Izutsu K, Abe Y, Yomota C*, Yoshida H: Morphological analysis of spherical adsorptive carbon granules using three-dimensional X-ray micro-computed tomography.

Chem Pharm Bull. 2020;68:179-180

The purpose of this study was to clarify applicability of three-dimensional X-ray micro-computed tomography (3D X-ray micro-CT) to elucidate interior morphology of spherical adsorptive carbon fine granules. Scanning of small single spherical granule hold on the rotating sample stage provided the structural information without particular preparation (e.g., slicing) that can affect the definite morphology. The three model formulations with similar appearance showed different internal structure in the 3D images, including large hollow in one of them. Other formulations showed some small empty or higher density area in the filled granules, suggesting uneven distribution of carbon. The results indicated relevance of the X-ray micro-CT analysis on the physical characterization of the spherical adsorptive carbon granule formulations.

Keywords: X-ray micro-computed tomography, spherical carbon granule, morphology

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宮崎玉樹，石田猛*1，茅野善行*2，山崎裕洋*3，若林啓造*4，大山国広*5，原章*6，野崎恵司*7，織田善純*8，久保田康裕*9，間和之助*10，岸本奈子*11，池田豊*12，松澤孝泰*13：経皮吸収型製剤のコールドフロー評価法に関する研究.

医薬品医療機器レギュラトリーサイエンス 2019;50:648-657

貼付剤のコールドフロー評価法に関する，外用製剤協議会技術委員会との共同試験結果を報告した．コールドフローの数値化手法につき，一般薬のニコチン製剤をモデルとした検討を行ったところ，はみ出した粘着剤の幅を指標とする測定法が妥当であると考えられた.

Keywords: cold flow, transdermal patches, evaluation method

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Watanabe A*1, Murayama S*2, Karasawa K*2, Yamamoto E, Morikawa S*3, Takita R*1, Murata S*1, Kato M*1,2: A simple and easy method of monitoring doxorubicin release from a liposomal drug formulation in the serum using fluorescence spectroscopy.

Chem Pharm Bull. 2019;67(4):367-371

Formulation of a drug as liposomes facilitates its delivery to the disease target. Rightly, liposomes are gaining popularity in the medical field. In order for the drug to show efficacy, release of the encapsulated drug from the liposome at the target site is required. However, the release is affected by the permeability of the lipid bilayer of the liposome, and it is important to examine the effect of the surrounding environment on the permeability. In this study, we showed the usefulness of fluorescence analysis, especially fluorescence fingerprint, for a rapid and simple monitoring of release of an encapsulated anticancer drug (doxorubicin) from its liposomal formulation (DOXIL). Our result indicated that the release is accelerated by the existence of membrane permeable ions, such as tris(hydroxymethyl)aminomethane, and blood proteins like albumin. Hence, monitoring of doxorubicin release by fluorescence analysis is useful for the efficacy evaluation of DOXIL in a biomimetic environment.

Keywords: liposomal drug, fluorescence fingerprint, doxorubicin release

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Akiyama K*, Horita K*, Sakamoto T, Satozono H*,

Takahashi H*, Goda Y: Monitoring the progress of Lactic acid fermentation in yogurt manufacturing using terahertz time-domain-attenuated total reflection spectroscopy

J Infrared, Millimeter and Terahertz Waves. 2019;40: 1160-7

Lactic acid fermentation in yogurt manufacturing can be monitored using terahertz (THz)-attenuated total-reflection (ATR) spectroscopy. Yogurt manufacturing was performed on an ATR prism. The THz absorption coefficient and pH were measured for the entire 1000 min of the fermentation process. The absorption spectra were similar to the spectrum of water at the THz range. Temporal changes in the absorption coefficient at 0.4, 1.0, and 1.6 THz all decreased during the fermentation process, with two inflection points. The absolute value of the change in temporal absorption was greater at high frequencies than at low frequencies. However, the normalized absorption coefficient was larger at 0.4 THz. Because temporal changes in absorption corresponded with temporal changes in pH, the absorption changes appeared to be caused by the decomposition of the milk ingredients during the lactic acid fermentation. THz measurements can therefore be applied to the nondestructive monitoring of lactic acid fermentation in yogurt manufacturing.

Keywords: terahertz spectroscopy, THz-ATR, lactic acid fermentation

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Ito M*1, Tokuda R*1, Suzuki H*1, Sakamoto T, Terada K*2, Noguchi S*1: Desolvation behavior of indinavir sulfate ethanol and follow-up by terahertz spectroscopy

Int J Pharm. 2019; Aug 15: 567: 118446

Active pharmaceutical ingredients are composed of single-component or multicomponent crystals. Multicomponent crystals include salts, co-crystals, and solvates. Indinavir sulfate is the ethanol solvate form of indinavir that is known to deliquesce through moisture absorption. However, the detailed behavior of solvent molecules in the crystal has not been investigated. In this study, we studied the desolvation mechanism of indinavir sulfate ethanol and investigated the behavior of solvent molecules in the solid from. Indinavir sulfate ethanol contained 1.7 molecules of ethanol, 0.7 of which desolvated at room temperature. They were originally two ethanol solvent molecules; one molecule of ethanol desolvated at room temperature, and the conformation of the remaining ethanol and t-butyl groups changed in conjunction with the removal of one ethanol molecule. Desolvation could hardly be detected by powder X-ray diffraction; however, it was detected using terahertz spectroscopy. Terahertz measurement of desolvation showed a high correlation with thermogravimetry data, suggesting that desolvation could be observed non-destructively using terahertz spectroscopy. We concluded that indinavir sulfate 1 ethanol deliquesced at 60% relative humidity, and it turned into an amorphous solid after drying.

Keywords: terahertz spectroscopy, indinavir sulfate ethanol, solvate

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Saito S*, Hattori Y*, Sakamoto T, Otsuka M*: Real-time monitoring of pharmaceutical properties of medical tablets during direct tableting process by hybrid tableting process parameter-time profiles

Biomed Mater Eng. 2020; 30: 509-24.

BACKGROUND: Real-time monitoring is required for the pharmaceutical manufacturing process to produce high-quality pharmaceutical products. OBJECTIVE: Changes in the critical tableting process parameters of single-punch tableting machine due to variability in the moisture content of the raw powders were monitored by hybrid tableting pressure-time profiles. METHODS: After mixing of the raw powders, which consisted of theophylline, anhydrous lactose, potato starch and crystalline cellulose, they were stored at 0%, 45%, or 75% relative humidity (RH) for 24 h, respectively. Continuous tablet productions were carried out using the mixed powder samples at 10%, 45%, or 75% RH, respectively. The critical process parameters, such as upper and lower puncture pressures, die wall pressures, and inter-punch distances were recoded with the tableting machine, and then, tablet hardness (H), weight (W) and disintegration time (DT) of the tablets were measured. RESULTS: Hybrid tableting pressure-time profiles were obtained from various critical process parameters, and calibration models

to predict pharmaceutical properties were calculated based on the hybrid profiles using a partial-least-squares regression (PLSR) method. In addition, the consistency of the calibration models was verified by constructing robust calibration models. CONCLUSION: Informetrical analysis for tablets based on hybrid tableting pressure-time profiles could evaluate the change of tablet properties dependent on the moisture content in the raw powders during the tableting process. The changes of tableting properties and elasticity were caused by agglomeration of powder particles at moisture content.

Keywords: tableting compression, effect of moisture content, hybrid tableting pressure–time profiles

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Yamamoto Y[*1], Fujii M[*2], Fukami T[*3], Koide T: Evaluation of the three-dimensional distribution of droplets in a droplet dispersion-type ointment using confocal Raman microscopy.

J Drug Deliv Sci Technol. 2019;51:639-42

We evaluated the three-dimensional distribution of liquid droplets in a droplet dispersion-type ointment using a confocal Raman microscope. In component 1, which extracted the most frequently detected information, although the domains were heterogeneous, domains with high intensity were observed. From the Raman spectrum, it was suggested that this component provided information on the distribution in the ointment base. In component 2, images with dispersed domains with diameters less than 10 μm were observed. In the Raman spectrum obtained from component 2, peaks derived from propylene glycol and benzyl alcohol were observed, suggesting that the domain of this component reflected liquid droplets. The distribution of droplets in the depth direction was also confirmed. From these results, it was suggested that confocal Raman microscopy enables the stereoscopic evaluation of microscopic properties in droplet dispersion-type ointments.

Keywords: droplet dispersion-type, ointment, confocal Raman microscope

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Hoshino T[*1], Azuma M[*1], Yamada Y[*2], Titapiwatanakun V[*3], Fujii M[*1], Yamamoto Y[*2], Koide T, Fukami T[*1]: Measurement of the Water Content in Semi-solid Formulations Used to Treat Pressure Ulcers and Evaluation of Their Water Absorption Characteristics.

Chem Pharm Bull. 2019;67:929-34

We investigated the water contents in commercial semi-solid preparations used for pressure ulcer (PU) treatment using near-IR spectroscopy (NIRS) and compared the results with those measured using the Karl Fischer (KF) method. The aim of this study was to determine a standard method and select the appropriate topical preparation with the optimal moisture for PU treatment. The water absorption properties of bases and formulations were evaluated with a time-dependent factor using Transwell as the model membrane. KF and NIRS were applicable as measurement methods of the water content in semi-solid formulations. NIRS was shown to be a useful, simple, nondestructive tool that is more advantageous than the KF method. The water absorption characteristics tested using Transwell revealed that the rate of and capacity for water absorption are determined not only by the absorption ability of the polymer base but also by other factors, such as the osmotic pressure exerted by additives. KF and NIR measurements can be used to choose external skin preparations to control the amount of water in PU treatment.

Keywords: pressure ulcer, near infrared, water content

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Shimamura R*, Koide T, Hisada H*, Inoue M*, Fukami T*, Katori N, Goda Y: Pharmaceutical Quantification with Univariate Analysis Using Transmission Raman Spectroscopy.

Drug Dev Ind Pharm. 2019;45:1430-6

The purpose of this study was to investigate the quantification performance of transmission Raman

spectroscopy with univariate analysis. Model dosage forms containing acetaminophen and an excipient, lactose monohydrate, were prepared. The Raman spectra of the tablets were obtained using the modes of transmission, backscattering micro-spectroscopy, and wide area illumination. Calibration curves for quantification of acetaminophen in the tablets were created using peak heights of the Raman spectra. Of the three modes of measurement, the quantitative results by transmission had the highest correlation with those by conventional UV–vis methods. In the validation of quantification by the transmission mode with univariate analysis, a certain degree of daily variation was confirmed. Additionally, quantitative results using peak heights were compared with those of partial least squares (PLSs) multivariate analysis. The root mean square error of prediction (RMSEP) suggested that quantification using PLS provided better precision than the peak height method as expected. However, content uniformity test using large sample sizes by the Raman spectra is not required to be very highly predictive because they usually employ non-parametric criteria and include wide specification ranges. Therefore, univariate analysis using transmission Raman spectroscopy was a suitable quantitative method for conducting content uniformity tests of large sample sizes.

Keywords: Raman spectroscopy, transmission, univariate analysis

* Meiji Pharmaceutical University

Fujii M[*1], Yamamoto Y[*2], Koide T, Hamaguchi M[*3], Onuki Y[*3], Suzuki N[*4], Suzuki T[*4], Fukami T[*5]: Imaging Analysis Enables Differentiate of the Distribution of Pharmaceutical Ingredients in Tacrolimus Ointments.

Appl Spectrosc. 2019;73:1183-92

We demonstrated the difference in the distribution state of pharmaceutical ingredients between tacrolimus (TCR) original ointment and six kinds of generic medicines. Two-dimensional imaging and depth analysis using attenuated total reflection Fourier transform infrared (ATR FT-IR) spectroscopy and confocal Raman microscopy were used, in addition to the evaluation of pharmaceutical properties, including spreading properties, rheological properties, and amount of solvent. The solvents, such as propylene carbonate and triacetin, in TCR ointments formed liquid droplets and dispersed in hydrocarbon oils. Waxes, white beeswax and beeswax, formed other domains. Confocal Raman microscopy could detect liquid droplet size without coalescence of that on germanium or glass surfaces. The combination of ATR FT-IR and confocal Raman imaging would be a powerful tool to reveal the size and shape of liquid droplets of pharmaceutical ingredients in semisolid formulations.

Keywords: ATR, confocal Raman microscopy, ointment

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Yamamoto Y[*1], Yamauchi R[*2], Ohno S[*2], Asai K[*2], Fukami T[*3], Koide T: Evaluation of the Water Content and Skin Permeability of Active Pharmaceutical Ingredients in Ketoprofen Poultice Formulations Removed from Their Airtight Containers and Left at Room Temperature.

Biol Pharm Bull. 2019;42:2102-8

The poultice formulation is a patch containing a large amount of water. It is known that the water contained in the adhesive polymer layer (ADPL) of poultice affects the cooling sensation and skin permeability of the active pharmaceutical ingredient (API). In this study, we evaluated the relationship between the water content in a ketoprofen poultice formulation and the amount of time the poultice was left out at room temperature after removal from the airtight container, as well as the influence of the decreasing water content on the skin permeability of the API. After removing the poultice from the container for 1 h, the mass of the ADPL decreased by approximately 40%. When the near-infrared (NIR) spectrum of the ADPL of poultice was measured, the peaks reflecting the hydroxyl group were attenuated depending on the time left out at room temperature. It is suggested that the changes in the mass and NIR spectrum of the ADPL are caused by

the change in the water content. Moreover, when the permeability of API was evaluated on hairless mouse skin, the cumulative skin permeation amount and flux decreased, while the lag time was prolonged as the time left out increased. These results suggest that the skin permeability of the API is impaired by water evaporation and that maintaining the water in the ADPL in poultice is very important from not only the viewpoint of cooling sensation, tackiness and moisturizing but also the skin permeability of the API.

Keywords: poultice formulation, near infrared, skin permeability

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Inoue M*1, Osada T*1, Hisada H*1, Koide T, Fukami T*1, Roy A*2, Carriere J*2, Heyler R*2: Solid-State Quantification of Cocrystals in Pharmaceutical Tablets Using Transmission Low-Frequency Raman Spectroscopy.

