Sasa veitchii* extract, methionine–choline-deficient diet

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Saito H, Furukawa Y, Sasaki T*, Kitajima S, Kanno J, Tanemura K*:Behavioral effects of adult male mice induced by low-level acetamiprid, imidacloprid, and nicotine exposure in early-life.

Front Neurosci. 2023;17:1239808. doi:10.3389/fnins.2023.1239808

Introduction:Acetamiprid (ACE) and imidacloprid (IMI), the neonicotinoid chemicals, are widely used as pesticides because of their rapid insecticidal activity. Although these neonicotinoids exert very low toxicity in mammals, the effects of early, low-level, chronic exposure on the adult central nervous system are largely unclear. This study investigated the effects of low-level, chronic neonicotinoids exposure in early life on the brain functions of adult mice, using environmentally relevant concentrations.

Methods:We exposed mice to an acceptable daily intake level of neonicotinoids in drinking water during the prenatal and postnatal periods. Additionally, we also exposed mice to nicotine (NIC) as a positive control. We then examined the effects on the central nervous system in adult male offspring.

Results:In the IMI and NIC exposure groups, we detected behavior that displayed impairment in learning and memory. Furthermore, immunohistochemical analysis revealed a decrease in SOX2 (as a neural stem cell marker) and GFAP (as an astrocyte marker) positive cells of the hippocampal dentate gyrus in the IMI and NIC exposure groups compared to the control group.

Discussion:These results suggest that exposure to neonicotinoids at low levels in early life affects neural circuit base formation and post-maturation behavior. Therefore, in the central nervous system of male mice, the effects of low-level, chronic neonicotinoids exposure during the perinatal period were different from the expected effects of neonicotinoids exposure in mature animals.

Keywords: developmental neurotoxicity, neonicotinoid, neurobehavioral effect

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Pu J*1, Kofuji S*1, Okamoto-Uchida Y*1, Danzaki K*1, Yu R*1, Suzuki A*2, Kitajima S, Nishina H*1:Lethal Phenotype-Based Database Screening Identifies Ceramide as a Negative Regulator of Primitive Streak Formation.

Stem Cells. 2023;41(12):1142-1156. doi.org/10.1093/stmcls/sxad071

In early embryogenesis, the primitive streak (PrS) generates the mesendoderm and is essential for organogenesis. However, because the PrS is a minute and transient tissue, elucidating the mechanism of its formation has been challenging. We performed comprehensive screening of 2 knockout mouse databases based on the fact that failure of PrS formation is lethal. We identified 812 genes involved in various cellular functions and responses that might be linked to PrS formation, with the category of greatest abundance being "Metabolism." In this study, we focused on genes of sphingolipid metabolism and investigated their roles in PrS formation using an *in vitro* mouse ES cell differentiation system. We show here that elevated intracellular ceramide negatively regulates gene expression essential for PrS formation and instead induces neurogenesis. In addition, sphingosine-1-phosphate (a ceramide derivative) positively regulates neural maturation. Our results indicate that ceramide regulates both PrS formation and the induction of neural differentiation.

Keywords: cardiac differentiation, neural differentiation, sphingosine-1-phosphate

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Yokota S, Wakayama T[*1], Miyaso H[*2], Suga K, Fujinoki M[*3], Kaneko S[*4], Kitajima S:Reactive blue 2 labels protamine in late-haploid spermatids and spermatozoa and can be used for toxicity evaluation.
Andrologia. 2023;2023:1-12. doi:10.1155/2023/7364862

Reactive blue 2 (RB2) dye specifically binds to the nuclei of human spermatozoa under weakly alkaline conditions, thereby providing a new method for assessing sperm quality. However, this technique has not yet been applied to other mammalian species, such as well-established rodent models, which would allow evaluation of the male reproductive toxicity of new drug candidates in nonclinical studies. We aimed to evaluate the usefulness of RB2 staining in assessing testicular and epididymal sperm toxicity in mice using a busulfan-induced infertility model. Male C57BL/6J mice were intraperitoneally administered 40 mg/kg of busulfan. After 28 days, the testes and epididymis were collected and stained with RB2 at pH 10. *In vitro* evaluations were conducted on uncoated glass slides with RB2 mixed with mouse synthetic protamines, protamines extracted from the human spermatozoa or intracellular protein components from somatic cells without protamines. Following peanut agglutinin lectin histochemistry, RB2-positive cells were observed in elongating and elongated spermatids at all stages except for stages IX–XI of the seminiferous epithelium. After busulfan administration, the proportion of RB2-positive germ cells in the seminiferous tubules was significantly decreased, and no RB2-positive spermatozoa were found in the caput epididymis of treated mice. Aggregates were observed in a mixture of RB2 dye (pH 10) and protamines but not in a mixture of intracellular protein components without protamines, and this specificity was lost at a neutral pH. Our study demonstrated that RB2 specifically stains steps 12–16 spermatids, indicating specific binding to the protamines expressed in these spermatids. The RB2 staining technique has potential as a biomarker for male reproductive toxicity, allowing for the rapid visualization of protamination in an animal model commonly used for the evaluation of male reproductive toxicity.

Keywords: reproductive toxicity, Reactive blue 2, protamine

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Shimizu M[*1], Hojo M[*1], Ikushima K[*1], Yamamoto Y[*1], Maeno A[*1], Sakamoto Y[*1], Ishimaru N[*2], Taquahashi Y, Kanno J, Hirose A[*3], Suzuki J[*1], Inomata A[*1], Nakae D[*4]:Continuous infiltration of small peritoneal macrophages in the mouse peritoneum through CCR2-dependent and -independent routes during fibrosis and mesothelioma development induced by a multiwalled carbon nanotube, MWNT-7.
J Toxicol Sci. 2023;48(12):617-639. doi:10.2131/jts.48.617.

Although toxicities of multiwalled carbon nanotube (MWCNT) have been found to be related with activities of macrophages phagocytosing the fibers, the exact relationship between macrophage population and pathogenesis of fibrosis and mesotheliomas induced by MWCNTs is largely unknown. CCL2-CCR2 axis, a major monocyte/macrophage infiltration route, is thought to be involved in not only acute inflammation but also the formation of tumor microenvironment. We therefore described a time-course of alteration of macrophage population in an attempt to clarify the contribution of the Ccr2 gene to mesotheliomagenesis. Wild-type (WT) C57BL/6 mice and Ccr2-knockout (KO) mice were intraperitoneally administered with MWNT-7 and were sequentially necropsied at 1, 7, 28, 90, and 245 day(s) after the injection. Peritoneal fibrosis was prominent in all MWCNT-treated mice, with a lower severity in the KO mice. No differences were observed in the incidences of neoplastic lesions of mesothelia between WT and KO mice. A flow cytometric analysis revealed that after gross disappearance of macrophages after MWCNT exposure, small peritoneal macrophages (SPMs) were exclusively refurbished by the CCR2-dependent route at day 1 (as Ly-6C+MHC class II- cells), followed by

additional CCR2-independent routes (as Ly-6C-MHC class II- cells);i.e., the only route in KO mice;with a delay of 1-7 days. The SPMs derived from both routes appeared to differentiate into maturated cells as Ly-6C-MHC class II+, whose ratio increased in a time-dependent manner among the total SPM population. Additionally, most macrophages expressed M1-like features, but a small fraction of macrophages exhibited an M1/M2 mixed status in MWCNT-treated animals. Our findings demonstrate a long-persistent activation of the CCL2-CCR2 axis after MWCNT exposure and enable a better understanding of the participation and potential roles of SPMs in fibrous material-induced chronic toxicities.

Keywords: Ccr2-knockout mice, MWCNT, mesothelioma

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Tominaga S*1, Yoshioka H*2, Yokota S, Tsukiboshi Y*2, Suzui M*3, Nagai M*1, Hara H*4, Maeda T*1, Miura N*5:Copper-induced diurnal hepatic toxicity is associated with Cry2 and Per1 in mice.

Environ Health Prev Med. 2023;28:78-86. doi:10.1265/ehpm.23-00205

Background:This study aimed to investigate diurnal variations in copper-induced hepatic toxicity and the molecular mechanisms underlying this chronotoxicity.

Methods:Male C57BL/6J mice were intraperitoneally injected with copper chloride (CuCl2) at zeitgeber time 2 (ZT2) or 14 (ZT14), twice per week for 5 or 8 weeks. Seventy-two hours after the final CuCl2 injection, the mice were euthanized, and plasma samples were collected. The livers and kidneys were collected and weighed. *In vitro* experiments were performed to assess cell viability and fluctuations in clock gene expression levels in Hepa1-6 cells after CuCl2 treatment. We examined copper homeostasis- and apoptosis-related genes under clock genes overexpression.

Results:Repeated CuCl2 administration for 8 weeks resulted in more severe toxicity at ZT14 compared to ZT2. CuCl2 administration at ZT14 elevated plasma aspartate aminotransferase, hepatic tumor necrosis factor-α, and interleukin-6 for 5 weeks, whereas the toxic effects of CuCl2 administration at ZT2 were weaker. Moreover, CuCl2 treatment inhibited Hepa1-6 cell viability in a dose-dependent manner. We observed increased expression of three clock genes (Ciart, Cry2, and Per1) after CuCl2 treatment. Among them, overexpression of Cry2 and Per1 accelerated CuCl2-induced inhibition of Hepa1-6 cell viability. Moreover, we found that the overexpression of Cry2 and Per1 regulates cleaved caspase-3 by modulating the copper transporter genes ATP7B and CTR1.

Conclusion:These results suggest that CuCl2-induced diurnal toxicity is associated with Cry2 and Per1 expression through the regulation of copper transporter genes in mice.

Keywords: chronotoxicity, copper, hepatotoxicity

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Takahashi Y, Wakabayashi R*, Kitajima S, Uchiyama H*:Epichordal vertebral column formation in Xenopus laevis.

Journal of Morphology 2023;285(2):e21664. doi:10.1002/jmor.21664

Although *Xenopus* laevis is the most widely used model amphibian, skeletal development of its vertebral column has not been well illustrated so far. Mode of vertebral column development in anurans has been classified into two modes:perichordal and epichordal. *Xenopus* vertebral column formation is believed to follow the epichordal mode, but this aspect has been underemphasized, and illustrative examples are currently unavailable to the scientific community. This study presents color photographs illustrating the entire process of vertebral column formation in *X*. laevis, from the initial neural arch formation to the completion of metamorphosis. These images reveal that the neural arch arises from the dorsal lamina and lateral pedicle primordia, with no strict adherence to

an anteroposterior sequence. Unlike other species, *Xenopus* centrum primordia exclusively form at the expanded ventral margins of neural arches, rather than from the cartilaginous layer surrounding the notochord. These paired centrum primordia then fuse at the ventral midline, dorsal to the notochord, and subsequently the notochord degenerates. This mode of centrum formation differs from the traditional epichordal mode, indicating that *Xenopus* might have lost the ability to form a cartilaginous layer around the notochord. Instead, the neural arch's ventral margin appears to have evolved to incorporate centrum precursor cells at its base, thereby forming a centrum-like structure compensating for the absence of a true centrum. It is widely accepted that postsacral vertebrae lack centra, only possessing neural arches, and eventually fuse with the hypochord to form the urostyle. However, we have shown that the paired ventral ends of the postsacral vertebrae also fuse at the midline to form a centrum-like structure. This process might extend to the trunk region during centrum formation. In addition to these findings, we offer evolutionary insights into the reasons why *Xenopus* retains centrum primordia at the base of neural arches.

Keywords: vertebral column, centrum, *Xenopus*

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Tsukiboshi Y*1, Noguchi A*1, Horita H*1, Mikami Y*2, Yokota S, Ogata K*2, Yoshioka H*1:Let-7c-5p associate with inhibition of phenobarbital-induced cell proliferation in human palate cells.

Biochem Biophys Res Commun. 2024;696:149516. doi:10.1016/j.bbrc.2024.149516

Cleft palate (CP) is one of the most common congenital diseases, and is accompanied by a complicated etiology. Medical exposure in women is among one of the reasons leading to CP. Recently, it has been reported that microRNA (miRNA) plays a crucial role in palate formation and the disruption of miRNA that influence the development of CP. Although association with pharmaceuticals and miRNAs were suggested, it has remained largely unknow. The aim of the current investigation is to elucidate upon the miRNA associated with the inhibition of phenobarbital (PB)-induced cell proliferation in human embryonic palatal mesenchymal (HEPM) cells. We showed that PB inhibited HEPM cell viability in a dose-dependent manner. We demonstrated that PB treatment suppressed cyclin-D1 expression in HEPM cells. Furthermore, PB upregulated let-7c-5p expression and downregulated the expression of two downstream genes (BACH1 and PAX3). Finally, we demonstrated that the let-7c-5p inhibitor alleviated PB-induced inhibition of cell proliferation and altered BACH1 and PAX3 expression levels. These results suggest that PB suppresses cell viability by modulating let-7c-5p expression.

Keywords: cleft palate, phenobarbital, miRNA

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Tsukiboshi Y*1, Horita H*1, Mikami Y*1, Yokota S, Ogata K*2, Yoshioka H*1:Involvement of *microRNA-4680-3p* against phenytoin-induced cell proliferation inhibition in human palate cells.

J Toxicol Sci. 2024;49(1):1-8. doi:10.2131/jts.49.1

Cleft palate (CP) is one of the most common birth defects and is caused by a combination of genetic and/or environmental factors. Environmental factors such as pharmaceutical exposure in pregnant women are known to induce CP. Recently, microRNA (miRNA) was found to be affected by environmental factors. The aim of the present study was to investigate the involvement of miRNA against phenytoin (PHE)-induced inhibition of proliferation in human embryonic palatal mesenchymal (HEPM) cells. We demonstrated that PHE inhibited HEPM cell proliferation in a dose-dependent manner. We found that treatment with PHE downregulated cyclin-D1 and cyclin-E expressions in HEPM cells. Furthermore, PHE increased *miR-4680-3p* expression and decreased two downstream genes (*ERBB2* and *JADE1*). Importantly, an *miR-4680-3p*-specific inhibitor restored HEPM cell proliferation and altered expression of *ERBB2* and *JADE1* in cells treated with PHE. These results suggest that PHE suppresses cell proliferation via modulation of *miR-4680-3p* expression.

Keywords: cleft palate, environmental factors,

phenytoin

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Hashimoto K*1,2, Arakawa H*3, Imamura R*3, Nishimura T, Kitajima S, Sato T*2, Makiyama K*1, Ogawa T*2, Yokota S:A novel alternative method for long-term evaluation of male reproductive toxicity and its recovery using a pre-pubertal mouse testis organ culture system.

J Appl Toxicol. 2024;44:784-793. doi:10.1002/jat.4584

Successful treatment of pediatric cancers often results in long-term health complications, including potential effects on fertility. Therefore, assessing the male reproductive toxicity of anti-cancer drug treatments and the potential for recovery is of paramount importance. However, *in vivo* evaluations are time-intensive and require large numbers of animals. To overcome these constraints, we utilized an innovative organ culture system that supports long-term spermatogenesis by placing the testis tissue between a base agarose gel and a polydimethylsiloxane ceiling, effectively mirroring the *in vivo* testicular environment. The present study aimed to determine the efficacy of this organ culture system for accurately assessing testicular toxicity induced by cisplatin, using acrosin-green fluorescent protein (GFP) transgenic neonatal mouse testes. The testis fragments were treated with different concentrations of cisplatin-containing medium for 24 h and incubated in fresh medium for up to 70 days. The changes in tissue volume and GFP fluorescence over time were evaluated to monitor the progression of spermatogenesis, in addition to the corresponding histopathology. Cisplatin treatment caused tissue volume shrinkage and reduced GFP fluorescence in a concentration-dependent manner. Recovery from testicular toxicity was also dependent on the concentration of cisplatin received. The results demonstrated that this novel *in vitro* system can be a faithful replacement for animal experiments to assess the testicular toxicity of anti-cancer drugs and their reversibility, providing a useful method for drug development.

Keywords: alternative method, testis organ culture, recovery from spermatogenic impairments

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Hase T*, Ghosh S*, Aisaki KI, Kitajima S, Kanno J, Kitano H*, Yachie A*:DTox:A deep neural network-based in visio lens for large scale toxicogenomics data.

J Toxicol Sci. 2024;49(3):105-115. doi:10.2131/jts.49.105

With the advancement of large-scale omics technologies, particularly transcriptomics data sets on drug and treatment response repositories available in public domain, toxicogenomics has emerged as a key field in safety pharmacology and chemical risk assessment. Traditional statistics-based bioinformatics analysis poses challenges in its application across multidimensional toxicogenomic data, including administration time, dosage, and gene expression levels. Motivated by the visual inspection workflow of field experts to augment their efficiency of screening significant genes to derive meaningful insights, together with the ability of deep neural architectures to learn the image signals, we developed DTox, a deep neural network-based in visio approach. Using the Percellome toxicogenomics database, instead of utilizing the numerical gene expression values of the transcripts (gene probes of the microarray) for dose-time combinations, DTox learned the image representation of 3D surface plots of distinct time and dosage data points to train the classifier on the experts' labels of gene probe significance. DTox outperformed statistical threshold-based bioinformatics and machine learning approaches based on numerical expression values. This result shows the ability of image-driven neural networks to overcome the limitations of classical numeric value-based approaches. Further, by augmenting the model with explainability modules, our study showed the potential to reveal the visual analysis process of human experts in toxicogenomics through the model weights. While the current work demonstrates the application of the

DTox model in toxicogenomic studies, it can be further generalized as an in visio approach for multi-dimensional numeric data with applications in various fields in medical data sciences.

Keywords: artificial intelligence, toxicogenomics data analysis, Percellome

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Ono R, Kuwagata M, Naruse M[*1], Watanabe A[*2], Takano M[*2], Hasegawa T[*2], Takashima H[*2], Yoshioka Y[*3], Ochiya T[*3], Hirabayashi Y, Kitajima S:Extracellular vesicle small RNAs secreted from mouse amniotic fluid induced by repeated oral administration of VPA to pregnant mice.
Fundam Toxicol Sci. 2024;11(1):37-56.

Extracellular vesicles (EVs) are particles released not only from blood cells but also from various organs. EVs, which are lipid bilayer vesicles, contain proteins, DNAs, and RNAs. The RNA and proteins within EVs display cell-specific characteristics. EVs derived from tumor cells are identified as biomarkers with diagnostic accuracy exceeding 90% for early cancer detection. Furthermore, EV RNA in serum has serves as a biomarker for toxicity. EVs have been found in various body fluids, including saliva, tears, urine, and amniotic fluid. In this study, we aimed to investigate the potential use of EV RNA in amniotic fluid as an indicator of developmental toxicity. Pregnant mice were exposed to valproic acid (VPA), a developmental toxicant, at concentrations of 0, 300, or 600 mg/kg/day on gestational days (GDs) 9–11. The study involved measuring VPA concentration in maternal plasma and fetuses on GD11, fetal weight on GD15 and 18, and assessing external morphological abnormalities on GDs11, 15 and 18. Additionally, EVs were collected from fetal amniotic fluid, and a comprehensive gene expression analysis of EV RNA was conducted on GD15. As a result, the concentration of VPA in the fetuses was not associated with the implantation location. Additionally, the VPA-treated group exhibited intrauterine growth retardation and teratogenic effects, including neural tube defects and digit malformations. EV RNA analysis identified differentially expressed EV small RNAs, both suppressed and induced, in the VPA-treated group compared with the control (vehicle, 0.5% Methylcellulose) group. These findings suggest that EV RNA in amniotic fluid serve as an indicator of developmental toxicity.

Keywords: extracellular vesicle (EV), exosome, teratogenicity

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Tsukiboshi Y[*1], Mikami Y[*1], Horita H[*1], Ogata A[*1], Noguchi A[*2], Yokota S, Ogata K[*3], Yoshioka H[*1]:Protective effect of *Sasa veitchii* extract against *all-trans*-retinoic acid-induced inhibition of proliferation of cultured human palate cells.
Nagoya J Med Sci. 2024;86:223–236. doi:10.18999/nagjms.86.2.223

Cleft palate is the most common facial birth defect worldwide. It is caused by environmental factors or genetic mutations. Environmental factors such as pharmaceutical exposure in women are known to induce cleft palate. The aim of the present study was to investigate the protective effect of *Sasa veitchii* extract against medicine-induced inhibition of proliferation of human embryonic palatal mesenchymal cells. We demonstrated that *all-trans*-retinoic acid inhibited human embryonic palatal mesenchymal cell proliferation in a dose-dependent manner, whereas dexamethasone treatment had no effect on cell proliferation. Cotreatment with *Sasa veitchii* extract repressed *all-trans*-retinoic acid-induced toxicity in human embryonic palatal mesenchymal cells. We found that cotreatment with *Sasa veitchii* extract protected *all-trans*-retinoic acid-induced cyclin D1 downregulation in human embryonic palatal mesenchymal cells. Furthermore, *Sasa veitchii* extract suppressed *all-trans*-retinoic acid-induced *miR-4680-3p* expression. Additionally, the expression levels of the genes that function downstream of the target genes (*ERBB2* and *JADE1*) of *miR-4680-3p* in signaling pathways were enhanced by cotreatment with *Sasa veitchii* extract and *all-trans*-retinoic acid compared to *all-trans*-retinoic acid treatment. These results suggest that *Sasa veitchii* extract suppresses *all-trans*-retinoic acid-induced inhibition of cell proliferation via modulation of *miR-4680-3p* expression.

Keywords: cleft palate, *all-trans*-retinoic acid, *miR-4680-3p*

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Mizoi K*1*2, Okada R*3, Mashimo A*1*4, Masuda N*5, Itoh M*3, Ishida S*6, Yamazaki D, Ogihara T*1. Novel Screening System for Biliary Excretion of Drugs Using Human Cholangiocyte Organoid Monolayers with Directional Drug Transport.
Biol Pharm Bull. 2024;47(2):427-433. doi: 10.1248/bpb.b23-00655

It has recently been reported that cholangiocyte organoids can be established from primary human hepatocytes. The purpose of this study was to culture the organoids in monolayers on inserts to investigate the biliary excretory capacity of drugs. Cholangiocyte organoids prepared from hepatocytes had significantly higher mRNA expression of CK19, a bile duct epithelial marker, compared to hepatocytes. The organoids also expressed mRNA for efflux transporters involved in biliary excretion of drugs, P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP). The subcellular localization of each protein was observed. These results suggest that the membrane-cultured cholangiocyte organoids are oriented with the upper side being the apical membrane side (A side, bile duct lumen side) and the lower side being the basolateral membrane side (B side, hepatocyte side), and that each efflux transporter is localized to the apical membrane side. Transport studies showed that the permeation rate from the B side to the A side was faster than from the A side to the B side for the substrates of each efflux transporter, but this directionality disappeared in the presence of inhibitor of each transporter. In conclusion, the cholangiocyte organoid monolayer system has the potential to quantitatively evaluate the biliary excretion of drugs. The results of the present study represent an unprecedented system using human cholangiocyte organoids, which may be useful as a screening model to directly quantify the contribution of biliary excretion to the clearance of drugs.

Keywords: P-glycoprotein, biliary excretion, cholangiocyte organoid

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In Vitro Cell Dev Biol Anim. 2024 Mar 12. doi: 10.1007/s11626-024-00863-w

Human pluripotent stem cells, such as human embryonic stem cells and human induced pluripotent stem cells, are used in basic research and various applied fields, including drug discovery and regenerative medicine. Stem cell technologies have developed rapidly in recent years, and the supply of culture materials has improved. This has facilitated the culture of human pluripotent stem cells and has enabled an increasing number of researchers and bioengineers to access this technology. At the same time, it is a challenge to share the basic concepts and techniques of this technology among researchers and technicians to ensure the reproducibility of research results. Human pluripotent stem cells differ from conventional somatic cells in many aspects, and many points need to be considered in their handling, even for those experienced in cell culture. Therefore, we have prepared this proposal, "Points of Consideration for Pluripotent Stem Cell Culture," to promote the effective use of human pluripotent stem cells. This proposal includes seven items to be considered and practices to be confirmed before using human pluripotent stem cells. These are laws/guidelines and consent/material transfer agreements, diversity of pluripotent stem cells, culture materials, thawing procedure, media exchange and cell passaging, freezing procedure, and culture management. We aim for the concept of these points of consideration to be

shared by researchers and technicians involved in the cell culture of pluripotent stem cells. In this way, we hope the reliability of research using pluripotent stem cells can be improved, and cell culture technology will advance.

Keywords: diversity, Good Cell Culture Practice, pluripotent stem cell

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Mitsuboshi S*1, Hamano H*2, Niimura T*3, Ozaki AF*4, Patel PM*4, Lin TJ*5, Tanaka Y*2, Kimura I*2, Iwata N*2, Shiromizu S*2, Chuma M*6, Koyama T*7, Yamanishi Y*8, Kanda Y, Ishizawa K*3,9, Zamami Y*2. Association between immune checkpoint inhibitor-induced myocarditis and concomitant use of thiazide diuretics.

Int J Cancer. 2023 Oct 15;153(8):1472-1476. doi: 10.1002/ijc.34616

Although an association has been reported between diuretics and myocarditis, it is unclear whether the risk of immune checkpoint inhibitor (ICI)-induced myocarditis is affected by concomitant diuretics. Thus, the aim of this work was to evaluate the impact of concomitant diuretics on ICI-induced myocarditis. This cross-sectional study used disproportionality analysis and a pharmacovigilance database to assess the risk of myocarditis with various diuretics in patients receiving ICIs via the analysis of data entered into the VigiBase database through December 2022. Multiple logistic regression analysis was performed to identify risk factors for myocarditis in patients who received ICIs. A total of 90 611 patients who received ICIs, including 975 cases of myocarditis, were included as the eligible dataset. A disproportionality in myocarditis was observed for loop diuretic use (reporting odds ratio 1.47, 95% confidence interval [CI] 1.02-2.04, P = .03) and thiazide use (reporting odds ratio 1.76, 95% CI 1.20-2.50, P < .01) in patients who received ICIs. The results of the multiple logistic regression analysis showed that the use of thiazides (odds ratio 1.67, 95% CI 1.15-2.34, P < .01) was associated with an increased risk of myocarditis in patients who received ICIs. Our findings may help to predict the risk of myocarditis in patients receiving ICIs.

Keywords: disproportionality analysis, immune checkpoint inhibitor, myocarditis

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Ogawa A*1, Ohira S*1,2, Kato Y*3, Ikuta T*4, Yanagida S, Mi X*3, Ishii Y*3, Kanda Y, Nishida M*3,5, Inoue A*4, Wei FY*1. Activation of the urotensin-II receptor by remdesivir induces cardiomyocyte dysfunction.

Commun Biol. 2023 May 12;6(1):511. doi: 10.1038/s42003-023-04888-x

Remdesivir is an antiviral drug used for COVID-19 treatment worldwide. Cardiovascular side effects have been associated with remdesivir; however, the underlying molecular mechanism remains unknown. Here, we performed a large-scale G-protein-coupled receptor screening in combination with structural modeling and found that remdesivir is a selective, partial agonist for urotensin-II receptor (UTS2R) through the Gαi/o-dependent AKT/ERK axis.