Anal Chem. 2019;91(21):13427-32

To enable the continuous production of cocrystal-containing pharmaceutical tablets, guaranteeing the cocrystal content of the final pharmaceutical tablets in the solid state is critical. This study demonstrates the quantification of caffeine-glutaric acid cocrystals in model tablets using transmission low-frequency Raman spectroscopy. Although distinguishing between cocrystals and raw materials using conventional Raman spectroscopy is difficult, the use of low-frequency Raman spectroscopy enables the discrimination of cocrystals and raw materials. Low-frequency Raman spectra were analyzed by the partial least-squares method (PLS) to obtain the predicted contents in the model tablets. To evaluate the quantitative ability of this method, the root means square error of cross-validation (RMSECV) was determined by comparing the actual concentration and predicted content with a calibration curve. For cocrystal-containing tablets, the quantitative ability of the transmission mode (RMSECV = 2.06-3.17) was 13.4-31.4% higher than that of the backscattering mode (RMSECV= 2.37-3.91). The coexistence of raw crystalline materials did not affect the quantitative ability for cocrystals.

Keywords: low-frequency Raman spectroscopy, transmission, cocrystal

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Koide T, Takeuch Y*1, Otaki T*2, Yamamoto K*2, Shimamura R*1, Ohashi R*1, Inoue M*1, Fukami T*1, Izutsu KI: Quantification of a cocrystal and its dissociated compounds in solid dosage form using transmission Raman spectroscopy.

J Pharm Biomed Anal. 2020;177:112886. doi:10.1016/j.jpba.2019.112886

The performance of transmission Raman spectroscopy (TRS) for quantifying a cocrystal and its dissociation in solid dosage form was investigated. Some tablets containing 0%-20% (w/w) of a cocrystal of carbamazepine (CBZ)/succinic acid (SUC), 0%-4% of CBZ, 0%-4% of SUC, and 75%-99% of D-mannitol were prepared. The Raman spectra of these tablets were preprocessed using the standard normal variate (SNV) or multiplicative scatter correction (MSC) as well as the Savitzky Golay second derivative, and then, these were used to generate calibration models using partial least squares (PLS) regression. The performance of the model was superior when the MSC preprocessing spectra of the cocrystal between 200 and 1800 cm^{-1} were used for calibration. The determination coefficient of the PLS calibration curve for the CBZ/SUC cocrystal between 200 and 1800 cm^{-1} with MSC was 0.97, root mean square error of cross validation (RMSECV) was 1.16, and root mean square error of prediction (RMSEP) was 1.10. As in the case of the CBZ/SUC cocrystal, the performance of the model was superior when the MSC preprocessing spectra of CBZ and SUC between 200 and 1800 cm^{-1} were used for calibration. These data suggest that TRS is useful for quantifying a cocrystal and its dissociation compounds in solid dosage forms.

Keywords: Raman spectroscopy, transmission, cocrystal

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Nomura K*1, Titapiwatanakun V*2, Hisada H*1, Koide T, Fukami T*1: In Situ Monitoring of

the Crystalline State of Active Pharmaceutical Ingredients during High-shear Wet Granulation Using a Low-frequency Raman Probe.

Euro J Pharm Biopharm. 2020;147:1-9

Optimization of manufacturing processes based on scientific evidence is important in the quality control of active pharmaceutical ingredients (APIs) and drug products, particularly when crystal forms change during production, which could affect subsequent drug performance. In this study, we verified crystalline states using various crystal faces and excipients during high-shear wet granulation based on non-contact low-frequency (LF) Raman probe monitoring. Four model drugs [indomethacin (IND), acetaminophen (APAP), theophylline (TP), and caffeine (CAF) polymorphs and cocrystals] were mixed with microcrystalline cellulose and hydroxypropyl cellulose with the addition of water over time. The LF Raman probe showed comparatively high sensitivity in monitoring 5–20% APAP and IND in a wet mass. Notably, as observed from the characteristic LF Raman peak shifts, form I TP and CAF and their cocrystals were more susceptible to transformation to the monohydrate form than form II. This method was also shown to be applicable in monitoring a commercial formulation of eight excipients and revealed crystalline transformations after 15 min of mixing. Therefore, probe-type LF Raman spectroscopy can be successfully employed to distinguish and monitor the crystalline state of APIs in real time during high-shear wet granulation, in which there is a risk of crystal transformation.

Keywords: low-frequency Raman spectroscopy, probe, high-shear wet granulation

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Hisada H[*1], Okayama A[*1], Hoshino T[*1], Carriere J[*2], Koide T, Yamamoto Y[*3], Fukami Y[*2]: Determining the distribution of active pharmaceutical ingredients in combination tablets using near-infrared and low-frequency Raman spectroscopy imaging.

Chem Pharm Bull. 2020;68:155-60

Combination tablets containing multiple active pharmaceutical ingredients (APIs) are expected to improve patient convenience by decreasing the number of tablets to be taken; thus, numerous formulations containing multiple APIs have recently been developed. To allow for dose adjustments based on patient conditions, many tablets have a bisection line to allow equal division of tablets. However, there have been no investigations regarding content uniformity among divided combination tablets. Therefore, in this study, the content uniformity of combination tablets after division was investigated using near IR and low-frequency (LF) Raman spectroscopy imaging as well as the Japanese Pharmacopoeia (JP) content uniformity tests. As model drugs, five tablets of three combination drugs containing 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) and benserazide hydrochloride (BNS) as APIs for treating Parkinson's disease were bisected; the resultant 10 samples were subjected to the JP content uniformity tests. We found that acceptance values of L-DOPA and BNS were 11.0–21.9% and 13.3–17.5%, respectively, with some non-conformity to the maximum allowed acceptance value (15.0%) as per the current JP. Image analyses by near IR showed that L-DOPA, BNS, lactose, and corn starch were uniformly distributed in each tablet; moreover, LF Raman spectroscopy imaging also supported the result that L-DOPA, BNS, and lactose were evenly distributed. Therefore, drug content in the tablets was uniform; thus, careful manipulation was recommended in the tablet bisection. However, the results of bisection line specifications and hardness tests revealed that the ease of division differed depending on the tablets, which warrants attention.

Keywords: near infrared, low-frequency Raman spectroscopy, imaging

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Fujii M[*1], Gato K[*2], Ozawa Y[*2], Hisada H[*2], Koide T, Inoue M[*2], Fukami T[*2]: In situ monitoring of lipid phase state make target lipid mixtures similar to intercellular lipid in the stratum corneum.

Euro J Lipid Sci Technol. 2020;122:1900171. doi:10.1002/ejlt.201900171.

In this study, lipid structural change is monitored using Raman spectroscopy during heat treatment, along with the impact of lipid states on the structural and physical properties during the preparation

process of the dried and hydrated lipid mixture (LM) similar to intercellular lipid in stratum corneum. The microstructures and thermal behavior of these LMs change depending on the melting of lipid ingredients in the preparation process. It is recognized that variable temperature Raman spectroscopy (VT-Raman) is a useful and attractive tool for the sensitive in situ monitoring of lipid state changes and lipid melting. The LMs can incorporate D_2O into their structures regardless of preparation temperature due to increasing lattice distance by hydration. These results suggest that monitoring lipid structural changes during the heating step is important to precisely prepare target LMs. Practical Applications: This study reveals that VT-Raman is a useful and attractive tool in in situ monitoring of lipid state change and lipid melting. The monitoring of the preparation process by VT-Raman is necessary to precisely prepare the target LM similar to intercellular lipid of stratum corneum because the microstructures and thermal properties of these LMs change depending on the melting of lipid ingredients during the preparation process.

Keywords: Raman spectroscopy, heat treatment, lipid mixture

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Sakai-Kato K*1, Yoshida K*1, Ohgita T*2, Takechi-Haraya Y, Demizu Y, Saito H*2: Refining calibration procedures of circular dichroism spectrometer to improve usability.

Analytical Sciences. 2019;35:1275-1278

Circular dichroism (CD) is a technique used for conformational studies of peptides and proteins. We studied the specific calibration procedures of CD spectrometers based on procedures specified in the European Pharmacopoeia. We aimed to develop procedures to improve the usability of CD, in addition to reducing adverse effects on users' health. The use of ethanol instead of 1,4-dioxane as the solvent for isoandrosterone was examined. Both solvents yielded the same maximum value of +3.3 for molar CD. We also studied a two-point calibration method using (1S)-(+)-ammonium 10-camphorsulfonate instead of (1S)-(+)-10-camphorsulfonic acid, which is a hygroscopic compound. Both compounds yielded similar results and the values for (1S)-(+)-ammonium 10-camphorsulfonate of 2.39 ± 0.04 and -4.92 ± 0.06 at 290.5 and 192.5 nm, respectively, were within the criteria defined in the European Pharmacopoeia. The inter-laboratory repeatability was also acceptable. These studies provide specific procedures for calibrating CD spectrometers for drug development.

Keywords: circular dichroism, calibration, usability

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Takechi-Haraya Y, Goda Y, Izutsu K, Sakai-Kato K*: Improved atomic force microscopy stiffness measurements of nanoscale liposomes by cantilever tip shape evaluation.

Analytical Chemistry. 2019;91:10432-10440

The stiffness of nanoscale liposomes, as measured by atomic force microscopy (AFM), was investigated as a function of temperature, immobilization on solid substrates, and cantilever tip shape. The liposomes were composed of saturated lipids and cholesterol, and the stiffness values did not change over the temperature range of 25-37℃ and were independent of immobilization methods. However, the stiffness varied with the tip shape of the cantilever. Therefore, 24 cantilevers were evaluated in terms of tip shape and aspect ratio (length/width) via a nonblind tip reconstruction (NBTR) method that used a tip characterizer with isolated line structures having specified dimensions. A standard for screening the tip geometry was established. A 24-fold improvement in stiffness precision in terms of relative standard deviation was demonstrated by using at least three cantilevers that meet the criteria of having a tip aspect ratio greater than 2.5 and a quadratic tip shape function. A significant difference in stiffness was subsequently revealed between dipalmitoylphosphatidylcholine-cholesterol (1:1 molar ratio) and egg yolk phosphatidylcholine-cholesterol (1:1 molar ratio) liposomes. Tip analysis using NBTR improved the precision of AFM stiffness measurements, which will enable the control of mechanical properties of nanoscale liposomes for various applications.

Keywords: atomic force microscopy, liposome stiffness, nonblind tip reconstruction method

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Aoyama M, Hashii N, Tsukimura W*, Osumi K*, Harazono A, Tada M, Kiyoshi M, Matsuda A*, Ishii-Watabe A: Effects of terminal galactose residues in mannose α1-6 arm of Fc-glycan on the effector functions of therapeutic monoclonal antibodies.
mAbs. 2019;11:826-836.

Typical crystallizable fragment (Fc) glycans attached to the CH2 domain in therapeutic monoclonal antibodies (mAbs) are core-fucosylated and asialo-biantennary complex-type glycans, e.g., G2F (full galactosylation), G1aF (terminal galactosylation on the Man α1-6 arm), G1bF (terminal galactosylation on the Man α1-3 arm), and G0F (non-galactosylation). Terminal galactose (Gal) residues of Fc-glycans are known to influence effector functions such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (CDC), but the impact of the G1F isomers (G1aF and G1bF) on the effector functions has not been reported. Here, we prepared four types of glycoengineered anti-CD20 mAbs bearing homogeneous G2F, G1aF, G1bF, or G0F (G2F mAb, G1aF mAb, G1bF mAb, or G0F mAb, respectively), and evaluated their biological activities. Interestingly, G1aF mAb showed higher C1q- and FcγR-binding activities, CDC activity, and FcγR-activation property than G1bF mAb. The activities of G1aF mAb and G1bF mAb were at the same level as G2F mAb and G0F mAb, respectively. Hydrogen-deuterium exchange/mass spectrometry analysis of dynamic structures of mAbs revealed the greater involvement of the terminal Gal residue on the Man α1-6 arm in the structural stability of the CH2 domain. Considering that mAbs interact with FcγR and C1q via their hinge proximal region in the CH2 domain, the structural stabilization of the CH2 domain by the terminal Gal residue on the Man α1-6 arm of Fc-glycans may be important for the effector functions of mAbs. To our knowledge, this is the first report showing the impact of G1F isomers on the effector functions and dynamic structure of mAbs.

Keywords: therapeutic monoclonal antibody, complement-dependent cytotoxicity, glycoengineering

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Tanaka Y*1, Yamada S*1, Connop SL*1, Hashii N, Sawada H*2, Shih Y*1, Nishida H*1: Vitelline membrane proteins promote left-sided nodal expression after neurula rotation in the ascidian, Halocynthia roretzi.
Dev Biol. 2019;449:52-61.

Stereotyped left-right asymmetry both in external and internal organization is found in various animals. Left-right symmetry is broken by the neurula rotation in the ascidian, Halocynthia roretzi. Neurula embryos rotate along the anterior-posterior axis in a counterclockwise direction, and the rotation stops when the left side of the embryo is oriented downwards, resulting in contact of the left-side epidermis with the vitelline membrane at the bottom of perivitelline space. Then, such contact induces the expression of nodal and its downstream Pitx2 gene in the left-side epidermis. Vitelline membrane is required for the promotion of nodal expression. Here, we showed that a chemical signal from the vitelline membrane promotes nodal gene expression, but mechanical stimulus at the point of contact is unnecessary since the treatment of devitellinated neurulae with an extract of the vitelline membrane promoted nodal expression on both sides. The signal molecules are already present in the vitelline membranes of unfertilized eggs. These signal molecules are proteins but not sugars. Specific fractions in gel filtration chromatography had the nodal promoting activity. By mass spectrometry, we selected 48 candidate proteins. Proteins that contain both a zona pellucida (ZP) domain and epidermal growth factor (EGF) repeats were enriched in the candidates of the nodal inducing molecules. Six of the ZP proteins had multiple EGF repeats that are only found in ascidian ZP proteins. These were considered to be the most viable candidates of the nodal-inducing molecules. Signal molecules are anchored to the entire vitelline membrane, and contact sites of signal-receiving cells are spatially and mechanically controlled by the neurula rotation. In this context, ascidians are unusual with respect to mechanisms for specification of the left-right axis. By suppressing formation of epidermis monocilia, we also showed that epidermal cilia drive the neurula rotation but are dispensable for sensing the signal from the vitelline membrane.