Functionally, remdesivir treatment induced prolonged field potential and APD90 in human induced pluripotent stem cell (iPS)-derived cardiomyocytes and impaired contractility in both neonatal and adult cardiomyocytes, all of which mirror the clinical pathology. Importantly, remdesivir-mediated cardiac malfunctions were effectively attenuated by antagonizing UTS2R signaling. Finally, we characterized the effect of 110 single-nucleotide variants in UTS2R gene reported in genome database and found four missense variants that show gain-of-function effects in the receptor sensitivity to remdesivir. Collectively, our study illuminates a previously unknown mechanism underlying remdesivir-related cardiovascular events and that genetic variations of UTS2R gene can be a potential risk factor for cardiovascular events during remdesivir treatment, which collectively paves the way for a therapeutic opportunity to prevent such events in the future.

Keywords: remdesivir, urotensin-II receptor, iPS-derived cardiomyocytes

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Nishimura A*1, Zhou L*1, Kato Y*2, Mi X*2, Ito T*1, Ibuki Y*3, Kanda Y, Nishida M*1. Supersulfide prevents cigarette smoke extract-induced mitochondria hyperfission and cardiomyocyte early senescence by inhibiting Drp1-filamin complex formation.

J Pharmacol Sci. 2024 Feb;154(2):127-135. doi: 10.1016/j.jphs.2023.12.008

Smoking is one of the most serious risk factors for cardiovascular diseases. Although cigarette mainstream and sidestream smoke are significant contributors to increased cardiovascular mortality and morbidity, the underlying mechanism is still unclear. Here, we report that exposure of rat neonatal cardiomyocytes to cigarette smoke extract (CSE) induces mitochondrial hyperfission-mediated myocardial senescence. CSE leads to mitochondrial fission and reactive oxygen species (ROS) production through the complex formation between mitochondrial fission factor Drp1 and actin-binding protein, filamin A. Pharmacological perturbation of interaction between Drp1 and filamin A by cilnidipine and gene knockdown of Drp1 or filamin A inhibited CSE-induced mitochondrial hyperfission and ROS production as well as myocardial senescence. We previously reported that Drp1 activity is controlled by supersulfide-induced Cys644 polysulfidation. The redox-sensitive Cys644 was critical for CSE-mediated interaction with filamin A. The administration of supersulfide donor, Na2S3 also improved mitochondrial hyperfission-mediated myocardial senescence induced by CSE. Our results suggest the important role of Drp1-filamin A complex formation on cigarette smoke-mediated cardiac risk and the contribution of supersulfide to mitochondrial fission-associated myocardial senescence.

Keywords: cardiomyocytes, myocardial senescence, supersulfides

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Drug Saf. 2023 Jun;46(6):545-552. doi: 10.1007/s40264-023-01300-9

Introduction: Recently, cases of cardiovascular toxicities, such as pericarditis, caused by anaplastic lymphoma kinase (ALK) inhibitors have been reported; however, whether these adverse events are common among all ALK inhibitors remains unclear.

Aims: This study aimed to clarify the cardiovascular toxicity profile of ALK inhibitors using an adverse event spontaneous report database.

Methods: We analyzed data from VigiBase, the WHO global database of individual safety reports, from its inception in 1968 to December 2021. We calculated the reporting odds ratio to evaluate the association between ALK inhibitors (crizotinib, ceritinib, alectinib, brigatinib, and lorlatinib) and 21 cardiovascular adverse events. Time to onset of pericarditis from ALK inhibitor administration was analyzed.

Results: Of the 27,994,584 reports, 19,911 involved treatment with ALK inhibitors. Among the 21 cardiovascular toxicities, only pericarditis signals were detected with all five ALK inhibitors (crizotinib [reporting odds ratios (ROR), 4.7; 95% CI 3.63-6.15], ceritinib [ROR, 12.9; 95% CI 9.37-17.79], alectinib [ROR, 4.8; 95% CI 3.15-7.42], brigatinib [ROR, 3.5; 95% CI 1.33-9.46], and lorlatinib [ROR, 6.4; 95% CI 3.60-11.22]). For torsade de pointes/QT prolongation, signals were detected with crizotinib (ROR, 5.0; 95% CI 3.72-6.77) and ceritinib (ROR, 4.2; 95% CI 2.17-8.05), whereas for hypertension, they were identified only with brigatinib (ROR, 3.9; 95% CI 2.88-5.20), and for heart failure, they were detected with alectinib (ROR, 2.2; 95% CI 1.60-2.90), crizotinib (ROR, 2.1; 95% CI 1.72-2.48), and lorlatinib (ROR, 2.0; 95% CI 1.27-3.23). Regarding time-to-onset analysis from drug administration to adverse event reporting, for pericarditis, it ranged from 52.5 days for alectinib to 166.5 days for crizotinib.

Conclusions: Systematic evaluation of ALK inhibitor-associated adverse events revealed differences in the cardiotoxicity profiles among ALK inhibitors. Understanding the differences in the cardiovascular toxicity profile of each ALK inhibitor will contribute to safe drug therapy when switching between ALK inhibitors.

Keywords: cardiovascular toxicities, ALK inhibitor, WHO Pharmacovigilance Database

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Tang X*1, 2, 3, Nishimura A*1, 2, 3, Ariyoshi K*4, Nishiyama K*4, Kato Y*4, Vasileva EA*5, Mishchenko NP*5, Fedoreyev SA*5, Stonik VA*5, Kim HK*6, Han J*6, Kanda Y, Umezawa K*7, Urano Y*8, 9, Akaike T*10, Nishida M*1, 2, 3, 4. Echinochrome Prevents Sulfide Catabolism-Associated Chronic Heart Failure after Myocardial Infarction in Mice. *Mar Drugs*. 2023, 21(1), 52. doi: 10.3390/md21010052

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

Keywords: cardiac remodeling, echinochrome, hydrogen sulfide

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Takahashi K, Ishibashi Y*, Chujo K, Suzuki I*, Sato K. Neuroprotective Potential of L-Glutamate Transporters in Human Induced Pluripotent Stem Cell-Derived Neural Cells against Excitotoxicity. doi: 10.3390/ijms241612605

Int J Mol Sci. 2023 Aug 9;24(16):12605.

Human induced pluripotent stem cell (hiPSC)-derived neural cells have started to be used in safety/toxicity tests at the preclinical stage of drug development. As previously reported, hiPSC-derived neurons exhibit greater tolerance to excitotoxicity than those of primary cultures of rodent neurons; however, the underlying mechanisms remain unknown. We here investigated the functions of L-glutamate (L-Glu) transporters, the most important machinery to maintain low extracellular L-Glu concentrations, in hiPSC-derived neural cells. We also clarified the contribution of respective L-Glu transporter subtypes. At 63 days *in vitro* (DIV), we detected neuronal circuit functions in hiPSC-derived neural cells by a microelectrode array system (MEA). At 63 DIV, exposure to 100μM L-Glu for 24 h did not affect the viability of neural cells. 100μM L-Glu in the medium decreased to almost 0 μM in 60 min. Pharmacological inhibition of excitatory amino acid transporter 1 (EAAT1) and EAAT2 suppressed almost 100% of L-Glu decrease. In the presence of this inhibitor, 100 μM L-Glu dramatically decreased cell viability. These results suggest that in hiPSC-derived neural cells, EAAT1 and EAAT2 are the predominant L-Glu transporters, and their uptake potentials are the reasons for the tolerance of hiPSC-derived neurons to excitotoxicity.

Keywords: excitotoxicity, human induced pluripotent stem cell, L-glutamate transporter

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Mizoi K*1,2, Okada R*3, Mashimo A*1,4, Masuda N*5, Itoh M*3, Ishida S*6, Yamazaki D, Ogihara T*1,7. Novel Screening System for Biliary Excretion of Drugs Using Human Cholangiocyte Organoid Monolayers with Directional Drug Transport.

Biol Pharm Bull. 2024;47:427-433 doi: 10.1248/bpb.b23-00655

It has recently been reported that cholangiocyte organoids can be established from primary human hepatocytes. The purpose of this study was to culture the organoids in monolayers on inserts to investigate the biliary excretory capacity of drugs. Cholangiocyte organoids prepared from hepatocytes had significantly higher mRNA expression of CK19, a bile duct epithelial marker, compared to hepatocytes. The organoids also expressed mRNA for efflux transporters involved in biliary excretion of drugs, P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP). The subcellular localization of each protein was observed. These results suggest that the membrane-cultured cholangiocyte organoids are oriented with the upper side being the apical membrane side (A side, bile duct lumen side) and the lower side being the basolateral membrane side (B side, hepatocyte side), and that each efflux transporter is localized to the apical membrane side. Transport studies showed that the permeation rate from the B side to the A side was faster than from the A side to the B side for the substrates of each efflux transporter, but this directionality disappeared in the presence of inhibitor of each transporter. In conclusion, the cholangiocyte organoid monolayer system has the potential to quantitatively evaluate the biliary excretion of drugs. The results of the present study represent an unprecedented system using human cholangiocyte

organoids, which may be useful as a screening model to directly quantify the contribution of biliary excretion to the clearance of drugs.

Keywords: biliary excretion, cholangiocyte organoid, directional drug transport

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Science Translational Medicine. 2023;15: eadi2623. doi: 10.1126/scitranslmed.adi2623

The Omicron variant continuously evolves under the humoral immune pressure exerted by vaccination and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, and the resulting Omicron subvariants display further immune evasion and antibody escape. An engineered angiotensin-converting enzyme 2 (ACE2) decoy composed of high-affinity ACE2 and an IgG1 Fc domain could offer an alternative modality to neutralize SARS-CoV-2. We previously reported its broad spectrum and therapeutic potential in rodent models. Here, we demonstrate that the engineered ACE2 decoy retains neutralization activity against Omicron subvariants, including the currently emerging XBB and BQ.1 strains, which completely evade antibodies currently in clinical use. SARS-CoV-2, under the suboptimal concentration of neutralizing drugs, generated SARS-CoV-2 mutants escaping wild-type ACE2 decoy and monoclonal antibodies, whereas no escape mutant emerged against the engineered ACE2 decoy. Furthermore, inhalation of aerosolized decoys improved the outcomes of rodents infected with SARS-CoV-2 at a 20-fold lower dose than that of intravenous administration. Last, the engineered ACE2 decoy exhibited therapeutic efficacy for cynomolgus macaques infected with SARS-CoV-2. These results indicate that this engineered ACE2 decoy represents a promising therapeutic strategy to overcome immune-evading SARS-CoV-2 variants and that liquid aerosol inhalation could be considered as a noninvasive approach to enhance the efficacy of COVID-19 treatments.

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Circulation. 2024 Apr 26. doi:10.1161/CIRCULATIONAHA.123.064876

Background: Clinical application of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) for cardiac repair commenced with the epicardial delivery of engineered cardiac tissue;

however, the feasibility of the direct delivery of hiPSC-CMs into the cardiac muscle layer, which has reportedly induced electrical integration, is unclear because of concerns regarding poor engraftment of cardiomyocytes (CMs) and post-transplant arrhythmias. Thus, in this study, we prepared purified hiPSC-derived cardiac spheroids (hiPSC-CSs) and confirmed whether their direct injection could regenerate infarcted non-human primate hearts.

Methods: We performed two separate experiments to explore the appropriate number of hiPSC-CMs. In the first experiment, 10 cynomolgus monkeys were subjected to myocardial infarction 2 weeks before transplantation and were designated as recipients of hiPSC-CSs containing 2×10^7CMs or the vehicle. The animals were euthanized 12 weeks after transplantation for histological analysis, and cardiac function and arrhythmia were monitored during the observational period. In the second study, we repeated the equivalent transplantation study using more CMs (6×10^7CMs).

Results: Recipients of hiPSC-CSs containing 2 × 107CMs showed limited CM grafts and transient increases in fractional shortening (FS) compared with those of the vehicle (FS at 4 weeks after transplantation: 26.2 ± 2.1%; 19.3 ± 1.8%, $p < 0.05$), with a low incidence of post-transplant arrhythmia. Transplantation of increased dose of CMs resulted in significantly greater engraftment and long-term contractile benefits (FS at 12 weeks after transplantation: 22.5 ± 1.0%; 16.6 ± 1.1%, $p < 0.01$, left ventricular ejection fraction at 12 weeks after transplantation: 49.0 ± 1.4%; 36.3 ± 2.9%, $p < 0.01$). The incidence of post-transplant arrhythmia slightly increased in recipients of hiPSC-CSs containing 6×10^7CMs.

Conclusions: We demonstrated that direct injection of hiPSC-CSs restores the contractile functions of injured primate hearts with an acceptable risk of post-transplant arrhythmia. Although the mechanism for the functional benefits is not fully elucidated, these findings provide a strong rationale for conducting clinical trials using the equivalent CM products.

Keywords : cardiac spheroid, heart regeneration, human iPS cell

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Fluids Barriers CNS. 2024 Apr 8;21(1):32. doi: 10.1186/s12987-024-00533-9.

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19), which is associated with various neurological symptoms, including nausea, dizziness, headache, encephalitis, and epileptic seizures. SARS-CoV-2 is considered to affect the central nervous system (CNS) by interacting with the blood–brain barrier (BBB), which is defined by tight junctions that seal paracellular gaps between brain microvascular endothelial cells (BMECs). Although SARS-CoV-2 infection of BMECs has been reported, the detailed mechanism has not been fully elucidated.

Methods: Using the original strain of SARS-CoV-2, the infection in BMECs was confirmed by a detection of intracellular RNA copy number and localization of viral particles. BMEC functions were evaluated by measuring transendothelial electrical resistance (TEER), which evaluates the integrity of tight junction dynamics, and expression levels of proinflammatory genes. BMEC signaling pathway was examined by comprehensive RNA-seq analysis.

Results: We observed that iPSC derived brain microvascular endothelial like cells (iPSC-BMELCs) were infected with SARS-CoV-2. SARS-CoV-2 infection resulted in decreased TEER. In addition, SARS-CoV-2 infection decreased expression levels of tight junction markers CLDN3 and CLDN11. SARS-CoV-2 infection also increased expression levels of proinflammatory genes, which are known to be elevated in patients with COVID-19. Furthermore, RNA-seq analysis revealed that SARS-CoV-2 dysregulated the canonical Wnt signaling pathway in iPSC-BMELCs. Modulation of the Wnt signaling by CHIR99021 partially inhibited

the infection and the subsequent inflammatory responses.

Conclusion: These findings suggest that SARS-CoV-2 infection causes BBB dysfunction via Wnt signaling. Thus, iPSC-BMELCs are a useful *in vitro* model for elucidating COVID-19 neuropathology and drug development.

Keywords: SARS-CoV-2, BBB, inflammation

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Mitsumoto T, Ishii Y, Takimoto N, Takasu S, Namiki M, Nohmi T, Umemura T*, Ogawa K: Site-specific genotoxicity of rubiadin: localization and histopathological changes in the kidneys of rats.
Arch Toxicol. 2023;97:3273-83. doi: 10.1007/s00204-023-03610-4.

Rubiadin (Rub) is a genotoxic component of madder color (MC) that is extracted from the root of Rubia tinctorum L. MC induces renal tumors and preneoplastic lesions that are found in the proximal tubule of the outer stripe of the outer medulla (OSOM), suggesting that the renal carcinogenicity of MC is site specific. To clarify the involvement of Rub in renal carcinogenesis of MC, we examined the distribution of Rub in the kidney of male *gpt* delta rats that were treated with Rub for 28 days. We used desorption electrospray ionization quadrupole time-of-flight mass spectrometry imaging (DESI-Q-TOF-MSI), along with the histopathological analysis, immunohistochemical staining, and reporter gene mutation assays of the kidney. DESI-Q-TOF-MSI revealed that Rub and its metabolites, lucidin and Rub-sulfation, were specifically distributed in the OSOM. Histopathologically, karyomegaly characterized by enlarged nuclear and microvesicular vacuolar degeneration occurred in proximal tubule epithelial cells in the OSOM. The γ-H2AX- and p21-positive cells were also found in the OSOM rather than the cortex. Although dose-dependent increases in *gpt* and Spi- mutant frequencies were observed in both the medulla and cortex, the mutant frequencies in the medulla were significantly higher. The mutation spectra of *gpt* mutants showed that A:T-T:A transversion was predominant in Rub-induced gene mutations, consistent with those of MC. Overall, the data showed that the distribution of Rub and its metabolites resulted in site-specific histopathological changes, DNA damage, and gene mutations, suggesting that the distribution of genotoxic components and metabolites is responsible for the site-specific renal carcinogenesis of MC.

Keywords: DESI-MSI, madder color, rubiadin

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Toyoda T, Kobayashi T*, Miyoshi N*, Matsushita K, Akane H, Morikawa T, Ogawa K: Mucosal damage and γ-H2AX formation in the rat urinary bladder induced by aromatic amines with structures similar to *o*-toluidine and *o*-anisidine.
Arch Toxicol. 2023;97:3197-207. doi: 10.1007/s00204-023-03606-0.

Although aromatic amines are widely used as raw materials for dyes, some, such as *o*-toluidine and *o*-anisidine, have shown concerning results regarding carcinogenicity in the urinary bladder. We have recently developed a short-term detection method for bladder carcinogens using immunohistochemistry for γ-H2AX, a DNA damage marker. Here, using this method, we evaluated aromatic amines with structures similar to *o*-toluidine and *o*-anisidine for bladder mucosal damage and potential carcinogenicity. In total, 17 aromatic amines were orally administered to male F344 rats for 28 days, and histopathological examination and γ-H2AX immunostaining of the urinary bladder were performed. Histopathological analysis revealed that seven aromatic amines, including 4-chloro-*o*-toluidine (4-CT), *o*-aminoazotoluene, 2-aminobenzyl alcohol (ABA), *o*-acetotoluidine (*o*-AT), 3,3'-dimethoxybenzidine, 4-aminoazobenzene (AAB), and 4,4'-methylenedianiline (MDA), induced various bladder lesions, such as hemorrhage, necrosis, and urothelial hyperplasia. The morphological characteristics of mucosal damage induced by these substances were divided into two major types: those resembling *o*-toluidine and those resembling *o*-anisidine. Six of these aromatic amines, excluding MDA, also caused significant increases in γ-H2AX formation in the bladder urothelium. Interestingly, 4-CT did not cause mucosal damage or γ-H2AX

formation at the lower dose applied in previous carcinogenicity studies. These results showed for the first time that *o*-AT and ABA, metabolites of *o*-toluidine, as well as AAB caused damage to the bladder mucosa and suggested that they may be bladder carcinogens. In addition, 4-CT, which was thought to be a noncarcinogen, was found to exhibit bladder toxicity upon exposure to high doses, indicating that this compound may contribute to bladder carcinogenesis.

Keywords: aromatic amine, γ-H2AX, urinary bladder

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Akagi J, Cho YM, Toyoda T, Mizuta Y, Ogawa K: EpCAM and APN expression in combination with γ-H2AX as biomarkers for detecting hepatocarcinogens in rats.

Cancer Sci. 2023;114:4763-9. doi: 10.1111/cas.15990.

The phosphorylated form of histone H2AX (γ-H2AX) serves as a commonly utilized biomarker for DNA damage. Based on our previous findings, which demonstrated the formation of γ-H2AX foci as a reliable biomarker for detecting bladder carcinogens in repeated dose 28-day study in rats, we hypothesized that γ-H2AX could also function as a biomarker for detecting hepatocarcinogens. However, we found that γ-H2AX foci formation was not effectively induced by hepatocarcinogens that did not stimulate hepatocyte proliferation. Therefore, we explored alternative biomarkers to detect chemical hepatocarcinogenicity and discovered increased expressions of epithelial cell adhesion molecule (EpCAM/CD326)- and aminopeptidase N (APN/CD13) in the hepatocytes of rats administered various hepatocarcinogens. Significant increases in EpCAM- and APN-positive hepatocytes were observed for eight and five of the 10 hepatocarcinogens, respectively. Notably, five and two of them, respectively, were negative for γ-H2AX foci. These results highlight the potential of EpCAM and APN as useful biomarkers in combination with γ-H2AX for the detection of chemical hepatocarcinogenicity.

Keywords: biomarker, carcinogenesis, γ-H2AX

Ishii Y, Shi L, Takasu S, Ogawa K, Umemura T: A 13-week comprehensive toxicity study with adductome analysis demonstrates the toxicity, genotoxicity, and carcinogenicity of the natural flavoring agent elemicin.

Food Chem Toxicol. 2023;179:113965. doi: 10.1016/j.fct.2023.113965.

Elemicin, an alkenylbenzene flavoring, exists naturally in foods, herbs, and spices. Some alkenylbenzenes are hepatotoxic and hepatocarcinogenic in rodents. However, few studies have examined the toxicology of elemicin. In the current study, we comprehensively evaluated the general toxicity, genotoxicity, and carcinogenicity of elemicin using *gpt* delta rats and DNA adductome analysis. Groups of 10 male F344 *gpt* delta rats were treated with elemicin by gavage at a dose of 0, 25, 100, or 400 mg/kg bw/day for 13 weeks. Liver weights were significantly increased with histopathological changes in groups receiving 100 mg/kg bw/day or more. Significant increases in serum hepatotoxic parameters were observed in the 400 mg/kg bw/day group. Based on the observed changes in liver weights, 18.6 mg/kg bw was identified as the low benchmark dose. Significant increases in the number and area of glutathione *S*-transferase placental form-positive foci and *gpt* mutant frequencies were apparent only in the 400 mg/kg/day group, although elemicin-specific DNA adducts were detected from the lowest dose, suggesting that elemicin exhibited hepatocarcinogenicity in rats only at higher doses. Because elemicin showed no mutagenicity at lower doses, there was an adequate safety margin between the acceptable daily intake and the estimated daily intake of elemicin.

Keywords: alkenylbenzene, comprehensive toxicity study, elemicin

Matsushita K, Toyoda T, Akane H, Morikawa T, Ogawa K: A 13-week subchronic toxicity study of heme iron in SD rats.

Food Chem Toxicol. 2023;175:113702. doi: 10.1016/j.fct.2023.113702.

Heme iron (HI) has been widely used as a food additive and supplement to support iron fortification. However, no sufficient toxicological data to evaluate the safety of HI have been reported. In the current study, we performed a 13-week subchronic toxicity study of HI in male and female Crl:CD(SD) rats. Rats were orally administered HI in the diet at

concentrations of 0%, 0.8%, 2%, and 5%. Observations of general condition, body weight (bw) and food consumption, urinalysis, hematology, serum biochemistry, and macroscopic and histopathological examination were performed. The results showed that HI had no adverse effects on any of the examined parameters. Therefore, we concluded that the no-observed-adverse-effect level (NOAEL) for HI was estimated to be 5% for both sexes (2,890 mg/kg bw/day for males and 3,840 mg/kg bw/day for females). Since the iron content of HI used in this study was in a range of 2.0-2.6%, iron content at NOAEL for HI was calculated to be 57.8-75.1 mg/kg bw/day for males and 76.8-99.8 mg/kg bw/day for females.

Keywords: food additive, heme iron, subchronic toxicity

Strupp C[*1], Corvaro M[*2], Cohen SM[*3], Corton JC[*4], Ogawa K, Richert L[*5], Jacobs MN[*6]: Increased cell proliferation as a key event in chemical carcinogenesis: application in an integrated approach for the testing and assessment of non-genotoxic carcinogenesis.

Int J Mol Sci. 2023;24:13246. doi: 10.3390/ijms241713246.

In contrast to genotoxic carcinogens, there are currently no internationally agreed upon regulatory tools for identifying non-genotoxic carcinogens of human relevance. The rodent cancer bioassay is only used in certain regulatory sectors and is criticized for its limited predictive power for human cancer risk. Cancer is due to genetic errors occurring in single cells. The risk of cancer is higher when there is an increase in the number of errors per replication (genotoxic agents) or in the number of replications (cell proliferation-inducing agents). The default regulatory approach for genotoxic agents whereby no threshold is set is reasonably conservative. However, non-genotoxic carcinogens cannot be regulated in the same way since increased cell proliferation has a clear threshold. An integrated approach for the testing and assessment (IATA) of non-genotoxic carcinogens is under development at the OECD, considering learnings from the regulatory assessment of data-rich substances such as agrochemicals. The aim is to achieve an endorsed IATA that predicts human cancer better than the rodent cancer bioassay, using methodologies that equally or better protect human health and are superior from the view of animal welfare/efficiency. This paper describes the technical opportunities available to assess cell proliferation as the central gateway of an IATA for non-genotoxic carcinogenicity.

Keywords: carcinogenicity, cell proliferation, new approach method

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Ishii Y, Namiki M, Takasu S, Nakamura K, Takimoto N, Mitsumoto T, Ogawa K: Lack of genotoxic mechanisms in isoeugenol-induced hepatocellular tumorigenesis in male B6C3F1 mice.

Jpn J Food Chem Safety. 2023;30:9-22. doi: 10.18891/jjfcs.30.1_9.

Isoeugenol (IEG) is a natural alkenylbenzene compound which is used as a flavoring additive in foods. However, it has been shown to be a hepatocarcinogen in male B6C3F1 mice. Although there are negative results in several genotoxicity tests, the genotoxicity of IEG in the livers of male mice has not been investigated. To determine whether a genotoxic mechanism is involved in hepatocarcinogenesis, we carried out histopathological analyses, comprehensive DNA adduct analyses, *in vivo* mutation assays and global gene expression analyses in the livers of male and female B6C3F1 *gpt* delta mice treated with IEG by gavage at doses of 0, 150, 300 or 600 mg/kg bw/day for 13 weeks. IEG induced slight hepatocyte hypertrophy along with liver weight gain in male mice treated with 300 mg/kg bw/day IEG and more, but not in similarly treated female mice. Comprehensive DNA adduct analyses by LC-MS/MS showed no specific DNA adduct formation in the liver, and there were no changes in *gpt* or Spi^- mutant frequencies in the livers. A pathway analysis of mRNA expression data as determined with a cDNA microarray suggested activation of pathways associated with peroxisome proliferator activated receptor (PPAR) α and γ in the livers of male mice. Overall, our data show a lack of genotoxicity in the

mechanisms leading to the hepatocarcinogenesis of IEG in mice, and they suggest the involvement of PPARα and γ pathway activation in this process.
Keywords: DNA adduct, isoeugenol, mutagenicity

Matsushita K, Toyoda T, Akane H, Morikawa T, Ogawa K: Role of CD44 expressed in renal tubules during maladaptive repair in renal fibrogenesis in an allopurinol-induced rat model of chronic kidney disease.
J Appl Toxicol. 2024;44:455-69. doi: 10.1002/jat.4554.