Keywords: ascidian, halocynthia roretzi, left–right asymmetry

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Shimizu Y*1, Yoneda K*2, Shirasago Y*1, Suzuki T*1, Tada M, Ishii-Watabe A, Sugiyama K*3, Suzuki T*4, Wakita T*1, Yagi K*2, Kondoh M*2, Fukasawa M*1: Human-rat chimeric anti-occludin monoclonal antibodies inhibit hepatitis C virus infection.
Biochem Biophys Res Commun. 2019;514(3):785-790.

Occludin (OCLN), an integral tetra-spanning plasma membrane protein, is a host entry factor essential for hepatitis C virus (HCV) infection, making it a promising host-targeting molecule for HCV therapeutic intervention. We previously generated rat anti-OCLN monoclonal antibodies (mAbs) that strongly prevented HCV infection in vitro and in vivo. In the present study, we attempted to improve the druggability of the extracellular loop domain-recognizing anti-OCLN mAbs, namely clones 1-3 and 37-5, using genetic engineering. To avoid adverse reactions induced by antibody-dependent cellular cytotoxicity and enhance the antibody stability, we developed human-rat chimeric immunoglobulin G4 S228P mutant (IgG4m) forms of clones 1-3 and 37-5 (named Xi 1-3 and Xi 37-5, respectively) by grafting the variable regions of the light and heavy chains of each rat anti-OCLN mAb into those of human IgG4m. The constructed Xi 1-3 and Xi 37-5 chimeras demonstrated levels of affinity and specificity similar to each parental rat anti-OCLN mAb, and the Fcγ receptor IIIa was not activated by the antigen-bound chimeric mAbs, as expected. Both chimeric mAbs inhibited in vitro infection with various HCV genotypes. These results indicate that the IgG4m forms of human-rat chimeric anti-OCLN mAbs may be potential candidate molecules of host-targeting antivirals with pan-genotypic anti-HCV activity.
Keywords: hepatitis C virus, occludin, monoclonal antibody

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森本和滋，小林哲，柴田寛子，石井明子：我が国初のバイオ医薬品（ホルモン，サイトカイン，酵素類等）のFDAとEMAでの承認の有無について．
臨床評価 2019;47(1):87-97.

Objective：To study whether 13 biopharmaceuticals originally developed in Japan, which consisted of 9 hormones and cytokines, and 3 enzymes, were approved by the FDA or EMA.

Methods: A total of 13 biopharmaceuticals first marketed in Japan from 1985 to 2016 were studied. The approval date and label of each medicine were obtained from the databases of the Pharmaceuticals and Medical Devices Agency (PMDA), FDA, and EMA.

Results and Discussion：Mecasermin was approved on Oct 5,1994 in Japan and on Aug 30, 2005 by the FDA as a treatment of growth failure in children with severe primary IGF-1 deficiency or with growth hormone deletion. Lenograstim was approved in 11 countries, including 6 European countries, as a granulocyte colony-stimulating factor (G-CSF) agent in 1993-1995. We further investigated why the other 11 biopharmaceuticals were not approved by the FDA or EMA. We also discussed the importance of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines in adapting new Good Clinical Practice (GCP) in 1998, and that of the quality of biotechnological/biological products. The establishment of the PMDA in 2004 and history of transparency enhancement in the drug review process were also discussed.
Keywords: japanese new biopharmaceuticals, ICH guidelines, transparency enhancement in the drug review process

Akiba H*1,2, Tamura H*1,3, Kiyoshi M, Yanaka S*4, Sugase K*4, Caaveiro JMM*1,5, Tsumoto K*1,2: Structural and thermodynamic basis for the recognition of the substrate-binding cleft on hen egg lysozyme by a single-domain antibody.
Sci Rep. 2019;9(1):15481. doi: 10.1038/s41598-019-50722-y

Single-domain antibodies (VHHs or nanobodies), developed from heavy chain-only antibodies of camelids, are gaining attention as next-generation therapeutic agents. Despite their small size, the high affinity and specificity displayed by VHHs for antigen molecules rival those of IgGs. How such

small antibodies achieve that level of performance? Structural studies have revealed that VHHs tend to recognize concave surfaces of their antigens with high shape-complementarity. However, the energetic contribution of individual residues located at the binding interface has not been addressed in detail, obscuring the actual mechanism by which VHHs target the concave surfaces of proteins. Herein, we show that a VHH specific for hen egg lysozyme, D3-L11, not only displayed the characteristic binding of VHHs to a concave region of the surface of the antigen, but also exhibited a distribution of energetic hot-spots like those of IgGs and conventional protein-protein complexes. The highly preorganized and energetically compact interface of D3-L11 recognizes the concave epitope with high shape complementarity by the classical lock-and-key mechanism. Our results shed light on the fundamental basis by which a particular VHH accommodate to the concave surface of an antigens with high affinity in a specific manner, enriching the mechanistic landscape of VHHs.

Keywords: single-domain antibody, VHH, nanobody

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Tada M, Aoyama M, Ishii-Watabe A: Fcγ Receptor Activation by Human Monoclonal Antibody Aggregates.

J Pharm Sci. 2020;109(1):576-583.

Protein aggregates are a potential risk factor for immunogenicity. The measurement, characterization, and control of protein aggregates in drug products are indispensable for the development of biopharmaceuticals, including therapeutic mAbs. In this study, Fcγ receptor (FcγR)-expressing reporter cell lines were used to analyze the FcγR-activation properties of mAb aggregates. Comparison of aggregates of mAbs harboring different IgG subclasses revealed that the FcγR-activation profiles of the mAb aggregates were dependent on IgG subclass. In addition, aggregates of Fc-engineered mAb with enhanced FcγR-activation properties exhibited stronger activation of FcγRs than was observed in the wild-type aggregates, whereas aggregates of Fc-engineered mAb with decreased FcγR-activation properties showed reduced activation. These results suggest that FcγR activation by mAb aggregates depends greatly on the Fc functions of the native (nonaggregated) mAbs. We also showed that aggregates of mAbs smaller than 1 μm in size have the potential to directly activate FcγRs. Unintended immune cell activation can be induced on account of FcγR activation by mAb aggregates and such FcγR activation may contribute to immunogenicity, and therefore, analysis of the FcγR-activation properties of mAb aggregates using FcγR-expressing reporter cell lines is a promising approach for the characterization of mAb aggregates.

Keywords: monoclonal antibody, immunogenicity, aggregation

Suzuki T, Tada M, Ishii-Watabe A: Development of anti-drug monoclonal antibody panels against adalimumab and infliximab.

Biologicals. 2020;63:39-47.

The generation of anti-drug antibodies (ADAs) is one of the most serious problems in therapy using monoclonal antibodies (mAbs), because ADAs can impact the pharmacokinetics, efficacy, and safety of mAbs. It is therefore important to detect the generated ADAs in patients. For the appropriate detection of ADAs, methods that detect various types of ADAs (e.g., low- and high-affinity ADAs) are needed, but since there are no adequate reference preparations of ADAs relevant to human ADAs in most cases, it is difficult to determine whether or not the developed methods have enough analytical performance. Here, we developed human-rat chimeric ADA panels against the anti-TNF-α therapeutic antibodies infliximab and adalimumab. The developed ADA panels consist of 7 (for infliximab) and 11 (for adalimumab) ADAs with various binding characters, and most of the ADAs are neutralizing antibodies. Using these ADA panels, we compared the detectability of model methods, i.e., binding assays using SPR, BLI, and ECL, and a cell-based assay to detect neutralization activity. Since we obtained ADAs showing low and high responses with the various methods, the ADA panels we developed were shown to be useful for the development of ADA assays.

Keywords: adalimumab, infliximab, anti-drug antibody

Hashii N, Tousaka Y, Arai K*1, Goda R*2, Inoue N*3, Murata K*4, Okuzono T*5, Sasahara S*3, Shigeyama T*4, Tachiki H*3, Yamane S*5, Saito Y, Ishii-Watabe A: Generic MS-based method for the bioanalysis of therapeutic monoclonal antibodies in nonclinical studies.

Bioanalysis. 2020;12:231-243.

Aim: A generic bioanalytical method was developed to quantify therapeutic IgG1 monoclonal antibodies (mAbs) in mouse sera by combining an easy sample preparation method with LC/MS using selected reaction monitoring. Materials & methods: Rituximab and trastuzumab were used as model mAbs. A synthetic stable isotope-labeled peptide or a stable isotope-labeled mAb was used as an internal standard. The method feasibility was evaluated by a collaborative study involving six laboratories. Results: The calibration curve ranged from 1.0 to 1000.0 μg/ml (correlation coefficient >0.99). The validation parameters including selectivity, linearity of calibration curve, accuracy and precision met the predefined acceptance criteria. Conclusion: Our method is a useful bioanalytical method for the quantification of therapeutic IgG mAbs in nonclinical animal studies.

Keywords: LC/MS, monoclonal antibody, selected reaction monitoring

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原園景，木吉真人，川崎ナナ*，石井明子：日本薬局方糖鎖試験法の国際調和に関する研究―日局参考情報「単糖分析及びオリゴ糖分析／糖鎖プロファイル法」への標準的な糖鎖試験の手順の追加に関する考察―.

*医薬品医療機器レギュラトリーサイエンス*2019;50:704-718.

In this study, we optimized sample preparation procedures and mixed-mode of hydrophilic interaction and anion-exchange chromatography of 2-aminobenzamide-labeled glycans. α1 Acid glycoprotein is used as a model for acidic glycans, and bovine ribonuclease B and two monoclonal antibodies derived from CHO and NS0 are used as models for neutral glycans. Furthermore, we considered the contents, description format, and useful analysis methods and techniques depending on characteristics of glycan structures in order to add representative procedures to the general information.

Keywords: glycosylation analysis, oligosaccharide profiling, Japanese pharmacopoeia

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Lee KH[*71], Sihlbom C[*75], Adamczyk B[*73], Jin C[*73], Karlsson NG[*73], Örnros J[*73], Larson G[*74], Nilsson J[*74], Meyer B[*75], Wiegandt A[*75], Komatsu E[*76], Perreault H[*76], Bodnar ED[*76],4, Said N[*77], Francois YN[*77], Leize-Wagner E[*77], Maier S[*78], Zeck A[*78], Heck AJR[*79], Yang Y[*79], Haselberg R[*80], Yu YQ[*81], Alley W[*81], Leone JW[*82], Yuan H[*82], Stein SE[*1].: NIST Interlaboratory Study on Glycosylation Analysis of Monoclonal Antibodies: Comparison of Results from Diverse Analytical Methods.

Mol Cell Proteomics. 2020;19(1):11-30.

Glycosylation is a topic of intense current interest in the development of biopharmaceuticals because it is related to drug safety and efficacy. This work describes results of an interlaboratory study on the glycosylation of the Primary Sample (PS) of NISTmAb, a monoclonal antibody reference material. Seventy-six laboratories from industry, university, research, government, and hospital sectors in Europe, North America, Asia, and Australia submitted a total of 103 reports on glycan distributions. The principal objective of this study was to report and compare results for the full range of analytical methods presently used in the glycosylation analysis of mAbs. Therefore, participation was unrestricted, with laboratories choosing their own measurement techniques. Protein glycosylation was determined in various ways, including at the level of intact mAb, protein fragments, glycopeptides, or released glycans, using a wide variety of methods for derivatization, separation, identification, and quantification. Consequently, the diversity of results was enormous, with the number of glycan compositions identified by each laboratory ranging from 4 to 48. In total, one hundred sixteen glycan compositions were reported, of which 57 compositions could be assigned consensus abundance values. These consensus medians provide community-derived values for NISTmAb PS. Agreement with the consensus medians did not depend on the specific method or laboratory type. The study provides a view of the current state-of-the-art for biologic glycosylation measurement and suggests a clear need for harmonization of glycosylation analysis methods.

Keywords: glycosylation analysis, interlaboratory study, NISTmAb

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生薬学雑誌 2020;74:35-45

Hampi（反鼻）is an animal crude drug obtained from *Gloydius blomhoffii* H. Boie and *G. brevicaudus* Stejneger, which is obtained after removal of the skin and internal organs. It is compounded into many crude drug products, mainly for analeptic effect, and its annual transaction amount in Japan reaches ca. five tons. Therefore, the drug has been standardized by listing it in Non-JP Crude Drug Standards 2018, where *Ptyas dhumnades* Cantor is defined as the source animal together with *G. blomhoffii* and *G. brevicaudus*. In this paper, we report the development of the identification test by TLC in preparation for the listing. Two indicator spots of the test were purified from *G. blomhoffii* and *P. dhumnades* using repeated column chromatography. The chemical structures of those 2 spots were elucidated as (i) the mixture of phosphatidylethanolamine (1) and its analogues and (ii) the mixture of lysophosphatidylethanolamine (2) and its analogues based on the comparison of results of TLC, LC/MS, and NMR with those of authentic compounds. The TLC used silica gel as chromatographic support and an ethyl acetate : ethanol (99.5) : water (1 : 1 : 1) mixture as developing solvent. The identification test had a developing length of 7 cm, used ninhydrin reagent for visualization, and reported an Rf of 0.7 for both the mixture of phosphatidylethanolamine and its analogues, and the mixture of lysophosphatidylethanolamine and its analogues.

Keywords: Hampi, identification test, glycerophospholipids

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堀井周文*，小此木明*，高橋隆二*，鎌倉浩之，袴塚高志，合田幸広：八味地黄丸エキス製剤および湯剤の同等性に関する研究.