The kidney is a major target organ for the adverse effects of pharmaceuticals; renal tubular epithelial cells (TECs) are particularly vulnerable to drug-induced toxicity. TECs have regenerative capacity; however, maladaptive repair of TECs after injury leads to renal fibrosis, resulting in chronic kidney disease (CKD). We previously reported the specific expression of CD44 in failed-repair TECs of rat CKD model induced by ischemia reperfusion injury. Here, we investigated the pathophysiological role of CD44 in renal fibrogenesis in allopurinol-treated rat CKD model. Dilated or atrophic TECs expressing CD44 in fibrotic areas were collected by laser microdissection and subjected to microarray analysis. Gene ontology showed that extracellular matrix (ECM)-related genes were upregulated and differentiation-related genes were downregulated in dilated/atrophic TECs. Ingenuity Pathway Analysis identified CD44 as an upstream regulator of fibrosis-related genes, including *Fn1*, which encodes fibronectin. Immunohistochemistry demonstrated that dilated/atrophic TECs expressing CD44 showed decreases in differentiation markers of TECs and clear expression of mesenchymal markers during basement membrane attachment. *In situ* hybridization revealed an increase in *Fn1* mRNA in the cytoplasm of dilated/atrophic TECs, whereas fibronectin was localized in the stroma around these TECs, supporting the production/secretion of ECM by dilated/atrophic TECs. Overall, these data indicated that dilated/atrophic TECs underwent a partial epithelial–mesenchymal transition (pEMT) and that CD44 promoted renal fibrogenesis via induction of ECM production in failed-repair TECs exhibiting pEMT. CD44 was detected in the urine and serum of APL-treated rats, which may reflect the expression of CD44 in the kidney.
Keywords: allopurinol, CD44, kidney

Toyoda T, Sone M, Matsushita K, Akane H, Akagi J, Morikawa T, Mizuta Y, Cho YM, Ogawa K: Early detection of hepatocarcinogens in rats by immunohistochemistry of γ-H2AX.
J Toxicol Sci. 2023;48:323-32. doi: 10.2131/jts.48.323.

We have developed an early detection method for bladder carcinogens with high sensitivity and specificity using immunohistochemistry of γ-H2AX, a well-known marker of DNA damage. To investigate the potential application of γ-H2AX as a biomarker for early detection of hepatocarcinogens, we examined γ-H2AX formation in the liver of rats treated with several different chemicals for 28 days. Six-week-old male F344 rats were orally treated for 28 days with five hepatocarcinogens: *N*-nitrosodiethylamine (DEN), di(2-ethylhexyl) phthalate, 1,4-dioxane (DO), 3,3'-dimethylbenzidine dihydrochloride, or thioacetamide (TAA), or with two non-hepatocarcinogens: 4-chloro-*o*-phenylenediamine and *N*-ethyl-*N*-nitrosourea. At the end of the treatment period, immunohistochemistry for γ-H2AX and Ki67 and expression analysis of DNA repair-related genes were performed. Significant increases in γ-H2AX-positive hepatocytes with upregulation of *Rad51* mRNA expression were induced by three of five hepatocarcinogens (DEN, DO, and TAA), whereas no changes were seen for the other two hepatocarcinogens and the two non-hepatocarcinogens. Significant increases in Ki67 expression with upregulation of *Brip1*, *Xrcc5*, and *Lig4* were observed in rats treated with TAA, a nongenotoxic hepatocarcinogen, suggesting that both direct DNA damage and secondary DNA damage due to cell replication stress may be associated with γ-H2AX formation. These results suggest that γ-H2AX immunostaining has potential value for early detection of hepatocarcinogens, but examination of the effects of more chemicals is needed, as is whether γ-H2AX immunostaining should be combined with other markers to increase sensitivity. γ-H2AX immunostaining using formalin-fixed paraffin-embedded specimens can be easily incorporated into existing 28-day repeated-dose toxicity studies, and further improvements in this method are expected.
Keywords: carcinogenicity, γ-H2AX, liver

Akagi J, Mizuta Y, Akane H, Toyoda T, Ogawa K: Oral toxicological study of titanium dioxide nanoparticles with a crystallite diameter of 6 nm in rats.

Part Fibre Toxicol. 2023;20:23. doi: 10.1186/s12989-023-00533-x.

Though titanium dioxide (TiO_2) is generally considered to have a low impact in the human body, the safety of TiO_2 containing nanosized particles (NPs) has attracted attention. We found that the toxicity of silver NPs markedly varied depending on their particle size, as silver NPs with a diameter of 10 nm exhibited fatal toxicity in female BALB/c mice, unlike those with diameters of 60 and 100 nm. Therefore, the toxicological effects of the smallest available TiO_2 NPs with a crystallite size of 6 nm were examined in male and female F344/DuCrlCrlj rats by repeated oral administration of 10, 100, and 1000 mg/kg bw/day (5/sex/group) for 28 days and of 100, 300, and 1000 mg/kg bw/day (10/sex/group) for 90 days. In both 28- and 90-day studies, no mortality was observed in any group, and no treatment-related adverse effects were observed in body weight, urinalysis, hematology, serum biochemistry, or organ weight. Histopathological examination revealed TiO_2 particles as depositions of yellowish-brown material. The particles observed in the gastrointestinal lumen were also found in the nasal cavity, epithelium, and stromal tissue in the 28-day study. In addition, they were observed in Peyer's patches in the ileum, cervical lymph nodes, mediastinal lymph nodes, bronchus-associated lymphoid tissue, and trachea in the 90-day study. Notably, no adverse biological responses, such as inflammation or tissue injury, were observed around the deposits. Titanium concentration analysis in the liver, kidneys, and spleen revealed that TiO_2 NPs were barely absorbed and accumulated in these tissues. Immunohistochemical analysis of colonic crypts showed no extension of the proliferative cell zone or preneoplastic cytoplasmic/nuclear translocation of β-catenin either in the male or female 1000 mg/kg bw/day group. Regarding genotoxicity, no significant increase in micronucleated or γ-H2AX positive hepatocytes was observed. Additionally, the induction of γ-H2AX was not observed at the deposition sites of yellowish-brown materials. No effects were observed after repeated oral administration of TiO_2 with a crystallite size of 6 nm at up to 1000 mg/kg bw/day regarding general toxicity, accumulation of titanium in the liver, kidneys, and spleen, abnormality of colonic crypts, and induction of DNA strand breaks and chromosomal aberrations.

Keywords: genotoxicity, nanomaterial, titanium dioxide

Akagi J, Yokoi M[*1], Miyake Y[*2], Shirai T[*3], Baba T[*2], Cho YM, Hanaoka F[*1,4], Sugasawa K[*1], Iwai S[*2], Ogawa K: A formamidopyrimidine derivative from the deoxyguanosine adduct produced by food contaminant acrylamide induces DNA replication block and mutagenesis. *J Biol Chem*. 2023;299:105002. doi: 10.1016/j.jbc.2023.105002.

Acrylamide, a common food contaminant, is metabolically activated to glycidamide, which reacts with DNA at the N7 position of dG, forming N7-(2-carbamoyl-2-hydroxyethyl)-dG (GA^7dG). Owing to its chemical lability, the mutagenic potency of GA^7dG has not yet been clarified. We found that GA^7dG undergoes ring-opening hydrolysis to form N^6-(2-deoxy-d-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-[*N*-(2-carbamoyl-2-hydroxyethyl) formamido]pyrimidine (GA-FAPy-dG), even at neutral pH. Therefore, we aimed to examine the effects of GA-FAPy-dG on the efficiency and fidelity of DNA replication using an oligonucleotide carrying GA-FAPy-9-(2-deoxy-2-fluoro-β-d-arabinofuranosyl)guanine (dfG), a 2'-fluorine substituted analog of GA-FAPy-dG. GA-FAPy-dfG inhibited primer extension by both human replicative DNA polymerase ε and the translesion DNA synthesis polymerases (Polη, Polι, Polκ, and Pol ζ) and reduced the replication efficiency by less than half in human cells, with single base substitution at the site of GA-FAPy-dfG. Unlike other formamidopyrimidine derivatives, the most abundant mutation was G:C > A:T transition, which was decreased in Polκ- or REV1-KO cells. Molecular modeling suggested that a 2-carbamoyl-2-hydroxyethyl group at the N^5 position of GA-FAPy-dfG can form an additional H-bond with thymidine, thereby contributing to the mutation. Collectively, our results provide further insight into the mechanisms underlying the mutagenic effects of acrylamide.

Keywords: acrylamide, DNA damage, mutagenesis mechanism

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Takimoto N, Ishii Y, Mitsumoto T, Takasu S, Namiki M, Shibutani M*, Ogawa K: Formation of hepatocyte cytoplasmic inclusions and their contribution to methylcarbamate-induced hepatocarcinogenesis in F344 rats.
Toxicol Sci. 2024;198:40-9. doi:10.1093/toxsci/kfad131.

Methylcarbamate (MC), a reaction product between dimethyl dicarbonate and ammonia or ammonium ion, is a potent hepatocarcinogen in F344 rats. Various genotoxicity tests have shown negative results for MC. Although previous studies have described the effects of MC on the liver, including the formation of characteristic basophilic cytoplasmic inclusions (CIs) in hepatocytes, the toxicological significance of CIs and their involvement in hepatocarcinogenesis remain unclear. In the current study, to elucidate the mechanisms of MC hepatocarcinogenesis, we examined hepatotoxicity and genotoxicity after 4 weeks of administration of MC using *gpt* delta rats with an F344 genetic background as a reporter gene transgenic animal model. Histopathologically, single-cell necrosis, karyomegaly, and the formation of CIs positive for Feulgen staining were observed in hepatocytes at the carcinogenic dose, demonstrating the hepatotoxicity of MC. CIs were also detected as large micronuclei in liver micronucleus tests but not in the bone marrow, suggesting that MC could cause chromosomal instability specifically in the livers of rats. Reporter gene mutation assays demonstrated that MC did not induce mutagenicity even in the liver. Immunofluorescence analyses revealed that CIs exhibited loss of nuclear envelope integrity, increased heterochromatinization, and accumulation of DNA damage. An increase in liver STING protein levels suggested an effect on the cyclic GMP-AMP synthase/stimulator of interferon genes innate immune pathway. Overall, these data demonstrated the possible occurrence of chromothripsis-like chromosomal rearrangements via CIs. Thus, the formation of CIs could be a crucial event in the early stage of MC-induced hepatocarcinogenesis in F344 rats.
Keywords: hepatocarcinogenesis, methylcarbamate, micronucleus test

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Mishima M*, Sugiyama K: Considerations for the genotoxicity assessment of middle size peptide drugs containing non-canonical amino acid residues.
Genes Environ. 2023;45:36. doi: 10.1186/s41021-023-00294-1.

Background: Middle size peptides (MSPs) have emerged as a promising new pharmaceutical modality. We are seeking the best way to assess the non-clinical safety of MSPs. Consideration: The requirements for assessing the genotoxicity of pharmaceuticals differ between small molecule drugs and biotherapeutics. Genotoxicity tests are necessary for small molecule drugs but not for biotherapeutics. MSPs, however, share similarities with both small molecule drugs and biotherapeutics. Here, we describe important points to consider in assessing the genotoxicity of MSP drugs. The current standard of genotoxicity assessment for small molecules may not be entirely appropriate for MSP drugs. MSP drugs need genotoxicity assessment mostly according to the current standard of small molecule drugs. Conclusion: We propose a few modifications to the standard test battery of genotoxicity tests, specifically, the inclusion of an *in vitro* gene mutation test using mammalian cells, and exclusion of (Q)SAR assessment on MSP-related impurities.
Keywords: genotoxicity, on-canonical amino acid residue, middle size peptide drug

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You X*1, Cao Y*1, Suzuki T, Shao J*2, Zhu B*2, Masumura K, Xi J*1, Liu W*1, Zhang X*1, Luan Y*1: Genome-wide direct quantification of *in vivo* mutagenesis using high-accuracy paired-end and complementary consensus sequencing.
Nucleic Acids Res. 2023;51:e109. doi: 10.1093/nar/gkad909.

Error-corrected next-generation sequencing (ecNGS) is an emerging technology for accurately measuring somatic mutations. Here, we report paired-end and complementary consensus sequencing (PECC-Seq), a high-accuracy ecNGS approach for genome-wide somatic mutation detection. We characterize a novel

2-aminoimidazolone lesion besides 7,8-dihydro-8-oxoguanine and the resulting end-repair artifacts originating from NGS library preparation that obscure the sequencing accuracy of NGS. We modify library preparation protocol for the enzymatic removal of end-repair artifacts and improve the accuracy of our previously developed duplex consensus sequencing method. Optimized PECC-Seq shows an error rate of <5 × 10-8 with consensus bases compressed from approximately 25 Gb of raw sequencing data, enabling the accurate detection of low-abundance somatic mutations. We apply PECC-Seq to the quantification of *in vivo* mutagenesis. Compared with the classic gpt gene mutation assay using gpt delta transgenic mice, PECC-Seq exhibits high sensitivity in quantitatively measuring dose-dependent mutagenesis induced by Aristolochic acid I (AAI). Moreover, PECC-Seq specifically characterizes the distinct genome-wide mutational signatures of AAI, Benzo[a]pyrene, N-Nitroso-N-ethylurea and N-nitrosodiethylamine and reveals the mutational signature of Quinoline in common mouse models. Overall, our findings demonstrate that high-accuracy PECC-Seq is a promising tool for genome-wide somatic mutagenesis quantification and for *in vivo* mutagenicity testing.

Keywords: next-generation sequencing, gpt gene mutation assay, *in vivo* mutagenicity testing

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Takeda-Nishikawa K*1, Rajaguru P*2, Miyazato N*3, Suzuki T: What samples are suitable for monitoring antimicrobial-resistant genes? Using NGS technology, a comparison between eDNA and mrDNA analysis from environmental water.

Front. Microbiol. 2023;14:954783. doi: 10.3389/fmicb.2023.954783.

Introduction: The rise in antimicrobial resistance (AMR) that is affecting humans, animals, and the environment, compromises the human immune system and represents a significant threat to public health. Regarding the impact on water sanitation, the risk that antimicrobial-resistant genes (ARGs) and antimicrobial-resistant bacteria in surface water in cities pose to human health remains unclear. To determine the prevalence of AMR in environmental surface water in Japan, we used DNA sequencing techniques on environmental water DNA (eDNA) and the DNA of multidrug-resistant bacteria (mrDNA). Methods: The eDNA was extracted from four surface water samples obtained from the Tokyo area and subjected to high-throughput next-generation DNA sequencing using Illumina-derived shotgun metagenome analysis. The sequence data were analyzed using the AmrPlusPlus pipeline and the MEGARes database. Multidrug-resistant bacteria were isolated using a culture-based method from water samples and were screened by antimicrobial susceptibility testing (for tetracycline, ampicillin-sulbactam, amikacin, levofloxacin, imipenem, and clarithromycin). Of the 284 isolates, 22 were identified as multidrug-resistant bacteria. The mrDNA was sequenced using the Oxford nanopore MinION system and analyzed by NanoARG, a web service for detecting and contextualizing ARGs. Results and discussion: The results from eDNA and mrDNA revealed that ARGs encoding beta-lactams and multidrug resistance, including multidrug efflux pump genes, were frequently detected in surface water samples. However, mrDNA also revealed many sequence reads from multidrug-resistant bacteria, as well as nonspecific ARGs, whereas eDNA revealed specific ARGs such as pathogenic OXA-type and New Delhi metallo (NDM)-beta-lactamase ARGs. Conclusion: To estimate potential AMR pollution, our findings suggested that eDNA is preferable for detecting pathogen ARGs.

Keywords: DNA sequencing, environmental water DNA, MinION

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Yamada M*1, Suzuki T, Kohara A*2, Honma M: Carcinogenic risk of food additive AF-2 banned in Japan: a case study on reassessment of genotoxicity.

Genes Environ. 2023;45:33. doi: 10.1186/s41021-023-00292-3.

Background: Carcinogenic risk assessment studies have been repeatedly improved and are still being debated to find a goal. Evaluation might be changed if new approaches would be applied to some chemicals

which means that new approaches may change the final assessment. In this paper, the risk assessment of a chemical, in particular the proper carcinogenicity, is examined using the long-banned food additive, 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, AF-2, as a case study. Results: First, Ames tests were carried out using strains TA1535, TA100, TA1538, and TA98 and their nitroreductase-deficient strains YG7127, YG7128, YG7129, and YG7130. The results showed that mutagenic activity was reduced by about 50% in the nitroreductase-deficient strains, indicating that part of the mutagenic activity shown in Ames test was due to bacterial metabolism. Second, *in vivo* genotoxicity tests were conducted, including the one that had not been developed in 1970's. Both a micronucleus test and a gene mutation assay using transgenic mice were negative. Third, assuming it is a genotoxic carcinogen, the virtual safety dose of 550 μg/day was calculated from the TD50 in rats with a probability of 10-5. Conclusion: AF-2 has been shown to be carcinogenic to rodents and has previously been indicated to be genotoxic *in vitro*. However, the present *in vivo* genotoxicity study, it was negative in the forestomach, a target organ for cancer, particularly in the gene mutation assay in transgenic mice. Considering the daily intake of AF-2 in the 1970s and its virtually safety dose, the carcinogenic risk of AF-2 could be considered acceptable.

Keywords: Ames test, AF-2, carcinogenicity

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Sugiyama K, Honma M. Evaluation of QSAR models for predicting mutagenicity: outcome of the Second Ames/QSAR international challenge project.
SAR QSAR Environ Res. 2023;34:983-1001. doi: 10.1080/1062936X.2023.2284902.

Quantitative structure-activity relationship (QSAR) models are powerful *in silico* tools for predicting the mutagenicity of unstable compounds, impurities and metabolites that are difficult to examine using the Ames test. Ideally, Ames/QSAR models for regulatory use should demonstrate high sensitivity, low false-negative rate and wide coverage of chemical space. To promote superior model development, the Division of Genetics and Mutagenesis, National Institute of Health Sciences, Japan (DGM/NIHS), conducted the Second Ames/QSAR International Challenge Project (2020-2022) as a successor to the First Project (2014-2017), with 21 teams from 11 countries participating. The DGM/NIHS provided a curated training dataset of approximately 12,000 chemicals and a trial dataset of approximately 1,600 chemicals, and each participating team predicted the Ames mutagenicity of each trial chemical using various Ames/QSAR models. The DGM/NIHS then provided the Ames test results for trial chemicals to assist in model improvement. Although overall model performance on the Second Project was not superior to that on the First, models from the eight teams participating in both projects achieved higher sensitivity than models from teams participating in only the Second Project. Thus, these evaluations have facilitated the development of QSAR models.

Keywords: ANEI-HOU new chemical, Ames mutagenicity prediction, Ames/QSAR International Challenge Projects

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Thakkar S*1, Slikker W Jr*2, Yiannas F*3, Silva P*4, Blais B*4, Chng KR*5, Liu Z*2, Adholeya A*6, Pappalardo F*7, Soares MDLC*8, Beeler PE*9, Whelan M*10, Roberts R*11, Borlak J*12, Hugas M*13, Torrecilla-Salinas C*10, Girard P*14, Diamond MC*15, Verloo D*13, Panda B*16, Rose MC*17, Jornet JB*18, Furuhama A, Fang H*2, Kwegyir-Afful E*19, Heintz K*19, Arvidson K*19, Burgos JG*18, Horst A*14, Tong W*2. Artificial intelligence and real-world data for drug and food safety - A regulatory science perspective.
Regul Toxicol Pharmacol. 2023;140:105388. doi: 10.1016/j.yrtph.2023.105388.

In 2013, the Global Coalition for Regulatory Science Research (GCRSR) was established with members from over ten countries (www.gcrsr.net). One of the main objectives of GCRSR is to facilitate communication among global regulators on the rise of new technologies with regulatory applications through the annual conference Global Summit on Regulatory Science (GSRS). The 11th annual GSRS conference (GSRS21) focused on "Regulatory Sciences for Food/Drug Safety with Real-World Data (RWD) and Artificial Intelligence (AI)." The conference discussed current advancements in both AI and RWD approaches with a specific emphasis on how they impact regulatory sciences and how regulatory agencies across the globe are pursuing the adaptation and oversight of these technologies. There were presentations from Brazil, Canada, India, Italy, Japan, Germany, Switzerland, Singapore, the United Kingdom, and the United States. These presentations highlighted how various agencies are moving forward with these technologies by either improving the agencies' operation and/or preparing regulatory mechanisms to approve the products containing these innovations. To increase the content and discussion, the GSRS21 hosted two debate sessions on the question of "Is Regulatory Science Ready for AI?" and a workshop to showcase the analytical data tools that global regulatory agencies have been using and/or plan to apply to regulatory science. Several key topics were highlighted and discussed during the conference, such as the capabilities of AI and RWD to assist regulatory science policies for drug and food safety, the readiness of AI and data science to provide solutions for regulatory science. Discussions highlighted the need for a constant effort to evaluate emerging technologies for fit-for-purpose regulatory applications. The annual GSRS conferences offer a unique platform to facilitate discussion and collaboration across regulatory agencies, modernizing regulatory approaches, and harmonizing efforts.

Keywords: artificial intelligence, regulatory science, real-world data

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Shimizu N*1, Hamada Y*1, Morozumi R*1, Yamamoto J*2, Iwai S*2, Sugiyama K, Ide H*1, Tsuda M: Repair of topoisomerase 1-induced DNA damage by tyrosyl-DNA phosphodiesterase 2 (TDP2) is dependent on its magnesium binding.

J Biol Chem. 2023;299:104988. doi: 10.1016/j.jbc.2023.

Topoisomerases are enzymes that relax DNA supercoiling during replication and transcription. Camptothecin, a topoisomerase 1 (TOP1) inhibitor, and its analogs trap TOP1 at the 3'-end of DNA as a DNA-bound intermediate, resulting in DNA damage that can kill cells. Drugs with this mechanism of action are widely used to treat cancers. It has previously been shown that tyrosyl-DNA phosphodiesterase 1 (TDP1) repairs TOP1-induced DNA damage generated by camptothecin. In addition, tyrosyl-DNA phosphodiesterase 2 (TDP2) plays critical roles in repairing topoisomerase 2 (TOP2)-induced DNA damage at the 5'-end of DNA and in promoting the repair of TOP1-induced DNA damage in the absence of TDP1. However, the catalytic mechanism by which TDP2 processes TOP1-induced DNA damage has not been elucidated. In this study, we found that a similar catalytic mechanism underlies the repair of TOP1- and TOP2-induced DNA damage by TDP2, with Mg^{2+}-TDP2 binding playing a role in both repair mechanisms. We show chain-terminating nucleoside analogs are incorporated into DNA at the 3'-end and abort DNA replication to kill cells. Furthermore, we found that Mg^{2+}-TDP2 binding also contributes to the repair of incorporated chain-terminating nucleoside analogs. Overall, these findings reveal the role played by Mg^{2+}-TDP2 binding in the repair of both 3'- and 5'-blocking DNA damage.

Keywords: tyrosyl-DNA phosphodiesterase, camptothecin, chain-terminating nucleoside analog

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Ahmad T*1, Kawasumi R*1, Taniguchi T*1, Abe T*1, Terada K*2, Tsuda M, Shimizu N*3, Tsurimoto T*4, Takeda S*5, Hirota K*1: The proofreading exonuclease of leading-strand DNA polymerase epsilon prevents replication fork collapse at broken template strands.

Nucleic Acids Res. 2023;51:12288-12302. doi: 10.1093/nar/gkad999.

Leading-strand DNA replication by polymerase epsilon (Polε) across single-strand breaks (SSBs) causes single-ended double-strand breaks (seDSBs), which are repaired via homology-directed repair (HDR) and suppressed by fork reversal (FR). Although previous studies identified many molecules required for hydroxyurea-induced FR, FR at seDSBs is poorly understood. Here, we identified molecules that specifically mediate FR at seDSBs. Because FR at seDSBs requires poly(ADP-ribose)polymerase 1 (PARP1), we hypothesized that seDSB/FR-associated molecules would increase tolerance to camptothecin (CPT) but not the PARP inhibitor olaparib, even though both anti-cancer agents generate seDSBs. Indeed, we uncovered that Polε exonuclease and CTF18, a Polε cofactor, increased tolerance to CPT but not olaparib. To explore potential functional interactions between Polε exonuclease, CTF18, and PARP1, we created exonuclease-deficient $POLE1^{exo-/-}$, $CTF18^{-/-}$, $PARP1^{-/-}$, $CTF18^{-/-}/POLE1^{exo-/-}$, $PARP1^{-/-}/POLE1^{exo-/-}$, and $CTF18^{-/-}/PARP1^{-/-}$cells. Epistasis analysis indicated that Polε exonuclease and CTF18 were interdependent and required PARP1 for CPT tolerance. Remarkably, $POLE1^{exo-/-}$ and HDR-deficient $BRCA1^{-/-}$ cells exhibited similar CPT sensitivity. Moreover, combining $POLE1^{exo-/-}$ with $BRCA1^{-/-}$ mutations synergistically increased CPT sensitivity. In conclusion, the newly identified PARP1-CTF18-Polε exonuclease axis and HDR act independently to prevent fork collapse at seDSBs. Olaparib inhibits this axis, explaining the pronounced cytotoxic effects of olaparib on HDR-deficient cells.

Keywords: polymerase epsilon, camptothecin, olaparib

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Tanaka M*1, Yamada M*2, Mushiake M*2, Tsuda M, Miwa M*2: Elucidating Differences in Early-Stage Centrosome Amplification in Primary and Immortalized Mouse Cells.
Int J Mol Sci. 2023;25:383. doi: 10.3390/ijms25010383.

The centrosome is involved in cytoplasmic microtubule organization during interphase and in mitotic spindle assembly during cell division. Centrosome amplification (abnormal proliferation of centrosome number) has been observed in several types of cancer and in precancerous conditions. Therefore, it is important to elucidate the mechanism of centrosome amplification in order to understand the early stage of carcinogenesis. Primary cells could be used to better understand the early stage of carcinogenesis rather than immortalized cells, which tend to have various genetic and epigenetic changes. Previously, we demonstrated that a poly(ADP-ribose) polymerase (PARP) inhibitor, 3-aminobenzamide (3AB), which is known to be nontoxic and nonmutagenic, could induce centrosome amplification and chromosomal aneuploidy in CHO-K1 cells. In this study, we compared primary mouse embryonic fibroblasts (MEF) and immortalized MEF using 3AB. Although centrosome amplification was induced with 3AB treatment in immortalized MEF, a more potent PARP inhibitor, AG14361, was required for primary MEF. However, after centrosome amplification, neither 3AB in immortalized MEF nor AG14361 in primary MEF caused chromosomal aneuploidy, suggesting that further genetic and/or epigenetic change(s) are required to exhibit aneuploidy. The DNA-damaging agents doxorubicin and γ-irradiation can cause cancer and centrosome amplification in experimental animals. Although doxorubicin and γ-irradiation induced centrosome amplification and led to decreased p27Kip protein levels in immortalized MEF and primary MEF, the phosphorylation ratio of nucleophosmin (Thr199) increased in immortalized MEF, whereas it decreased in primary MEF. These results suggest that there exists a yet unidentified pathway, different from the nucleophosmin phosphorylation pathway, which can cause centrosome amplification in primary MEF.