生薬学雑誌 2020;74:46-57

Our previous studies [Horii, C. *et al., Shoyakugaku Zasshi*, **68**(1), 9-12 (2014); *Shoyakugaku Zasshi*, **69**(2), 59-65 (2015); *Shoyakugaku Zasshi*, **68**(2), 65-69, (2014); *Shoyakugaku Zasshi*, **73**(2), 73-83 (2019)], in which bioequivalence between the Kakkonto /

Shoseiryuto decoction and its extract preparation was evaluated, revealed that some components can be marker compounds for bioequivalence but not others. In this study, we selected Hachimijiogan containing benzoylmesaconine, benzoylhypaconine, and 14-anisoylaconine specified as marker compounds by the Japanese Pharmacopoeia for quantification for quality control, and evaluated these components as possible marker compounds for bioequivalence.

Six healthy adult males were randomly divided into two groups, and an oral administration crossover study was performed. Changes in the plasma concentrations of 10 components (benzoylmesaconine, benzoylhypaconine, 14-anisoylaconine, alisol A, alisol A monoacetate, alisol B, alisol B monoacetate, loganin, morroniside, and paeoniflorin) were evaluated. As a result, the plasma concentration of each component in both the decoction and extract preparation varied among blood collection sites.

A t-test revealed a significant difference ($p<0.01$) in the plasma concentration of benzoylhypaconine 4 h after administration, a significant difference ($p<0.05$) in the plasma concentration of alisol A monoacetate 1 h after administration, and a significant difference ($p<0.05$) in the plasma concentration of loganin 4 h after administration, for the decoction and the extract. However, significant differences in the plasma concentrations of other constituents were not noted for the decoction and extract.

Alisol B and alisol B monoacetate could not be quantified due to an inadequate SN ratio (SN rate 10 or more). Analysis of variance for 8 components after excluding alisol B and alisol B monoacetate showed a significant difference ($p<0.05$) in the area under the blood concentration-time curve ($AUC_{0\text{-}8}$) for benzoylmesaconine in the subjects' neck.

The preparation, time and subjects did not differ significantly as a factor, so the statistical power (1-β) was calculated (except for alisol B and alisol B).

Both the peak plasma concentration (C_{max}) and $AUC_{0\text{-}8}$ values for all 8 components had inadequate (< 80%) statistical powers (1-β).

Next, the number of subjects needed to achieve sufficient statistical power was estimated based on the obtained results. The statistical powers of both C_{max} and $AUC_{0\text{-}8}$ were adequate (≧ 80%) when the number of subjects (1 group) was ≧ 24 (1 group) for benzoylmesaconine, ≧ 25 for 14-anisoylaconine, and ≧ 24 for alisol A. On the other hand, the statistical power was inadequate even when the number of subjects was 61 (1 group) for benzoylhypaconine, alisol A monoacetate, loganin, paeoniflorin, or morroniside.

The contents of alisols have been reported to vary in Alisma Tuber. Considering conversion due to metabolism, alisol A is also difficult to use as a marker compound. Therefore, in this prescription, benzoylmesaconine and 14-anisoylaconine may be appropriate marker compounds.

Keywords: bioequivalence, Hachimijiogan, changes in plasma concentration

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Yoshitomi T, Wakana D*[1], Uchiyama N, Tsujimoto T, Kawano N*[2], Yokokura T*[3], Yamamoto Y*[4], Fuchino H*[2], Hakamatsuka T, Komatsu K*[5], Kawahara N*[2], Maruyama T: ^{1}H-NMR based metabolomic analysis coupled with reversed-phase solid phase extraction for sample preparation of Saposhnikovia roots and related crude drugs.

J. Nat. Med. 2020;74:65-75

^{1}H NMR-based metabolomics has been applied in research on food, herbal medicine, and natural products. Although excellent results were reported, samples were directly extracted with a deuterated solvent (e.g., methanol-d_4 or D_2O) in most reports. As primary metabolites account for most of the results, data for secondary metabolites are partially reflected. Consequently, secondary metabolites tend to be excluded from factor loading analysis, serving as a significant unfavorable feature of ^{1}H NMR-based metabolomics when investigating biologically active or functional components in natural products and health foods. Reversed-phase solid-phase extraction column (RP-SPEC) was applied for sample preparation in ^{1}H NMR-based metabolomics to overcome this feature. The methanol extract from Saposhnikoviae radix (SR), an important crude drug, was fractionated with RP-SPEC into 5% methanol-eluting fractions, and the remaining fraction was collected. Each fraction was subjected to ^{1}H NMR-based metabolomics and compared to results from conventional ^{1}H NMR-based metabolomics. Based on principal component analysis (PCA) and partial least squares projections

to latent structures discriminant analysis (PLS-DA), the 5% methanol fraction and conventional method reflected the amount of saccharides such as sucrose on the PC1/PLS1 axes, and wild and cultivated samples were discriminated along those axes. The remaining fraction clearly distinguished SR from *Peucedanum ledebourielloides* root. The compounds responsible for this discrimination were deemed falcarindiol derivatives and other unidentified secondary metabolites from the s-plot on PLS-DA. The secondary metabolites from original plants were, therefore, presumed to be concentrated in the remaining fraction by RP-SPEC treatment and strongly reflected the species differences. The developed series is considered effective to performquality evaluation of crude drugs and natural products.

Keywords: Saposhnikoviae radix, *Peucedanum ledebourielloides* root, ^{1}H-NMR metabolome

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生薬学雑誌 2019;73:84-88

Ligustrum Fruit is a crude drug derived from the fruit of *Ligustrum lucidum* W.T. Aiton or *L. japonicum* Thunb. (Oleaceae). It has several pharmaceutical activities including hepatoprotective, antioxidant and improvement of bone turnover and therefore, it is used as an ingredient in many crude drug products for nutrition fortification. In preparation for the listing of the drug to Non-JP Crude Drug Standards (Non-JPS), we designed the identification test using TLC and identified the marker spot as nuzhenide based on the spectroscopic data including ^{1}H-, ^{13}C-NMR and MS together with the comparison of TLC, LC-MS and NMR with those of the authentic compound.

The established TLC conditions are as follows: chromatographic support, silica gel; developing solvent, EtOAc/MeOH/H_2O (7/2/1); developing length, 7 cm; detection, UV (254 nm) and 1-naphthol-sulphuric acid reagent; R_f value, 0.4 (nuzhenide).

Keywords: Ligustrum fruit, identification test, nuzhenide

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堀井周文*，小此木明*，高橋隆二*，鎌倉浩之，袴塚高志，合田幸広：小青竜湯エキス製剤および湯剤の同等性に関する研究（Ⅱ）．

生薬学雑誌 2019;73:73-83

In a previous study (Horii, C., *et al., Shoyakugaku Zasshi*, 68 (2), 65-69, 2014), the current authors examined the bioequivalence of Shoseiryuto decoction and its extract preparation, and findings from that study indicated that ephedrine and pseudoephedrine from plants in the genus Ephedra could serve as characteristic constituents with which to evaluate the bioequivalence of preparations. As in the previous study, we examined bioequivalence; the current study used the Shoseiryuto formula of the decoction and the product. The change in concentration of 9 constituents, paeoniflorin, gomisin A, scizandrin, glycyrrhizicacid, liquiritin, liquiritigenin, asarinin, [6]-shogaol, and zingerone, was observed. These characteristic constituents can be used to evaluate the bioequivalence of preparations after their oral administration.

A cross-over study was conducted by randomly dividing 6 healthy adult men into 2 groups and then orally administering the preparations. Results revealed variations in the plasma concentration of each constituent depending on when blood samples were taken, and this result was true for both the decoction and the extract. Analysis of variance did not reveal significant differences in constituents (except for zingerone) in the decoction or extract. Analysis of variance indicated that the preparation was a significant factor for variability in the C_{max} of zingerone. The plasma concentration of zingerone was low and measurements were not obtained with sufficient sensitivity, which presumably explains the

results obtained. Analysis of variance indicated that the subject was a significant factor for variability in the peak plasma concentration (C_{max}) and the area under the curve for the plasma concentration ($AUC_{0\text{-}8}$) of paeoniflorin, and in the peak plasma concentration (C_{max}) of gomisin A, schizandrin, and [6] - shogaol.

The statistical power (1-β) of the C_{max} and the $AUC_{0\text{-}8}$ was deemed to be insufficient (less than 80%) for all of the constituents. Therefore, based on the data obtained in this study, we estimated the sample size needed to obtain sufficient power. For liquiritin, a sample size of 9 or more subjects per group would yield a C_{max} and $AUC_{0\text{-}8}$ with sufficient power (80% or more). For [6]-shogaol, a sample size of 5 or more subjects per group would do so. For gomisin A, a sample size of 18 or more subjects per group would do so. For schizandrin, a sample size of 15 or more subjects per group would suffice. However, a sample size of 61 or more subjects per group would not yield sufficient power for paeoniflorin, glycyrrhizic acid, or liquiritigenin.

The 9 constituents of Shoseiryuto are known to be representative compounds with active ingredients. Results suggested that increasing the sample size for gomisin A, schizandrin, liquiritin, asarinin and [6] -shogaol might allow those 5 compounds to serve as characteristic constituents with which to evaluate the equivalence of the prescribed preparations. The current results indicated that 4 compounds of zingerone, paeoniflorin, glycyrrhizic acid and liquiritigenin could not, at the current point in time, acceptably serve as characteristic constituents with which to evaluate the equivalence of preparations.

Keywords: equivalence, Shoseiryuto, changes in plasma concentrations

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生薬学雑誌 2019;73:68-72

Saffron is the stigma of *Crocus sativus* L. and widely used as a spice and a crude drug. Because the price of saffron is very high, it suffers from various adulterations including coloration with synthetic dyes. In the Japanese Pharmacopoeia 17th edition (JP17), a purity test for aniline dyes is described for saffron. However, the target aniline dyes are not specified, and chloroform, a hazardous solvent which can be used only if no alternative method is available in newly listed JP monographs, is used in this test. Therefore, a new method using thin layer chromatography (TLC) to replace the purity test was examined. From literature search for the synthetic pigments which were reported to be detected from saffron, five pigments, sudan III (**1**), sudan red G (**2**), methyl orange (**3**), auramine (**4**) and sunset yellow (**5**), were picked up as target pigments. In addition, tartrazine (**6**) and naphthol yellow (**7**), which are used in the purity test of saffron in European Pharmacopoeia, are also included as the target pigments. TLC conditions to separate these pigments from the yellow pigments (crosins) contained in saffron, together with suitable detection methods to distinguish the synthetic dyes from crosins, were investigated and a TLC method suitable to replace the current purity test of saffron for aniline dyes in JP was established. In addition, as three yellow spots of crosins are clearly observed under this condition, this TLC method was also suitable for an identification test of saffron for JP.

Keywords: saffron, purity test, clean analysis

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Yoshimura M[*1,2], Amakura Y[*1], Hyuga S[*2], Hyuga M, Nakamori S[*3], Maruyama T, Oshima N[*4], Uchiyama N, Yang J[*5], Oka H[*6], Ito H[*7], Kobayashi Y[*3], Odaguchi H[*2], Hakamatusuka T, Hanawa T[*2], Goda Y: Quality evaluation and characterization of

fractions with biological actibity from Ephedra Herb extract and ephedrine alkaloids-free Ephedra Herb extract.

Chem. Pharm. Bull. 2020;68:140-149

Previously, we reported that the c-Met inhibitory effect of Ephedra Herb extract (EHE) is derived from ingredients besides ephedrine alkaloids. Moreover, analgesic and anti-influenza activities of EHE and ephedrine alkaloids-free Ephedra Herb extract (EFE) have been reported recently. In this study, we examined the fractions containing c-Met kinase inhibitory activity from EHE and the fractions with analgesic and anti-influenza activities from EFE, and elucidated the structural characteristics of the active fractions. Significant c-Met kinase activity was observed in 30, 40, and 50% methanol (MeOH) eluate fractions obtained from water extract of EHE using Diaion HP-20 column chromatography. Similarly, 20 and 40% MeOH, and MeOH eluate fractions obtained from water extract of EFE were found to display analgesic and anti-influenza activities. Reversed phase-HPLC analysis of the active fractions commonly showed broad peaks characteristic of high-molecular mass condensed tannin. The active fractions were analyzed using ^{13}C-NMR and decomposition reactions; the deduced structures of active components were high-molecular mass condensed tannins, which were mainly procyanidin B-type and partly procyanidin A-type, including pyrogallol- and catechol-type flavan 3-ols as extension and terminal units. HPLC and gel permeation chromatography (GPC) analyses estimated that the ratio of pyrogallol- and catechol-type was approximately 9 : 2, and the weightaverage molecular weight based on the polystyrene standard was >45000. Furthermore, GPC-based analysis was proposed as the quality evaluation method for high-molecular mass condensed tannin in EHE and EFE.

Keywords: ephedrine alkaloids-free Ephedra Herb extract (EFE), condensed tannin, c-Met

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Tsujimoto T*1, Nishihara M*2, Osumi Y*2, Hakamatsuka T, Goda Y, Uchiyama N, Ozeki Y*1: Structural analysis of polygalaxanthones, *C*-glucosyl xanthones of *Polygala tenuifolia* roots. *Chem. Pharm. Bull.* 2019;67:1242-1247

Polygalaxanthone III, a xanthone glycoside that is a major constituent of "Polygala Root" (*Polygala tenuifolia* roots, Onji in the Japanese Pharmacopoeia), has been used as a standard in the quality control of crude drugs. However, we previously noted differences in the chromatographic properties of one of three samples of polygalaxanthone III. Therefore, standardization of the standard itself is extremely important. The structures of three standard samples commercially available as polygalaxanthone III were characterized by LC/MS and NMR. LC/MS analysis revealed that two molecular types exist. Both types are chromatographically separable but have an identical mass number with distinguishable MS/MS spectra. One dimensional (1D)-NMR analyses demonstrated that both had the same xanthone moiety and heteronuclear multiple bond correlation (HMBC) analyses revealed that they are structural isomers at the connecting position of glucose to apiose 1-position. Consequently, the isomers were identified as polygalaxanthone III and its regioisomer, polygalaxanthone XI. Based on the findings, we recommend using the LC-MS/MS detection method, which discriminates polygalaxanthone III and XI, to confirm the quality of the standard.