Keywords: centrosome, PARP, nucleophosmin

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Izawa K, Tsuda M, Suzuki T, Honma M, Sugiyama K: Detection of *in vivo* mutagenicity in rat liver samples using error-corrected sequencing techniques.
Genes Environ. 2023;45:30. doi: 10.1186/s41021-023-00288-z

Mutagenicity, the ability of chemical agents to cause mutations and potentially lead to cancer, is a critical aspect of substance safety assessment for protecting human health and the environment. Metabolic enzymes activate multiple mutagens in living organisms, thus *in vivo* animal models provide highly important information for evaluating mutagenicity in human. Rats are considered suitable models as they share a similar metabolic pathway with humans for processing toxic chemical and exhibit higher responsiveness to chemical carcinogens than mice. To assess mutagenicity in rats, transgenic rodents (TGRs) are widely used for *in vivo* gene mutation assays. However, such assays are labor-intensive and could only detect transgene mutations inserted into the genome. Therefore, introducing a technology to directly detect *in vivo* mutagenicity in rats would be necessary. The next-generation sequencing (NGS) based error-corrected sequencing technique is a promising approach for such purposes. We investigated the applicability of paired-end and complementary consensus sequencing (PECC-Seq), an error-corrected sequencing technique, for detecting *in vivo* mutagenicity in the rat liver samples. PECC-Seq allows for the direct detection of ultra-rare somatic mutations in the genomic DNA without being constrained by the genomic locus, tissue, or organism. We tested PECC-Seq feasibility in rats treated with diethylnitrosamine (DEN), a mutagenic compound. Interestingly, the mutation and mutant frequencies between PECC-Seq and the TGR assay displayed a promising correlation. Our results also demonstrated that PECC-Seq could successfully detect the A:T > T:A mutation in rat liver samples, consistent with the TGR

assay. Furthermore, we calculated the trinucleotide mutation frequency and proved that PECC-Seq accurately identified the DEN treatment-induced mutational signatures. Our study provides the first evidence of using PECC-Seq for *in vivo* mutagenicity detection in rat liver samples. This approach could provide a valuable alternative to conventional TGR assays as it is labor- and time-efficient and eliminates the need for transgenic rodents. Error-corrected sequencing techniques, such as PECC-Seq, represent promising approaches for enhancing mutagenicity assessment and advancing regulatory science.

Keywords: *in vivo* mutagenicity, next-generation sequencing, rat liver sample

Morozumi R[*1], Shimizu N[*1], Tamura K[*1], Nakamura K[*1], Suzuki A[*1], Ishiniwa H[*2], Ide H[*1], Tsuda M: Changes in repair pathways of radiation-induced DNA double-strand breaks at the midblastula transition in *Xenopus* embryo.

J Radiat Res. [Epub ahead of print]. doi: 10.1093/jrr/rrae012

Ionizing radiation (IR) causes DNA damage, particularly DNA double-strand breaks (DSBs), which have significant implications for genome stability. The major pathways of repairing DSBs are homologous recombination (HR) and nonhomologous end joining (NHEJ). However, the repair mechanism of IR-induced DSBs in embryos is not well understood, despite extensive research in somatic cells. The externally developing aquatic organism, Xenopus tropicalis, serves as a valuable model for studying embryo development. A significant increase in zygotic transcription occurs at the midblastula transition (MBT), resulting in a longer cell cycle and asynchronous cell divisions. This study examines the impact of X-ray irradiation on Xenopus embryos before and after the MBT. The findings reveal a heightened X-ray sensitivity in embryos prior to the MBT, indicating a distinct shift in the DNA repair pathway during embryo development. Importantly, we show a transition in the dominant DSB repair pathway from NHEJ to HR before and after the MBT. These results suggest that the MBT plays a crucial role in altering DSB repair mechanisms, thereby influencing the IR sensitivity of developing embryos.

Keywords: *Xenopus tropicalis*, ionizing radiation, DNA double-strand break

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Abdallah A[*1], Adel N[*2], Elkerdawy AM[*3,4], Tanabe S, Andres F[*5], Pester A[*1], Ali HH[*6]: Geom-SAC: Geometric multi-discrete soft actor critic with applications in *de novo* drug design.

IEEE Access. 2024;12:45519-45529. doi: 10.1109/ACCESS.2024.3377289

Finding new molecules with desirable properties has high computational and overhead costs. Much research has focused on generating candidate molecules in one- and two-dimensional spaces, which has produced some favorable results. However, extending these approaches to molecules in three-dimensional space would be far more useful because the representation of molecules is more realistic, although three-dimensional methods have much higher computational costs. In this work, we developed a geometric deep reinforcement learning agent that generates and optimizes molecules that could interact with a biochemical target. The agent can be used for generating molecules from scratch or for lead optimization when it enhances the properties of a given molecule, whether by enhancing its drug-likeness or increasing its activity toward the target via implicit learning. Thus, the agent works with molecules in three-dimensional space without high computational costs.

Keywords: *de novo* drug design, geometric deep learning, molecule optimization

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Tanabe S, Boonstra E[*1], Hong T[*1], Quader S[*2], Ono R, Cabral H[*1], Aoyagi K[*3], Yokozaki H[*4], Perkins EJ[*5], Sasaki H[*3]: Molecular Networks of Platinum Drugs and Their Interaction with microRNAs in Cancer.

Genes. 2023;14(11):2073. doi: 10.3390/genes14112073

The precise mechanism of resistance to anti-cancer drugs such as platinum drugs is not fully revealed. To reveal the mechanism of drug resistance, the molecular networks of anti-cancer drugs such as cisplatin, carboplatin, oxaliplatin, and arsenic trioxide were analyzed in several types of cancers. Since diffuse-type stomach adenocarcinoma, which has epithelial-mesenchymal transition (EMT)-like characteristics, is more malignant than intestinal-type stomach adenocarcinoma, the gene expression and molecular networks in diffuse- and intestinal-type stomach adenocarcinomas were analyzed. Analysis of carboplatin revealed the causal network in diffuse large B-cell lymphoma. The upstream regulators of the molecular networks of cisplatin-treated lung adenocarcinoma included the anti-cancer drug trichostatin A (TSA), a histone deacetylase inhibitor. The upstream regulator analysis of cisplatin revealed an increase in FAS, BTG2, SESN1, and CDKN1A, and the involvement of the tumor microenvironment pathway. The molecular networks were predicted to interact with several microRNAs, which may contribute to the identification of new drug targets for drug-resistant cancer. Analysis of oxaliplatin, a platinum drug, revealed that the SPINK1 pancreatic cancer pathway is inactivated in ischemic cardiomyopathy. The study showed the importance of the molecular networks of anti-cancer drugs and tumor microenvironment in the treatment of cancer resistant to anti-cancer drugs.

Keywords: cisplatin, drug resistance, microRNA

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Kawashima A, Inoue K, Ushida K, Kai K, Suzuki H, Yoshida-Yamashita LS, Hirose A, Masumura K: Derivation of human health hazard assessment values of 1,2-dichloroethane under the Japanese Chemical Substances Control Law.
Fundam Toxicol Sci. 2023;10(3):91-103. doi: 10.2131/fts.10.91

1,2-Dichloroethane, a priority assessment chemical substance under the Japan Chemical Substances Control Law (CSCL), required a detailed human health hazard assessment under Assessment II. We evaluated its general, reproductive, and developmental toxicities, genotoxicity, and carcinogenicity, based on the hazard information provided by domestic and international risk assessment organizations, and the hazard assessment values (HAVs) for oral and inhalation exposure were proposed. For oral exposure, a 78-week gavage carcinogenicity study (US NCI, 1978) with incidence data of hemangiosarcoma in male rats was selected as a significant toxicological endpoint and the lower confidence limit of benchmark dose (BMD) at 10% benchmark response ($BMDL_{10}$) of 9.3 mg/kg/day was obtained as a point of departure (POD). A slope factor of 1.07×10^{-2} $(mg/kg/day)^{-1}$ from which a carcinogenic 10^{-5} risk of 0.93 μg/kg/day was derived as an oral HAV. For inhalation exposure, a 104-week inhalation exposure carcinogenicity study (Nagano et al., 2006) with a $BMDL_{10}$ of 11.5 ppm based on the incidence data of mammary gland tumors (adenocarcinoma + adenoma + fibroadenoma, combined) in female rats was obtained, and the human equivalent $BMDL_{10}$ of 15.7 mg/m^3 was calculated. Therefore, a unit risk of 6.40×10^{-6} $(\mu g/m^3)^{-1}$ from which a carcinogenic 10^{-5} risk of 1.6 $\mu g/m^3$ (0.00039 ppm) was derived as an inhalation HAV.

Keywords: 1,2-dichloroethane (CAS No. 107-06-2), Chemical Substance Control Law (CSCL), Assessment II for human health effects

Murata Y, Natsume M*1, Iso T, Shigeta Y*2, Hirose N, Umano T, Horibata K, Sugiyama K, Masumura K, Hirose A*3, Matsumoto M: *In vivo* mutagenicity assessment of styrene in MutaMouse liver and lung.
Genes and Environment. 2023;45:12. doi: 10.1186/s41021-023-00270-9

Background Styrene (CAS 100-42-5) is widely used as polystyrene and acrylonitrile–butadiene–styrene resin such as plastic, rubber, and paint. One of the primary uses of styrene is food utensils and containers, but a small amount of styrene transferred into food can be ingested by eating. Styrene is metabolized into styrene 7,8-oxide (SO). SO is mutagenic in bacteria and mouse lymphoma assays. It is clastogenic in cultured mammalian cells. However, styrene and SO are not clastogenic/aneugenic in rodents, and no

rodent *in vivo* gene mutation studies were identified. Methods To investigate the mutagenicity of orally administered styrene, we used the transgenic rodent gene mutation assay to perform an *in vivo* mutagenicity test (OECD TG488). The transgenic MutaMouse was given styrene orally at doses of 0 (corn oil; negative control), 75, 150, and 300 mg/kg/day for 28 days, and mutant frequencies (MFs) were determined using the lacZ assay in the liver and lung (five male mice/group). Results There were no significant differences in the MFs of the liver and lung up to 300 mg/kg/day (close to maximum tolerable dose (MTD)), when one animal with extremely high MFs that were attributed to an incidental clonal mutation was omitted. Positive and negative controls produced the expected results. Conclusions These findings show that styrene is not mutagenic in the liver and lung of MutaMouse under this experimental condition.
Keywords: TG488, MutaMouse, styrene, *in vivo* mutagenicity

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Iso T, Suzuki K*1, Murata Y, Hirose N, Umano T, Horibata K, Sugiyama K, Hirose A*2, Masumura K, Matsumoto M: Lack of *in vivo* mutagenicity of carbendazim in the liver and glandular stomach of MutaMice.
Genes and Environment. 2024;46:7. doi: 10.1186/s41021-024-00299-4

Background Carbendazim (methyl 2-benzimidazolecarbamate, CASRN: 10605-21-7) exhibits spindle poisoning effects and is widely used as a fungicide. With respect to genotoxicity, carbendazim is deemed to be non-mutagenic *in vitro*, but it causes indicative DNA damage *in vivo* and chromosome aberrations *in vitro* and *in vivo*. In this study, we examined the mutagenicity of carbendazim *in vivo*. Results MutaMice were treated with carbendazim orally at doses of 0 (corn oil), 250, 500, and 1,000 mg/kg/day once a day for 28 days. A *lacZ* assay was used to determine the mutant frequency (MF) in the liver and glandular stomach of mice. MutaMice were administered up to the maximum dose recommended by the Organization for Economic Co-operation and Development Test Guidelines for Chemicals No. 488 (OECD TG488). The *lacZ* MFs in the liver and glandular stomach of carbendazim-treated animals were not significantly different from those in the negative control animals. In contrast, positive control animals exhibited a significant increase in MFs in both the liver and glandular stomach. Conclusions Carbendazim is non-mutagenic in the liver and glandular stomach of MutaMice following oral treatment.
Keywords: carbendazim, *in vivo* mutagenicity, transgenic rodent gene mutation assay

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Murata Y, Suzuki K*1, Shigeta Y*2, Iso T, Hirose N, Umano T, Horibata K, Sugiyama K, Hirose A*3, Masumura K, Matsumoto M: *In vivo* mutagenicity assessment of orally treated *tert*-butyl hydroperoxide in the liver and glandular stomach of MutaMouse.
Genes and Environment. 2023;45:29. doi: 10.1186/s41021-023-00285-2

Background tert-Butyl hydroperoxide (TBHP; CAS 75–91-2), a hydroperoxide, is mainly used as a polymerization initiator to produce polyethylene, polyvinyl chloride, and unsaturated polyester. It is a high-production chemical, widely used in industrial countries, including Japan. TBHP is also used as an additive for the manufacturing of food utensils, containers, and packaging (UCP). Therefore, there could be consumer exposure through oral intake of TBHP eluted from UCPs. TBHP was investigated in various *in vitro* and *in vivo* genotoxicity assays. In Ames tests, some positive results were reported with and/or without metabolic activation. As for the mouse lymphoma assay, the positive result was reported, regardless of the presence or absence of metabolic activation enzymes. The results of some chromosomal aberrations test and comet assay *in vitro* also demonstrated the genotoxic positive results. On the other hand, in *in vivo* tests, there are negative results in the bone marrow micronucleus test of TBHP-administered mice by single intravenous injection and

the bone marrow chromosomal aberration test using rats exposed to TBHP for 5 days by inhalation. Also, about dominant lethal tests, the genotoxic positive results appeared. In contrast, there is little information about *in vivo* mutagenicity and no information about carcinogenicity by oral exposure. Methods To investigate the mutagenicity of orally administered TBHP, we used the transgenic rodent gene mutation assay to perform an *in vivo* mutagenicity test (OECD TG488). The transgenic MutaMouse was given TBHP orally at doses of 0 (0.5% MC; negative control), 75, 150, and 300 mg/kg/day for 28 days, and mutant frequencies (MFs) were determined using the *lacZ* assay in the liver and glandular stomach (five male mice/group). Results We conducted *in vivo* gene mutation assay using MutaMice according to the OECD Guidelines for the Testing of Chemicals No. 488 to investigate *in vivo* mutagenicity of TBHP through oral exposure. After repeated dosing for 28 days, there were no significant differences in the mutant frequencies (MFs) of the liver and glandular stomach up to 300 mg/kg/day (close to the maximum tolerable dose (MTD)). The positive and negative controls produced the expected responses. Conclusions These findings show that orally administrated TBHP is not mutagenic in the mouse liver and glandular stomach under these experimental conditions.
Keywords: TG488, MutaMouse, TBHP

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Matsumoto, M, Murata, Y, Hirose, N, Shigeta, Y*1, Iso, T, Umano, T, Hirose, A*2: Derivation of subacute guidance values for chemical contaminants of drinking water quality standard in Japan.
Regulatory Toxicology and Pharmacology. 2024;141: 105401. doi: 10.1016/j.yrtph.2023.105401

The concentration of chemicals in drinking water may transiently and accidently exceed the Drinking Water Quality Standard (DWQS). If the level of a contaminant is not expected to cause adverse effects for a limited period of exposure, immediate suspension of the water supply may not be necessary. Assessments should be conducted using subacute guidance values (SGVs). In this study, we assessed 26 chemicals for the DWQS to establish the SGVs. Principally, a key study was selected from subacute studies to derive a Subacute Reference Dose (saRfD). The SGV was calculated from the saRfD for adults (drinking water intakes: 40 mL/kg/day) and children (drinking water intakes: 150 mL/kg/day). No allocation factor was applied to derive the SGV. We established the SGV for 20 chemicals, which were 2–38 times higher than the corresponding DWQS. However, SGVs for six chemicals were the same as the corresponding DWQS. Therefore, immediate action will be required for these six accidental contaminants. Our established SGVs are useful for assessing accidental contamination.
Keywords: drinking water quality standard, subacute reference dose, subacute guidance value

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Myden A*1, Stalford SA*1, Fowkes A*1, White E*1, Hirose A*2, Yamada T: Enhancing developmental and reproductive toxicity knowledge: A new AOP stemming from glutathione depletion.
Curr Res Toxicol. 2023;5:100124. doi: 10.1016/j.crtox.2023.100124

Integrated approaches to testing and assessments (IATAs) have been proposed as a method to organise new approach methodologies in order to replace traditional animal testing for chemical safety assessments. To capture the mechanistic aspects of toxicity assessments, IATAs can be framed around the adverse outcome pathway (AOP) concept. To utilise AOPs fully in this context, a sufficient number of pathways need to be present to develop fit for purpose IATAs. *In silico* approaches can support IATA through the provision of predictive models and also through data integration to derive conclusions using a weight-of-evidence approach. To examine the maturity of a developmental and reproductive toxicity (DART) AOP network derived from the literature, an assessment of its coverage was performed against a novel toxicity dataset. A dataset of diverse compounds, with data from studies performed according to OECD test guidelines TG-421 and TG-422, was curated to test

the performance of an *in silico* model based on the AOP network - allowing for the identification of knowledge gaps within the network. One such gap in the knowledge was filled through the development of an AOP stemming from the molecular initiating event 'glutathione reaction with an electrophile' leading to male fertility toxicity. The creation of the AOP provided the mechanistic rationale for the curation of pre-existing structural alerts to relevant key events. Integrating this new knowledge and associated alerts into the DART AOP network will improve its coverage of DART-relevant chemical space. In addition, broadening the coverage of AOPs for a particular regulatory endpoint may facilitate the development of, and confidence in, robust IATAs.

Keywords: adverse outcome pathway, glutathione, male fertility toxicity

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Yamazoe Y*1, Murayama N*2, Kawamura T, Yamada T: Application of fused-grid-based CYP-Template systems for genotoxic substances to understand the metabolisms.

Genes Environ. 2023;45:22. doi: 10.1186/s41021-023-00275-4

Understanding of metabolic processes is a key factor to evaluate biological effects of carcinogen and mutagens. Applicability of fused-grid Template systems of CYP enzymes (Drug Metab Pharmacokinet 2019, 2020, 2021, and 2022) was tested for three phenomena. (1) Possible causal relationships between CYP-mediated metabolisms of β-naphthoflavone and 3-methylcholanthrene and the high inducibility of CYP enzymes were examined. Selective involvement of non-constitutive CYP1A1, but not constitutive CYP1A2, was suggested on the oxidative metabolisms of efficient inducers, β-naphthoflavone and 3-methylcholanthrene. These results supported the view of the causal link of their high inducibility with their inefficient metabolisms due to the lack of CYP1A1 in livers at early periods after the administration of both inducers. (2) Clear differences exist between human and rodent CYP1A1 enzymes on their catalyses with heterocyclic amines, dioxins and polyaromatic hydrocarbons (PAHs). Reciprocal comparison of simulation results with experimental data suggested the rodent specific site and distinct sitting-preferences of ligands on Template for human and rodent CYP1A1 enzymes. (3) Enhancement of metabolic activation and co-mutagenicity have been known as phenomena associated with Salmonella mutagenesis assay. Both the phenomena were examined on CYP-Templates in ways of simultaneous bi-molecule bindings of distinct ligands as trigger and pro-metabolized molecules. α-Naphthoflavone and norharman served consistently as trigger-molecules to support the oxidations of PAHs and arylamines sitting simultaneously as pro-metabolized molecules on Templates of CYP1A1, CYP1A2 and CYP3A4. These CYP-Template simulation systems with deciphering capabilities are promising tools to understand the mechanism basis of metabolic activations and to support confident judgements in safety assessments.

Keywords: CYP-mediated activation, co-mutagenicity, fused hexagonal-grid template system

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Ashikaga T, Narita K*1, Kobayashi M*1, Tachibana S*1, Murasaki W*2, Suzuki M*2, Ambe K*2, Tohkin M*2: Skin Sensitization Potency Prediction of Ingredients in Hair Colorants Using *in silico* Models of Machine Learning.

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Skin sensitization caused by cosmetics, including quasi-drugs, is an extremely serious problem and can become a health hazard. Although several *in vitro* methods for skin sensitization have been established by the Organization for Economic Cooperation and Development (OECD), no practical method for predicting skin sensitization potency has been found which can serve as an alternative to animal testing. In this study, an *in silico* model is developed which predicts skin sensitization potency by combining *in vitro* OECD test guidelines and information of the physical properties of chemicals to evaluate hair dye ingredients without animal testing. A dataset published by Cosmetics Europe was used to develop the *in silico* model. The EC3 value of the mouse local lymph node assay (LLNA), which is used as an indicator of skin

sensitization potency, was the objective variable; *in vitro* test values, physical properties, and chemical information obtained from the molecular descriptor calculation software MOE constituted the explanatory variables. CatBoost was adopted as the machine-learning approach. Bandrowski's base (BB), a trimer of the typical oxidative dye para-phenylenediamine (pPD), was used as the test material. In the predictive model, the mean-predicted EC3 value for BB was 0.33%, which meant a strong sensitizer. Furthermore, it was lower than the mean model prediction EC3 value (0.66%) of pPD, which is considered weaker sensitization potency than BB in LLNA. This indicates that BB was correctly predicted as a stronger skin sensitizer than pPD in the proposed *in silico* model.

Keywords: skin sensitization, potency prediction, alternatives

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Strickland J*1, Haugabrooks E*2, Allen DG*1, Balottin LB*3, Hirabayashi Y, Kleinstreuer NC*4, Kojima H, Nishizawa C*5, Prieto P*6, Ratzlaff DE*7, Jeong J*8, Lee J*8, Yang Y*9, Lin P*10, Sullivan K*2, Casey W*4: International regulatory uses of acute systemic toxicity data and integration of new approach methodologies.

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Chemical regulatory authorities around the world require systemic toxicity data from acute exposures via the oral, dermal, and inhalation routes for human health risk assessment. To identify opportunities for regulatory uses of non-animal replacements for these tests, we reviewed acute systemic toxicity testing requirements for jurisdictions that participate in the International Cooperation on Alternative Test Methods (ICATM): Brazil, Canada, China, the European Union, Japan, South Korea, Taiwan, and the USA. The chemical sectors included in our review of each jurisdiction were cosmetics, consumer products, industrial chemicals, pharmaceuticals, medical devices, and pesticides. We found acute systemic toxicity data were most often required for hazard assessment, classification, and labeling, and to a lesser extent quantitative risk assessment. Where animal methods were required, animal reduction methods were typically recommended. For many jurisdictions and chemical sectors, non-animal alternatives are not accepted, but several jurisdictions provide guidance to support the use of test waivers to reduce animal use for specific applications. An understanding of international regulatory requirements for acute systemic toxicity testing will inform ICATM's strategy for the development, acceptance, and implementation of non-animal alternatives to assess the health hazards and risks associated with acute toxicity.

Keywords: acute systemic toxicity, animal alternatives, regulatory toxicology

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Tanabe I*1, Yoshida K*1, Ishikawa S*1, Ishimori K*1, Hashizume T*1, Yoshimoto T*2, Ashikaga T: Development of an *In Vitro* Sensitisation Test Using a Coculture System of Human Bronchial Epithelium and Immune Cells.

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Chemical respiratory sensitisation is a serious health problem. However, to date, there are no validated test methods available for identifying respiratory sensitisers. The aim of this study was to develop an *in vitro* sensitisation test by modifying the human cell line activation test (h-CLAT) to detect respiratory sensitisers and distinguish them from skin sensitisers. THP-1 cells were exposed to the test chemicals (two skin sensitisers and six respiratory sensitisers), either as monocultures or as cocultures with air-liquid interface-cultured reconstructed human bronchial epithelium. The responses were analysed by measuring the expression levels of surface markers on THP-1 cells (CD86, CD54 and OX40L) and the concentrations of cytokines in the culture media (interleukin (IL)-8, IL-33 and thymic stromal lymphopoietin (TSLP)). The cocultures exhibited increased CD54 expression on THP-1 cells; moreover, in the cocultures but not in the monocultures, exposure to two uronium salts (i.e. respiratory sensitisers) increased CD54 expression on THP-1 cells to levels above the criteria for a positive h-CLAT result. Additionally, exposure to the respiratory sensitiser abietic acid, significantly increased IL-8 concentration in the culture medium, but only in the cocultures. Although further optimisation of the method is needed to distinguish respiratory from skin sensitisers by using these potential markers (OX40L, IL-33 and TSLP), the coculture of THP-1 cells with bronchial epithelial cells offers a potentially useful approach for the detection of respiratory sensitisers.

Keywords: air-liquid interface, bronchial epithelial cells, coculture

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The Epidermal Sensitization Assay (EpiSensA) is a reconstructed human epidermis (RhE)-based gene expression assay for predicting the skin sensitization potential of chemicals. Since the RhE model is covered by a stratified stratum corneum, various kinds of test chemicals, including lipophilic ones and pre-/pro-haptens, can be tested with a route of exposure akin to an *in vivo* assay and human exposure. This article presents the results of a formally managed validation study of the EpiSensA that was carried out by three participating laboratories. The purpose of this validation study was to assess transferability of the EpiSensA to new laboratories along with its within- (WLR) and between-laboratory reproducibility (BLR). The validation study was organized into two independent stages. As demonstrated during the first stage, where three sensitizers and one non-sensitizer were correctly predicted by all participating laboratories, the EpiSensA was successfully transferred to all three participating laboratories. For Phase I of the second stage, each participating laboratory performed three experiments with an identical set of 15 coded test chemicals resulting in WLR of 93.3%, 93.3%, and 86.7%, respectively. Furthermore, when the results from the 15 test chemicals were combined with those of the additional 12 chemicals tested in Phase II of the second stage, the BLR for 27 test chemicals was 88.9%. Moreover, the predictive capacity among the three laboratories showed 92.6% sensitivity, 63.0% specificity, 82.7% accuracy, and 77.8% balanced accuracy based on murine local lymph node assay (LLNA) results. Overall, this validation study concluded that EpiSensA is easily transferable and sufficiently robust for assessing the skin sensitization potential of chemicals.

Keywords: reconstructed human epidermis, skin sensitisation, validation study

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Yamamoto N*1,2, Hiramatsu N*1, Kato Y*3, Sato A*3, Kojima H: Development of an Eye Irritation Test Method Using an In House Fabrication of a Reconstructed Human Cornea like Epithelium Model for Eye Hazard Identification.
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In a previous study, a novel human corneal-like epithelium model utilizing an immortalized human corneal epithelial cell line (iHCE-NY1) was developed as an alternative to animal models to identify chemicals not classified under the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and was evaluated following the criteria of Test Guideline 492 of the Organization for Economic Co-operation and Development (OECD). In the present study, our aim was to establish an eye irritation test protocol using the iHCE-NY1 model to classify liquid chemicals under the GHS ocular hazard categories: no effect, no classification (No Cat.), Category 2 (Cat. 2) reversible effects, and Category 1 (Cat. 1) irreversible eye damage. The protocol involved exposing the iHCE-NY1 model to 31 liquid test chemicals for 5 min, followed by observation at post-incubation periods (PIPs) to assess recovery. Classification was based on cell viability, and histopathological findings on PIP days 7, 14, and 21. The outcomes were compared with an established database of classifications. All Cat. 1 liquid chemicals, 62.5% of No Cat., and 63.2% of Cat. 2 were correctly categorized. This study demonstrates that the iHCE-NY1 model can not only distinguish No Cat. test liquid chemicals but also differentiate between Cat. 2 and Cat. 1 liquid chemicals.

Keywords: eye irritation, human corneal epithelial cell, reconstructed human cornea-like epithelium model

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