Keywords: polygalaxanthone, *C*-glycoside, *Polygala tenuifolia*

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Hashimoto M*1,2, Ichijo H*1, Fujiwara K*1, Sugasawa H*1, Abo S*1, Matsudo K*1, Uchiyama N, Goda Y,

Fujii I[*1]: Functional expression of a highly-reducing polyketide synthase of *Emericella variecolor* IFM42010, an asteltoxin-producing strain, resulted in production of two polyenoic β-ketolactones with opposite stereochemistry.

Bioorg. Med. Chem. Lett. 2019;29:126686

The asteltoxin-producing fungus *Emericella variecolor* IFM42010 possesses 22 highly-reducing polyketide synthase (HR-PKS) genes. Of these, an HR-PKS with a methyltransferase domain but lacking an enoylreductase domain could be involved in the biosynthesis of asteltoxin and related compounds. From six such candidate HR-PKS genes, *Ev460pks* was analyzed by gene disruption in E. variecolor and heterologous expression in *Aspergillus oryzae*. The *Ev460pks*-disrupted strain retained asteltoxin production ability, indicating that *Ev460pks* is not involved in asteltoxin biosynthesis. The A. oryzae transformant harboring the *Ev460pks* gene produced compounds **1** and **2**, along with several unidentified products possibly decomposed from **2**. Spectroscopic analyses revealed that **1** was a 4-methyl-β-ketolactone with a methylheptatriene side-chain at the C-5 position, and **2** was also a 4-methyl-β-ketolactone, bearing a dimethyltetradecahexaene side-chain at the same position. The relative configuration at C-4 in compounds **1** and **2** was opposite.

Keywords: highly-reducing polyketide synthase, fungi, polyketide

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局外生規2018に新規収載された単味生薬エキスの確認試験及び定量法の設定について.

生薬学雑誌 2020;74:20-34

In December 2015, the "Application guidance for OTC (non-Kampo) single crude drug extract products" was published by the Ministry of Health, Labour and Welfare (Japan) for the quality control of crude drugs. To further expand on this guidance, we designed and verified methods for the identification tests and assays of three single crude drug extracts: Epimedium Herb Extract (**1**), Uncaria Hook Extract (**2**), and Ginger Extract (**3**) specified in Non-JP Crude Drug Standards (Non-JPS) 2018. Magnoflorine and icariin were selected as the marker compounds of **1** for the identification test using TLC and the assay using HPLC, respectively, and [6]-gingerol was selected for both tests of **3**. The marker compounds of **2** were total alkaloids for the identification test using filter paper and rhynchophylline and hirsutine for the assay using HPLC. Based on the results from this study, we determined both identification tests and assays for these three single crude drug extracts listed in the Non-JPS 2018.

Keywords: single crude drug extract, identification test, assay

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Nakamori S[*1,2], Takahashi J[*1,2], Hyuga S[*2], Yang J[*3], Takemoto H[*1,2], Maruyama T, Oshima N[*4], Uchiyama N, Amakura Y[*5], Hyuga M, Hakamatsuka T, Goda Y, Odaguchi H[*2], Hanawa T[*2], Kobayashi Y[*1,2]: Analgesic effects of Ephedra Herb extract, Ephedrine alkaloids-free Ephedra Herb extract, Ephedrine, and Pseudoephedrine on Formalin-induced pain.

Biol. Pharm. Bull. 2019;42:1538-1544

The analgesic effect of Ephedra Herb (EH) is believed to be derived from the anti-inflammatory action of pseudoephedrine (Pse). We recently reported that ephedrine alkaloids-free EH extract (EFE) attenuates formalin-induced pain to the same level as that achieved by EH extract (EHE), which suggests that the analgesic effect of EH may not be due to ephedrine alkaloids (EAs). To examine the contribution of EAs to the analgesic effect of EH, mice were injected with formalin to induce a biphasic pain reaction (first phase, 0-5 min; second phase, 10-45 min) at various time points after oral administration of the following test drugs: ephedrine (Eph), Pse, "authentic" EHE from Tsumura & Co. (EHE-Ts), EFE, and EHE that was used as the source of EFE (EHE-To). Biphasic pain was suppressed at 30 min after administration of Eph, EHETs, and EHE-To. At 6 h after administration of EFE, EHE-To, and Pse-and at 4 to 6 h after administration of EHE-Ts-only second-phase pain was suppressed; however, the effect of Pse at 6 h was not significant. These results suggested that EHE has a biphasic analgesic effect against biphasic formalin-induced pain: in the first phase of analgesia (30 min after administration), biphasic pain is suppressed by Eph; in the second phase of analgesia (4-6 h after administration), second-phase pain is alleviated by constituents other than EAs, although Pse may partially contribute to the relief of second-phase pain.

Keywords: Ephedra Herb, ephedrine, analgesic effect

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Tsujimoto T[*1], Yoshitomi T, Maruyama T, Yamamoto Y[*2], Hakamatsuka T, Uchiyama N: High resolution LC-MS based metabolomic discrimination of *Citrus*-type crude drugs and comparison with NMR-based metabolomics.

J. Nat. Prod. 2019;82:2116-2123

Five Citrus-type crude drugs (40 samples) were classified using liquid chromatography－mass spectrometry (LCMS)-based metabolomics. The following six flavonoid derivatives were identified as contributors from the loading plots of multivariate analysis: naringin (1), neohesperidin (2), neoeriocitrin (3), narirutin (9), hesperidin (10), and 3,5,6,7,8,3′,4′heptamethoxyflavone (12). Three coumarin derivatives, namely, meranzin (6), meranzin hydrate (7), and meranzin glucoside (8), were also identified as contributors. Furthermore, compared with our previous studies on proton (1H) and 13C NMR spectroscopy-based metabolomics, the present study revealed that the Citrus-type crude drugs were distinguished with the same pattern; however, the contributors differed between the 1H and 13C NMR spectroscopy-based metabolomics. The high dynamic range of NMR spectroscopy provided broad coverage of the metabolomes including the primary and secondary metabolites. However, LC-MS appeared to be superior in detecting secondary metabolites with high sensitivity, some of which occurred in quantities that were undetectable using NMR spectroscopy.

Keywords: metabolomics, LC-MS, NMR

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Masada S, Tsuji G, Arai R, Uchiyama N, Demizu Y, Tsutsumi T, Abe Y, Akiyama H, Hakamatsuka T, Izutsu KI, Goda Y, Okuda, H: Rapid and efficient high-performance liquid chromatography analysis of *N*-nitrosodimethylamine impurity in valsartan drug substances and its products.

Sci. Rep. 2019;9:11852

In July 2018, certain valsartan-containing drugs were voluntary recalled in Japan owing to contamination with *N*-nitrosodimethylamine (NDMA), a probable

human carcinogen. In this study, an HPLC method was developed for the quantitative detection of NDMA simultaneously eluted with valsartan. When the recalled valsartan samples were subjected to this method, the observed NDMA contents were in agreement with the reported values, indicating that our method achieved sufficient linearity, accuracy, and precision to detect NDMA in valsartan drug substances and products. Moreover, six samples (valsartan drug substances and tablet formulations), which had a possibility for NDMA contamination, were analyzed; none of the samples contained NDMA at detectable levels. Our method would be useful for the rapid screening and quantification of NDMA impurity in valsartan drug substances and products.

Keywords: *N*-nitrosodimethylamine, valsartan, HPLC

政田さやか，水野沙稀*，小谷彩加*，藤原裕未*，内山奈穂子，袴塚高志，永津明人*：ピペリン及びモノグルコシルヘスペリジンを機能性関与成分とする機能性表示食品の製剤学的品質評価と溶出試験法の検討.

日本食品化学学会誌 2019;26:147-152

In April 2015, the system of Foods with Functional Claims (FFC) was launched and consumers expected health benefits from the FFC whose function was supported by scientific evidence. As the FFC guideline requires food manufacturers to keep the amount of a functional component in the product more than the labeling amount, it is also desirable to test its disintegration and dissolution in order to ensure the effectiveness of the FFC products. In this study, we focused on FFC products containing piperine and monoglucosyl hesperidin as functional substances to perform the weigh variation, disintegration, and dissolution tests. The results indicated that some FFCs products were put on the market with evidence-based functions despite the lack of disintegration. Additionally, more investigation and discussion must be done for developing an adequate dissolution test method and criteria for FFC products.

Keywords: foods with functional claims, disintegration test, dissolution test

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Nose M*[1], Tada M*[1], Kato A*[1], Hisaka S*[1], Masada S, Homma M*[2], Hakamatsuka T: Effect of Schisandrae Fructus on glycyrrhizin content in Kampo extracts containing Glycyrrhizae Radix used clinically in Japan.

J. Nat. Med. 2019;73:834-840

Glycyrrhizae Radix is an important crude drug in Japan and is the most frequently prescribed drug in Kampo medicines for the treatment of a wide range of diseases. Glycyrrhizin (GL), the major active ingredient of Glycyrrhizae Radix, has various pharmacological actions but causes adverse effects such as pseudoaldosteronism. In the present study, we investigated the extraction efficiency of GL from Glycyrrhizae Radix in decoctions comprising Glycyrrhizae Radix and five different fruit-derived crude drugs. Among the five fruit-derived crude drugs tested, Schisandrae Fructus markedly decreased both the pH value of the decoction and the extraction efficiency of GL. A comparison of the pH value of the decoction and the GL content of 12 Kampo prescriptions (containing at least Glycyrrhizae Radix and Schisandrae Fructus) showed that the GL content per daily dose was proportional to the compounding amount of Glycyrrhizae Radix, and that the extraction efficiency of GL from Glycyrrhizae Radix was strongly correlated with the pH value of the decoction. These results suggested that the GL content in Glycyrrhizae Radix-containing Kampo products can be estimated from both the compounding amounts of Glycyrrhizae Radix and the pH value documented in their interview forms. Knowledge of GL content will help avoid adverse reactions due to Glycyrrhizae Radix.

keywords: Glycyrrhizae Radix, Glycyrrhizin, pH

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Sogame M*, Naraki Y*, Sasaki T*, Seki M*, Yokota K*, Masada S, Hakamatsuka T: Quality Assessment of Medicinal Product and Dietary Supplements Containing *Vitex agnus-castus* by HPLC Fingerprint and Quantitative Analyses.

Chem. Pharm. Bull. 2019;67:527-533

In this study, we aimed to evaluate the quality of 11 products sold in Japan (one medicinal product and 10 dietary supplements) containing/claiming to contain chasteberry extract (fruit of *Vitex agnus-castus* L.) using HPLC fingerprint (15 characteristic

peaks), quantitative determination of chemical marker compounds, and a disintegration test. The HPLC profile of the medicinal product was similar to that of the reference standard of *V. agnus-castus* fruit dry extract obtained from European Directive for the Quality of Medicines (EDQM), whereas the profiles of some dietary supplements showed great variability, such as different proportions of peaks or lack of peaks. Results of the principal component analysis of the fingerprint data were consistent with those of the HPLC profile analysis. The contents of two markers, agnuside and casticin, in dietary supplements showed wide variability; this result was similar to that achieved with the HPLC fingerprint. Results of the disintegration test showed poor formulation quality of two dietary supplements. These results call attention to the quality problems of many dietary supplements, such as incorrect or poor-quality origin, different contents of the active ingredient, and/or unauthorized manufacturing procedures.

Keywords: *Vitex agnus-castus*, dietary supplement, incorrect origin

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Kitajima M*, Yamaguchi Y*, Yanagisawa T*, Kogure N*, Ogata J, Kikura-Hanajiri R, Takayama H*: Biphenyl quinolizidine lactone alkaloids from "sinicuichi" (*Heimia salicifolia*).

Tetrahydron 2019;75:3733-3739

Six new biphenyl quinolizidine lactone alkaloids: 14 *α*-hydroxydecodine (**1**), 14β-hydroxydecodine (**2**), 4″-*O*-demethylheimidine (**3**), 4″-*O*-demethyl-9β-hydroxyvertine (**4**), 4″-*O*-demethylvertine *N*-oxide (**5**), and 4″-*O*-demethyl-9β-hydroxyvertine *N*-oxide (**6**) were isolated from so-called "sinicuichi" (origin: *Heimia salicifolia*) together with 18 known alkaloids. Their structures were determined by spectroscopic analyses and chemical conversions.

Keywords: alkaloid, *Heimia salicifolia*, sinicuichi

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Mitsuoka T*[1,2], Hanamura K*[1], Koganezawa N*[1], Kikura-Hanajiri R, Sekino Y*[2], Shirao T*[1]: Assessment of NMDA receptor inhibition of phencyclidine analogues using a high-throughput drebrin immunocytochemical assay.

Journal of Pharmacological and Toxicological Methods 2019;99:106583

In recent years, new psychoactive substances (NPS) have been widely distributed for abuse purposes. Effective measures to counter the spread of NPS are to promptly legislate them through the risk assessment. Phencyclidine analogues having inhibitory effects toward NMDA receptor (NMDAR) have recently emerged in Japan. Therefore, it is important to establish a high-throughput system for efficiently detecting NPS that can inhibit NMDAR activity.

Hippocampal neurons prepared from embryonic rats were incubated in 96-well microplates. After 3 weeks in vitro, cultured neurons were preincubated with phencyclidine (PCP) or PCP-analogues, including 3-methoxyphencyclidine (3-MeO-PCP) and 4-[1-(3-methoxyphenyl)cyclohexyl]morpholine (3-MeO-PCMo), and then treated with 100 μM glutamate for 10 min. After fixation, cultured neurons were immunostained with anti-drebrin and anti-MAP2 antibodies. The linear cluster density of drebrin along the dendrites was automatically quantified using a protocol that was originally developed by us.

The high-throughput immunocytochemical assay, measuring drebrin cluster density of cultured neurons, demonstrated that glutamate-induced reduction of drebrin cluster density in 96-well plates is competitively inhibited by NMDAR antagonist, APV. The reduction was also antagonized by PCP, 3-MeO-PCP and 3-MeO-PCMo. The inhibitory activity of 3-MeO-PCMo was lower than that of PCP or 3-MeO-PCP, with IC_{50} values of 26.67 μM (3-MeO-PCMo), 2.02 μM (PCP) and 1.51 μM (3-MeO-PCP).

The relative efficacy among PCP, 3-MeO-PCP and 3-MeO-PCMo calculated from IC_{50} are similar to those from Ki values. This suggests that the high-throughput imaging analysis is useful to speculate the Ki values of new PCP analogues without performing the kinetic studies.

Keywords: dendritic spine, drebrin, high-throughput analysis

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Li RS[*1,2], Fukumori R[*3], Takeda T[*1], Song Y[*1], Morimoto S[*1], Kikura-Hanajiri R, Yamaguchi T[*3], Watanabe K[*4], Aritake K[*4], Tanaka Y[*1], Yamada H[*1], Yamamoto T[*3], Ishii Y[*1]: Elevation of endocannabinoids in the brain by synthetic cannabinoid JWH-018: mechanism and effect on learning and memory.

Sci. Rep. 2019;9:9621

The impairment of learning and memory is a well-documented effect of both natural and synthetic cannabinoids. In the present study, we aimed to investigate the effect of acute administration of JWH-018, a synthetic cannabinoid, on the hippocampal metabolome to assess biochemical changes *in vivo*. JWH-018 elevated levels of the endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The increase of endocannabinoid levels in response to JWH-018 could be inhibited by co-administration of AM251, a CB1 receptor antagonist. Biochemical analyses revealed that this was the result of suppression of two hydrolases involved in endocannabinoid degradation (fatty acid amide hydrolase [FAAH] and monoacylglycerol lipase [MAGL]). Additionally, we showed that JWH-018 causes a reduction in the levels of brain-derived neurotrophic factor (BDNF), which is known to modulate synaptic plasticity and adaptive processes underlying learning and memory. The decrease of BDNF following JWH-018 treatment was also rescued by co-administration of AM251. As both endocannabinoids and BDNF have been shown to modulate learning and memory in the hippocampus, the alteration of their levels in response to JWH-018 may explain the contribution of synthetic cannabinoids to impairment of memory.

Keywords: synthetic cannabinoids, endocannabinoids, impairment of learning and memory

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Kawahara G[*], Nakayashiki MS[*], Maeda H[*], Kikura-Hanajiri R, Yoshida K[*], Hayashi YK[*]: Antagonists for serotonin receptors ameliorate rhabdomyolysis induced by 25D-NBOMe, a psychoactive designer drug.

Forensic Toxicol. 2020;38:122-128

N-Benzyl-substituted phenethylamines (NBOMes) are psychoactive drugs, which induce various symptoms like serotonin syndrome, even at low doses. Recently, we reported the first lethal case of a designer drug, 2-(4-bromo-2, 5-dimethoxyphenyl)-NBOMe (25B-NBOMe) intoxication with severe rhabdomyolysis, evaluated by clinical, pathological, and toxicological analyses. We also confirmed that 25B-NBOMe can induce rhabdomyolysis using a zebrafish model. To further elucidate pathomechanism of NBOMes-induced rhabdomyolysis, we treated zebrafish with a similar designer drug, 2-(4-methyl-2, 5-dimethoxyphenyl)-NBOMe (25D-NBOMe).

Zebrafish treated with a designer drug, 25D-NBOMe, were examined survival rate and were analyzed skeletal muscle degeneration by birefringence. They were also analyzed expression levels of 5-HT_{2A} and 5-HT_{2C} receptors and ryanodine receptor.

The 25D-NBOMe-treated fish showed decreased survival rate and skeletal muscle degeneration detected by birefringence mimicking to those of 25B-NBOMe-treated fish. We revealed that 25D-NBOMe induced up-regulation of the expression of 5-HT_{2A} and 5-HT_{2C} receptors, and rhabdomyolysis was inhibited by the 5-HT_{2A} receptor antagonist, aripiprazole and 5-HT_{2C} receptor antagonist, SB242084. Moreover, ryanodine receptor expression was up-regulated in 25D-NBOMe-treated fish as compared to that of untreated fish, and the up-regulated expression of ryanodine receptor in 25D-NBOMe-treated fish was improved with co-treatment aripiprazole and SB242084.

Our findings suggest that multiple 5-HT receptors have important roles in NBOMes-induced rhabdomyolysis together with other serotonin-related symptoms. Zebrafish is a highly useful model for therapeutic studies of rhabdomyolysis induced by psychoactive designer drugs.

Keywords: 5-HT_{2A} and 5-HT_{2C} receptors, NBOMes induced rhabdomyolysis, zebrafish

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Kuroda T, Yasuda S, Tachi S[*1], Matsuyama S[*2], Kusakawa S, Tano K, Miura T, Matsuyama A[*2], Sato Y: SALL3 expression balance underlies lineage biases in human induced pluripotent stem cell differentiation.

Nat. Commun., 2019;10:2175. doi: 10.1038/s41467-019-09511-4

Clinical applications of human induced pluripotent stem cells (hiPSCs) are expected, but hiPSC lines vary in their differentiation propensity. For efficient selection of hiPSC lines suitable for differentiation into desired cell lineages, here we identify SALL3 as a marker to predict differentiation propensity. SALL3 expression in hiPSCs correlates positively with ectoderm differentiation capacity and negatively with mesoderm/endoderm differentiation capacity. Without affecting self-renewal of hiPSCs, SALL3 knockdown inhibits ectoderm differentiation and conversely enhances mesodermal/endodermal differentiation. Similarly, loss- and gain-of-function studies reveal that SALL3 inversely regulates the differentiation of hiPSCs into cardiomyocytes and neural cells. Mechanistically, SALL3 modulates DNMT3B function and DNA methyltransferase activity, and influences gene body methylation of Wnt signaling-related genes in hiPSCs. These findings suggest that SALL3 switches the differentiation propensity of hiPSCs toward distinct cell lineages by changing the epigenetic profile and serves as a marker for evaluating the hiPSC differentiation propensity.

Keywords: human induced pluripotent stem cell, propensity, SALL3

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Sato Y, Bando H[*1], Di Piazza M[*2], Gowing G[*3], Herberts C[*4], Jackman S[*5], Leoni G[*6], Libertini S[*7], MacLachlan T[*7], McBlane JW[*8], Pereira Mouriès L[*9], Sharpe M[*6], Shingleton W[*10], Surmacz-Cordle B[*6], Yamamoto K[*11], van der Laan JW[*4]: Tumorigenicity assessment of cell therapy products: The need for global consensus and points to consider.

Cytotherapy, 2019;21:1095-111. doi: 10.1016/j.jcyt.2019.10.001

Pluripotent stem cells offer the potential for an unlimited source for cell therapy products. However, there is concern regarding the tumorigenicity of these products in humans, mainly due to the possible unintended contamination of undifferentiated cells or transformed cells. Because of the complex nature of these new therapies and the lack of a globally accepted consensus on the strategy for tumorigenicity evaluation, a case-by-case approach is recommended for the risk assessment of each cell therapy product. In general, therapeutic products need to be qualified using available technologies, which ideally should be fully validated. In such circumstances, the developers of cell therapy products may have conducted various tumorigenicity tests and consulted with regulators in respective countries. Here, we critically review currently available in vivo and in vitro testing methods for tumorigenicity evaluation against expectations in international regulatory guidelines. We discuss the value of those approaches, in particular the limitations of in vivo methods, and comment on challenges and future directions. In addition, we note the need for an internationally harmonized procedure for tumorigenicity assessment of cell therapy products from both regulatory and technological perspectives.

Keywords: human pluripotent stem cell, international guidelines, tumorigenicity

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Suganya N[*1], Mani KP[*2], Sireesh D[*1], Rajaguru P[*2], Vairamani M[*1], Suresh T, Suzuki T, Chatterjee S[*2], Ramkumar KM[1]: Establishment of pancreatic microenvironment model of ER stress: Quercetin attenuates β-Cell apoptosis by invoking nitric oxide-cGMP signaling in endothelial cells.

J Nutr Bioche. 2018;55:142-156.doi: 10.1016/j.jnutbio.2017.12.012.

The involvement of endoplasmic reticulum (ER) stress in endothelial dysfunction and diabetes-associated complications has been well documented. Inhibition of ER stress represents a promising therapeutic strategy to attenuate endothelial dysfunction in diabetes. Recent attention has focused on the development of small molecule inhibitors of ER stress to maintain endothelial homeostasis in diabetes. Here we have developed a reliable, robust co-culture system that allows a study on the endothelial cells and pancreatic β-cells crosstalk under ER stress and validated using a known ER stress modulator, quercetin. Furthermore, sensitizing of endothelial cells by quercetin (25 μM) confers protection of pancreatic β-cells against ER stress through nitric oxide (NO·) signaling. In addition, increased intracellular insulin and NO-mediated cyclic 3',5'-guanosine monophosphate (cGMP) levels in pancreatic β-cells further confirmed the mechanism of protection under co-culture system. In addition, the potential protein targets of quercetin against ER stress in the endothelial cells were investigated through proteomic profiling and its phosphoprotein targets through Bioplex analysis. On the whole, the developed in vitro co-culture set up can serve as a platform to study the signaling network between the endothelial and pancreatic β-cells as well as provides a mechanistic insight for the validation of novel ER stress modulators.

Keywords: ER stress, Nitric oxide, Quercetin

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Furuta-Hanawa B, Yamaguchi T*, Uchida E : Two-dimensional droplet digital PCR as a tool for titration and integrity evaluation of recombinant adeno-associated viral vectors.
Human Gene Therapy Methods. 2019;30:127-136.

Recombinant adeno-associated virus (rAAV) vectors have recently been widely utilized for in in vivo gene therapy. The clinical dose definition of AAV vector requires the exact quantification as starting doses and for dose escalation studies. Vector genome (vg) copies measured by quantitative PCR (qPCR) are commonly used for rAAV vector titration, and rAAV vector plasmids DNA is often used for qPCR standards, although the rAAV reference standard materials (RSMs) for serotypes 2 and 8 (rAAV2RSM and rAAV8RSM) are available from American Type Culture Collection. However, qPCR-based determination of the AAV vg is affected by the selection of the qPCR standard and the amplification target sites. In this study, we have developed a new PCR method, two dimensional droplet digital PCR (2D ddPCR), for the absolute quantitation of target DNA and for evaluating the stability of the rAAV vector. The number of vg copies of rAAV2RSM determined by qPCR dramatically changed when standard plasmid DNAs with different conformations were treated with restriction enzymes, suggesting that qPCR amplification is significantly affected by the secondary structure of the standard. In contrast, the number of vg copies determined by ddPCR was unaffected by using primer probes for different positions of target sites or by the secondary structure conformation of the vg. Furthermore, the integrity of the AAV vg can be monitored using 2D ddPCR with fluorescein- and hexachloro-6 carboxy-fluorescine–labeled probes targeting different positions in the same rAAV genome. The titer of intact rAAV was highly correlated with rAAV activity in an accelerated (37℃) stability study. 2D ddPCR is a useful tool for rAAV vector quantitation and quality evaluation.

Keywords: gene therapy, AAV, ddPCR

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Yoshida T, Naito Y*1,2, Yasuhara H*3, Sasaki K, Kawaji H*4,5, Kawai J*5, Naito M, Okuda H, Obika S*3, Inoue T: Evaluation of off-target effects of gapmer antisense oligonucleotides using human cells.
Genes Cells. 2019;24:827-835

Antisense oligonucleotide (ASO) has the potential to induce off-target effects due to complementary binding between the ASO and unintended RNA with a sequence similar to the target RNA. Conventional animal studies cannot be used to assess toxicity induced by off-target effects because of differences in the genome sequence between humans and other animals. Consequently, the assessment of off-target effects with in silico analysis using a human RNA database and/or in vitro expression analysis using human cells has been proposed. Our previous study

showed that the number of complementary regions of ASOs with mismatches in the human RNA sequences increases dramatically as the number of tolerated mismatches increases. However, to what extent the expression of genes with mismatches is affected by off-target effects at the cellular level is not clear. In this study, we evaluated off-target effects of gapmer ASOs, which cleave the target RNA in an RNase H-dependent manner, by introducing the ASO into human cells and performing microarray analysis. Our data indicate that gapmer ASOs induce off-target effects depending on the degree of complementarity between the ASO and off-target candidate genes. Based on our results, we also propose a scheme for the assessment of off-target effects of gapmer ASOs.

Keywords: gapmer antisense oligonucleotide, off-target, pre-mRNA

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Komura F*1, Takahashi Y*1, Inoue T, Takakura Y*1, Nishikawa M*2: Development of a Nanostructured RNA/DNA Assembly as an Adjuvant Targeting Toll-Like Receptor 7/8.

Nucleic Acid Ther. 2019;29:335-342.

Adjuvants are essential for efficiently inducing an antigen-specific immune response in vaccine therapy. Single-stranded RNA (ssRNA) containing guanosine- and uridine-rich sequences is recognized by Toll-like receptor (TLR)7 and/or TLR8 and induces strong immune responses; thus, the application of ssRNA as an adjuvant is desirable. The development of a ssRNA-based adjuvant, however, requires the efficient delivery of ssRNA into the endosomes of antigen-presenting cells, where the TLRs exist. To achieve this, we developed a nanostructured RNA/DNA assembly using DNA nanotechnology, which can be efficiently recognized by antigen-presenting cells. The nanostructured RNA/DNA assembly, named tetrapodRD3, was designed using a 40-mer phosphorothioate-stabilized RNA and three 40-mer phosphodiester DNAs. TetrapodRD3 was more stable than ssRNA under serum conditions. The secreted alkaline phosphatase assay using HEK-Blue hTLR cells showed that tetrapodRD3 triggered human TLR8-specific responses. Fluorescently labeled tetrapodRD3 was efficiently taken up by murine dendritic DC2.4 cells and induced a high level of tumor necrosis factor-α release from the cells. Antigen presentation by the major histocompatibility complex class I on bone marrow-derived dendritic cells was significantly increased by the addition of an antigen along with tetrapodRD3. These results indicate that tetrapodRD3 constructed using DNA nanotechnology can be a useful adjuvant targeting human TLR8.

Keywords: Toll-like receptor 7/8, adjuvant, nanostructure

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Nishikawa S*1, Itoh Y*1,2, Tokugawa M*1, Inoue Y*1, Nakashima KI*3, Hori Y*1, Miyajima C*1, Yoshida K*1, Morishita D*1, Ohoka N, Inoue M*3, Mizukami H*1, Makino T*1, Hayashi H*1: Kurarinone from *Sophora Flavescens* Roots Triggers ATF4 Activation and Cytostatic Effects Through PERK Phosphorylation.

Molecules. 2019;24:3110.

In response to cellular stresses, activating transcriptional factor 4 (ATF4) regulates the expression of both stress-relieving genes and apoptosis-inducing genes, eliciting cell fate determination. Since pharmacological activation of ATF4 exerts potent anti-tumor effects, modulators of ATF4 activation may have potential in cancer therapy. We herein attempted to identify small molecules that activate ATF4. A cell-based screening to monitor *TRB3* promoter activation was performed using crude drugs used in traditional Japanese Kampo medicine. We found that an extract from *Sophora flavescens* roots exhibited potent *TRB3* promoter activation. The activity-guided fractionation revealed that kurarinone was identified as the active ingredient. Intriguingly, ATF4 activation in response to kurarinone required PKR-like endoplasmic reticulum kinase (PERK). Moreover, kurarinone induced the cyclin-dependent kinase inhibitor p21 as well as

cytostasis in cancer cells. Importantly, the cytostatic effect of kurarinone was reduced by pharmacological inhibition of PERK. These results indicate that kurarinone triggers ATF4 activation through PERK and exerts cytostatic effects on cancer cells. Taken together, our results suggest that modulation of the PERK-ATF4 pathway with kurarinone has potential as a cancer treatment.

Keywords: cancer, Sophora flavescens, kurarinone

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Fukuura K*1, Inoue Y*1, Miyajima C*1, Watanabe S*1, Tokugawa M*1, Morishita D*1, Ohoka N, Komada M*2, Hayashi H*1: The ubiquitin-specific protease USP17 prevents cellular senescence by stabilizing the methyltransferase SET8 and transcriptionally repressing *p21*.

Journal of Biological Chemistry. 2019;294:16429-16439.

Su(var)3-9, Enhancer-of-zeste, and Trithorax (SET) domain-containing protein 8 (SET8) is the sole enzyme that monomethylates Lys-20 of histone H4 (H4K20). SET8 has been implicated in the regulation of multiple biological processes, such as gene transcription, the cell cycle, and senescence. SET8 quickly undergoes ubiquitination and degradation by several E3 ubiquitin ligases; however, the enzyme that deubiquitinates SET8 has not yet been identified. Here we demonstrated that ubiquitin-specific peptidase 17-like family member (USP17) deubiquitinates and therefore stabilizes the SET8 protein. We observed that USP17 interacts with SET8 and removes polyubiquitin chains from SET8. USP17 knockdown not only decreased SET8 protein levels and H4K20 monomethylation but also increased the levels of the cyclin-dependent kinase inhibitor p21. As a consequence, USP17 knockdown suppressed cell proliferation. We noted that USP17 was down-regulated in replicative senescence and that USP17 inhibition alone was sufficient to trigger cellular senescence. These results reveal a regulatory mechanism whereby USP17 prevents cellular senescence by removing ubiquitin marks from and stabilizing SET8 and transcriptionally repressing *p21*.

Keywords: cell cycle, deubiquitylation, histone methylation

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Ohoka N, Tsuji G, Shoda T, Fujisato T, Kurihara M, Demizu Y, Naito M: Development of Small Molecule Chimeras That Recruit AhR E3 ligase to Target Proteins.

ACS Chemical Biology. 2019;14:2822-2832.

Targeted protein degradation using chimeric small molecules such as proteolysis-targeting chimeras (PROTACs) and specific and nongenetic inhibitors of apoptosis protein [IAP]-dependent protein erasers (SNIPERs) is an emerging modality in drug discovery. Here, we expand the repertoire of E3 ligases capable of ubiquitylating target proteins using this system. By incorporating β-naphthoflavone (β-NF) as a ligand, we developed a novel class of chimeric molecules that recruit the arylhydrocarbon receptor (AhR) E3 ligase complex. β-NF-ATRA, a chimeric degrader directed against cellular retinoic acid binding proteins (CRABPs), induced the AhR-dependent degradation of CRABP-1 and CRABP-2 via the ubiquitin-proteasome pathway. A similar compound ITE-ATRA, in which an alternative AhR ligand was used, also degraded CRABP proteins. Finally, we developed a chimeric compound β-NF-JQ1 that is directed against bromodomain-containing (BRD) proteins using β-NF as an AhR ligand. β-NF-JQ1 induced the interaction of AhR and BRD proteins and displayed effective anticancer activity that correlated with protein knockdown activity. These results demonstrate a novel class of chimeric degrader molecules based on the ability to bring a target protein and an AhR E3 ligase into close proximity.

Keywords: targeted protein degradation, E3 ligase, proteasome

Miyajima C*1, Kawarada Y*1, Inoue Y*1, Suzuki C*1, Mitamura K*1, Morishita D*1, Ohoka N, Imamura T*2, Hayashi H*1: Transcriptional Coactivator TAZ Negatively Regulates Tumor Suppressor p53 Activity and Cellular Senescence.

Cells. 2019;9:171.

Transcriptional coactivator with a PDZ-binding motif (TAZ) is one of the mammalian orthologs of *Drosophila* Yorkie, a transcriptional coactivator of the Hippo pathway. TAZ has been suggested to function as a regulator that modulates the expression of cell proliferation and anti-apoptotic genes in order to stimulate cell proliferation. TAZ has also been associated with a poor prognosis in several cancers, including breast cancer. However, the physiological role of TAZ in tumorigenesis remains unclear. We herein demonstrated that TAZ negatively regulated the activity of the tumor suppressor p53. The overexpression of TAZ down-regulated p53 transcriptional activity and its downstream gene expression. In contrast, TAZ knockdown up-regulated p21 expression induced by p53 activation. Regarding the underlying mechanism, TAZ inhibited the interaction between p53 and p300 and suppressed the p300-mediated acetylation of p53. Furthermore, TAZ knockdown induced cellular senescence in a p53-dependent manner. These results suggest that TAZ negatively regulates the tumor suppressor functions of p53 and attenuates p53-mediated cellular senescence.

Keywords: TAZ, cellular senescence, oncogene

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Müller D*1,2, Shin S*1,2, Goullet de Rugy T*1, Samain R*1,2, Baer R*1, Strehaiano M*1,2, Masvidal-Sanz L*3, Guillermet-Guibert J*1, Jean C*1,2, Tsukumo Y, Sonenberg N*4, Marion F*5, Guilbaud N*5, Hoffmann JS*1, Larsson O*3, Bousquet C*1,2, Pyronnet S*1,2, Martineau Y*1,2: eIF4A inhibition circumvents uncontrolled DNA replication mediated by 4E-BP1 loss in pancreatic cancer.

JCI Insight. 2019;4:doi:10.1172/jci.insight.121951.

Pancreatic ductal adenocarcinoma (PDAC) relies on hyperactivated protein synthesis. Consistently, human and mouse PDAC lose expression of the translational repressor and mTOR target 4E-BP1. Using genome-wide polysome profiling, we here explore mRNAs whose translational efficiencies depend on the mTOR/4E-BP1 axis in pancreatic cancer cells. We identified a functional enrichment for mRNAs encoding DNA replication and repair proteins, including RRM2 and CDC6. Consequently, 4E-BP1 depletion favors DNA repair and renders DNA replication insensitive to mTOR inhibitors, in correlation with a sustained protein expression of CDC6 and RRM2, which is inversely correlated with 4E-BP1 expression in PDAC patient samples. DNA damage and pancreatic lesions induced by an experimental pancreatitis model uncover that 4E-BP1/2-deleted mice display an increased acinar cell proliferation and a better recovery than WT animals. Targeting translation, independently of 4E-BP1 status, using eIF4A RNA helicase inhibitors (silvestrol derivatives) selectively modulates translation and limits CDC6 expression and DNA replication, leading to reduced PDAC tumor growth. In summary, 4E-BP1 expression loss during PDAC development induces selective changes in translation of mRNA encoding DNA replication and repair protein. Importantly, targeting protein synthesis by eIF4A inhibitors circumvents PDAC resistance to mTOR inhibition.

Keywords: translation, pancreatic cancer, mTOR

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Suzuki T, Tsukumo Y, Furihata C, Naito M, Kohara A*: Preparation of the standard cell lines for reference mutations in cancer gene-panels by genome editing in HEK 293 T/17 cells.

Genes Environ. 2020;42:8.doi:10.1186/s41021-020-0147-2.

BACKGROUND:

Next Generation Sequencer (NGS) is a powerful tool for a high-throughput sequencing of human genome. It is important to ensure reliability and sensitivity of the sequence data for a clinical use of the NGS. Various cancer-related gene panels such as Oncomine™ or NCC OncoPanel have been developed and used for clinical studies. Because these panels contain multiple genes, it is difficult to ensure the performance of mutation detection for every gene. In addition, various platforms of NGS are developed and their cross-platform validation has become necessity. In order to create mutant standards in a defined background, we have used CRISPR/Cas9 genome-editing system in

HEK 293T/17 cells.
RESULTS:

Cancer-related genes that are frequently used in NGS-based cancer panels were selected as the target genes. Target mutations were selected based on their frequency reported in database, and clinical significance and on the applicability of CRISPR/Cas9 by considering distance from PAM site, and off-targets. We have successfully generated 88 hetero- and homozygous mutant cell lines at the targeted sites of 36 genes representing a total of 125 mutations.
CONCLUSIONS:

These knock-in HEK293T/17 cells can be used as the reference mutant standards with a steady and continuous supply for NGS-based cancer panel tests from the JCRB cell bank. In addition, these cell lines can provide a tool for the functional analysis of targeted mutations in cancer-related genes in the isogenic background.
Keywords: NGS, CRISPR, standard cell lines

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* National Institutes of Biomedical Innovation, Health and Nutrition

Tsukumo Y, Naito M, Suzuki T: Influence of EGFR-activating mutations on sensitivity to tyrosine kinase inhibitors in a KRAS mutant non-small cell lung cancer cell line.
PLoS One 2020: 15.e0229712. doi: 10.1371/journal.pone.0229712.

In non-small cell lung cancer (NSCLC), oncogenic driver mutations including those in KRAS and EGFR are typically mutually exclusive. However, recent reports indicate that multiple driver mutations are found in a certain percentage of cancers, and that the therapeutic responses of such cases with co-mutations of driver genes are largely unclear. Here, using CRISPR-Cas9-mediated genome editing, we generated isogenic cell lines harboring one or two copies of an EGFR-activating mutation from the human NSCLC cell line A549, which is known to harbor a homozygous KRAS gene mutation. In comparison with parent cells with KRAS mutation alone, cells with concomitant EGFR mutation exhibited higher sensitivity to EGFR-tyrosine kinase inhibitors (TKIs) but not to conventional anti-cancer drugs. In particular, cells with two copies of EGFR mutation were markedly more sensitive to EGFR-TKIs compared with parent cells. Thus, the presence of concomitant EGFR mutation can affect the TKI response of KRAS-mutated cells, implying that EGFR-TKI may represent an effective treatment option against NSCLC with EGFR/KRAS co-mutation.
Keywords: EGFR, lung cancer, CRISPR

Furihata C, You X*, Toyoda T, Ogawa K, Suzuki T: Using FFPE RNA-seq with 12 marker genes to evaluate genotoxic and non-genotoxic rat hepatocarcinogens.
Genes Environ. 2020;42:15.doi:10.1186/s41021-020-00152-4

Previously we published results detailing targeted mRNA sequencing (RNA-Seq) using intact liver RNA. In this paper, we performed FFPE RNA-Seq to compare a typical genotoxic hepatocarcinogen (GTHC), 2-acetylaminofluorene (AAF) to genotoxicity equivocal p-cresidine (CRE). RNA-Seq was used to examine liver FFPE samples obtained from male F344 rats that were fed with chemicals (AAF: 0.025% and CRE: 1% in food) for 4 weeks or from controls that were fed a basal diet. AAF induced remarkable differences in the expression of eight genes (Aen, Bax, Btg2, Ccng1, Gdf15, Mbd1, Phlda3 and Tubb4b) from that in the control group, while CRE only induced expression changes in Gdf15. Gene expression profiles for nine genes (Aen, Bax, Btg2, Ccng1, Cdkn1a, Gdf15, Mbd1, Phlda3, and Plk2) differed between samples treated with AAF and CRE. Finally, principal component analysis (PCA) of 12 genes (Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2, and Tubb4b) using our previous Open TG-GATE data plus FFPE-AAF and FFPE-CRE successfully differentiated FFPE-AAF, as GTHC, from FFPE-CRE, as non gentotoxic.
Keywords: 2-acetylaminofluorene, FFPE RNA-Seq, p-cresidine

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迫田秀行，岡本吉弘，菅野伸彦*：ダイナミック超微小硬度計により測定した超高分子量ポリエチレン製コンポーネント内部の力学特性分布.
臨床バイオメカニクス 2019;40:167-170.
人工関節の超高分子量ポリエチレン（UHMWPE）製

部材に加わる力学的負荷や生体脂質等による化学的負荷は，材料表面近傍の微小領域における力学特性に影響する可能性があるが，打抜試験等従来の方法では，その評価が困難であった．そこで，ミクロトームで平滑に仕上げた試料断面を用い，ダイナミック超微小硬度計で評価した．

未使用のUHMWPEの力学特性は，深さ方向に均一だったが，スクアレンを用いて脂質劣化を模擬した試料では，表面近傍での酸化度の上昇に伴い，弾性率と硬度が上昇していた．抜去した人工股関節ライナー10例の摺動面及び背面の表面近傍では，弾性率と硬度が顕著に低下していた．本試験法によりUHMWPE内部の力学特性分布をミクロレベルで評価可能であることを確認すると共に，人工股関節の摺動面では，脂質劣化による影響は見られず，力学的負荷又は脂質の浸入によると思われる軟化が顕著であることがわかった．

Keywords: indentation test, hardness, elastic modulus, lipids, wear

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Yagi T[*1], Ishida F[*2], Shojima M [*3], Anzai H [*4], Fujimura S[*5,6], Sano T [*7], Shinozaki S[*1], Yamanaka Y[*6], Yamamoto Y[*8], Okamoto Y, Ohta M[*4], Nakamura M[*8]: on behalf of the CFD-BIO study group: Systematic review of hemodynamic discriminators for ruptured intracranial aneurysms.
Journal of Biorheology, 2019, 33 (2), 53-64

Researchers have aimed to identify unruptured intracranial aneurysms at a higher risk of rupture during follow-up for a long time. Computational fluid dynamics has been used widely to identify a hemodynamic discriminator between ruptured and unruptured aneurysms. However, this method has yet to reach a consensus between groups, which may be due, in part, to the significant degrees of freedom in hemodynamic indexes and computational workflows. The present review aims to characterize the degree of association between ruptured aneurysms and hemodynamic indexes, as well as the degree of variability between groups. A PubMed search identified 588 relevant studies. Thirteen met our criteria, yielding a total of 3,692 aneurysms. The definition of hemodynamic indexes were first carefully assessed and then classified accordingly. The variability of hemodynamic indexes between groups displayed a significant index-dependent nature. Normalizing hemodynamic indexes was an effective measure of reducing variability. Hemodynamic indexes were evaluated for associability and quantifiability. Overall, in an attempt to advance the diagnostic performance of hemodynamic indexes, these results shed light on the poor ability to interpret hemodynamic states pathologically. Future studies should incorporate the pathological significance of hemodynamic states into the design of hemodynamic indexes.

Keywords: aneurysm, rupture, computational fluid dynamics

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小林憲弘，宮本紫織[*1,2]，佐藤学[*3]，木下輝昭[*4]，高木総吉[*5]，岩間紀知[*6]，粕谷智浩[*7]，古川浩司[*8]，堀池秀樹[*9]，齊藤香織[*10]，京野完[*11]，髙原玲華[*12]，五十嵐良明：液体クロマトグラフィータンデム質量分析による水道水中の140農薬の一斉分析法の妥当性評価．
水環境学会誌 2019;42:247–58.

前報で確立した水道水中の140農薬のLC/MS/MS一斉分析法が全国の水道水質検査に適用できるかどうかを検証するために，国立衛研以外に新たに11機関において水道水を用いた添加回収試験を行い，これら12機関の試験結果を合わせて解析および評価した．各機関は，採取した水道水にアスコルビン酸ナトリウムを加えて脱塩素処理した後，140農薬の混合標準液を添加し，各機関で最適化したLC/MS/MS測定条件を用いて試料を測定した．その結果，48農薬は目標値の1/10と1/100の両方の添加濃度において全12機関が厚生労働省の「水道水質検査方法の妥当性評価ガイドライン」の真度・併行精度の両方の目標を満たし，69農薬は過半数（≧7）の機関が同ガイドラインの真度・併行精度の両方の目標を満たしたことから，本分析法は迅速・簡便な農薬一斉分析法として全国の水道水質検査に適用できると考えられる．

Keywords: agricultural chemical, drinking water, LC/MS/MS

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Tanaka-Kagawa T*1, Saito I*2, Onuki A*2, Tahara M, Kawakami T, Sakai S, Ikarashi Y, Oizumi S*3, Chiba M S*3, Uemura H*4, Miura N*1, Kawamura I*1, Hanioka N*1, Jinno H*5: Method validation for the determination of phthalates in indoor air by GC-MS with solid-phase adsorption/solvent extraction using octadecyl silica filter and styrene–divinylbenzene copolymer cartridge.

BPB Reports, 2019; 2:86-90.

This study proposes and evaluates a precise and labor-saving method for quantifying phthalic-acid esters (PAEs) in indoor air based on solid-phase extraction. Three different adsorbents were evaluated; i.e., two types of octadecyl silica (ODS) filter and a styrene–divinylbenzene (SDB) copolymer cartridge. Calibration curves for five PAEs [diethyl phthalate (DEP), diisobutyl phthalate, di-*n*-butyl phthalate (DBP), benzyl butyl phthalate (BBP), and di(2-ethylhexyl) phthalate (DEHP)] were created using an internal standard (DBP-d_4). Values of the coefficient of determination (R^2) indicated good linearity of the calibration curves (R^2>0.9953). Among the three adsorbents, the SDB cartridge was easiest to handle because it can be used without cleaning and has the lowest blank value. The recovery of deuterated PAEs (DEP-d_4, DBP-d_4, BBP-d_4, and DEHP-d_4) did not differ significantly among the three adsorbents; values were consistently > 89.7% for an air volume of 2.88 m^3. During simultaneous indoor air sampling, PAE concentrations were very similar for the three adsorbents. Interlaboratory validation studies were conducted in five laboratories to validate the proposed method for two PAEs (DBP and DEHP). The mean recoveries of the two PAEs added to two types of adsorbent were 91.3-99.9%, the reproducibility relative standard deviations (RSD_R) were 5.1-13.1%, and the Horwitz ratio (HorRat) values were 0.31-0.79. The proposed method using solid-phase extraction with two types of adsorbents provides accurate estimates of PAEs in ambient air.

Keywords: phthalic acid esters, indoor air, validation

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Kawakami T, Isama K*, Ikarashi Y: Chromium and cobalt concentrations in textile products and the amounts eluted into artificial sweat.

J Environ Chem, 2020; 30:23-8.

Metal allergy due to accessories, dental implants, and other metal-based household products is one of the most common causes of contact dermatitis. Meanwhile, nylon, wool, and silk textile products are often dyed with mordant dyes and metal complex acid dyes that contain chromium and cobalt, which are recognized as allergic metals. In this study, elements present in 78 textile products (106 samples) made of nylon, wool, and silk were analyzed by X-ray fluorescence using a fundamental parameter method. Twenty elements were detected in one or more samples, and Cr and Co were detected in 66 and 40 samples, respectively. The Cr concentration was found to be high, and exceeded 1,000 μg/g in 49 samples, among which, five samples showed >10,000 μg/g of Cr. On the other hand, the Co concentration exceeded 1,000 μg/g in three samples, and no sample showed >10,000 μg/g of Co. Both Cr and Co were detected in dark-toned samples (black, gray, and navy blue), and were hardly detected in light-toned samples (pink and red). Elution tests using seven samples which contained Cr and Co at high concentrations (>10,000 and >1,000 μg/g, respectively) were performed using artificial sweat. The Cr concentrations in acidic sweat (pH 5.5) and alkaline sweat (pH 8.0) were found to be 0.17-170 and 0.36-82 ng/mL, respectively, while the Co concentrations were found to be 0.042-130 and 0.028-130 ng/mL, respectively. The differences in the elution tendencies observed from each textile might be due to differences in the chemical structures of dyes containing Cr or Co. In the case of samples investigated in this study, it is

deemed that Cr and Co are not likely to cause contact dermatitis at concentrations eluted into the artificial sweat.

Keywords: allergic contact dermatitis, textile, chromium and cobalt

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花谷祐未[*1]，村山直也[*2]，竹中基[*2]，室田浩之[*2]，田原麻衣子，河上強志，鈴木加余子[*3]：リストバンドによるアレルギー性接触皮膚炎.

皮膚病診療 2020;42:34-7.

リストバンドによりアレルギー性接触皮膚炎を生じた症例を経験した．製品分析，パッチテストにより，2-（2-hydroxy-5-methylphenyl）benzotriazole（Tinuvin-P®）が原因であることが判明した．Tinuvin-P®は紫外線吸収剤としてさまざまな有機高分子化合物に添加されており，注意が必要である．

Keywords: allergic contact dermatitis, wrist band, Tinuvin-P®

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Saito-Shida S, Kashiwabara N, Shiono K, Nemoto S, Akiyama H：Development of an analytical method for determination of total ethofumesate residues in foods by gas chromatography-tandem mass spectrometry.

Food Chem 2020;313:126-132

Analytical method was developed for determining the total residue of ethofumesate (ET) herbicide using GC-MS/MS. The ET residues were analyzed as a sum of ET, 2-keto-ethofumesate (KET), and open-ring-2-keto-ethofumesate (OKET) and its conjugate. The extracted samples were partitioned with hexane and NaOH solution. For ET analysis, the hexane layer was cleaned up by a silica gel cartridge prior to GC-MS/MS analysis. For the analyses of the metabolites, the aqueous layer was heated with HCl to hydrolyze the conjugates, thereafter, heated in acetic anhydride to convert OKET to KET, and cleaned up by a silica gel cartridge prior to GC-MS/MS analysis. The method was validated for ET, KET, and OKET in garlic, onion, and sugar beet at 0.3 and 0.01 mg/kg. The recoveries were 94–113%, with relative standard deviations of <6%. The limits of detection were 0.0005 mg/kg for all analytes. The proposed method is suitable for regulatory analysis.

Keywords: ethofumesate, hydrolysis, GC-MS/MS

Saito-Shida S, Nagata M*, Nemoto S, Akiyama H：Quantitative analysis of pesticide residues in tea by gas chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization.

J Chromatogr B. 2020;1143:122057. doi: 10.1016/j.jchromb.2020.122057

In this study, gas chromatography–tandem mass spectrometry (GC-MS/MS) using an atmospheric pressure chemical ionization (APCI) source was applied for the quantitative analysis of pesticide residues in tea. To determine the optimum ionization conditions for multiresidue analysis, the full-scan mass spectra and peak intensities of pesticides were compared in the presence and absence of water as a modifier. When water was added as a modifier in the ion source, most of the target compounds formed $[M + H]^+$ ions and exhibited enhanced intensities. However, compounds consisting of only carbon, hydrogen, and chlorine, such as aldrin, γ-hexachlorocyclohexane, and *p,p′*-dichlorodiphenyldichloroethane, typically formed $M^{+\cdot}$ or fragment ions, whose intensities were significantly decreased by the addition of water. GC-MS/MS methods using APCI (without modifier addition) and electron ionization (EI) were validated for 16 pesticides in tea at spiking levels of 0.01 and 0.1 mg/kg. Unlike EI, signal suppression was observed for most compounds at a spiking level of 0.01 mg/kg using APCI; however, dilution of the samples minimized this effect. Using APCI, the trueness of the target compounds ranged from 77% to 121% at both spiking levels, except for pyrethrins owing to matrix effects, with relative standard deviations of less than 14%. For most compounds, these results were comparable with those obtained using EI. However, because the use of APCI limited fragmentation, this ionization technique offered significantly higher sensitivity and specificity than EI. Using APCI, linear calibration curves with coefficients of determination greater than 0.998 were obtained in the range of 0.0005–0.5 μg/mL for all compounds. These findings indicated that GC-MS/MS with APCI is applicable for the routine monitoring of pesticide residues, even in

complex samples such as tea.

Keywords: pesticide, gas chromatography–tandem mass spectrometry, atmospheric pressure chemical ionization

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Kikuchi H, Sakai T, Okura T, Nemoto S, Akiyama H: A simple and sensitive LC-MS/MS method for determining residues of the tranquilizer chlorpromazine in livestock products, seafood, and honey.

Jpn J Food Chem Safety. 2019;26:125-131

A simple and sensitive analytical method for determining residues of the tranquilizer chlorpromazine in foods such as livestock products, seafood, and honey was developed. The method involves solvent extraction with acetone, clean-up using InertSep MC-1 strong cation-exchange solid-phase extraction cartridges, and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis with selective reaction monitoring in positive ionization mode. Because chlorpromazine was gradually degraded in the extraction step when using methanol or ethanol as the extraction solvent, we examined the stability of chlorpromazine in the presence of various solvents. Acetone was selected as the extraction solvent because chlorpromazine was not degraded over time in acetone extracts. Because chlorpromazine adsorbs onto glass surfaces, polypropylene tubes were used for the extraction step to prevent loss of the recovery. The developed method was validated using eight food products spiked with chlorpromazine at 0.1 μg/kg. The validation results exhibited excellent recovery (range, 86-106%) and precision (variation <10%). The limit of quantification (S/N ≥10) of the developed method was 0.1 μg/kg. The proposed method would be very useful for regulatory monitoring of the illegal use of chlorpromazine in foods.

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

朝倉敬行*，北村真理子*，石川孝明*，飯田智成*，中里光男*，安田和男*，根本了：LC-MS/MSによる畜産物中のジルパテロールの分析法.

食品衛生学雑誌 2019;60(5):127-133

An analytical method for the determination of zilpaterol in livestock products was developed. The sample was stirred with *n*-hexane and *n*-hexane saturated acetonitrile, and zilpaterol in the sample was extracted with acetonitrile. The extract was cleaned up on a ODS cartridge column (1 g) and SCX cartridge column (500 mg). The LC separation was carried out using an Inertsil ODS-4 column and linear gradient elution with 0.1% formic acid and acetonitrile containing 0.1% formic acid as mobile phase. Detection of MS was carried out positive ion electrospray ionization mode. Average recoveries (n = 5) of zilpaterol from 6 kinds of livestock products fortified at the MRLs (0.01 mg/kg) were 87.0-99.4%, and the relative standard deviations were 2.4-6.3%. The limits of quantitation were 0.01 mg/kg.

Keywords: zilpaterol, β 2 -agonist, LC-MS/MS

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鍋師裕美，堤智昭，松田りえ子，蜂須賀暁子，穐山浩：ストロンチウム抽出カラムを用いた緊急時に適用可能な食品中のストロンチウム90迅速分析法の確立.

食品衛生学雑誌 2018;60(2):7-15

To ensure food safety during emergency events such as nuclear disasters, we developed a practical rapid determination method for strontium-90 (Sr-90) in foods. Purification of Sr from foods was simplified using a commercial Sr-extraction column. We also reduced the waiting time to achieve radiative equilibrium between Sr-90 and Y-90. Finally, we developed a rapid determination method for Sr-90 that can be completed in about a week. Using the new method, stable Sr recoveries exceeded 85%. The trueness of the method ranged from 109 to115% and the detection limit of Sr-90 was estimated to be 0.07 Bq/kg fresh weight according to a performance evaluation using standard materials. Sr-90 radioactivity concentrations in food samples determined by the new method were highly correlated and nearly equal to concentrations determined by the conventional method. The present study suggests that the new method offers highly sensitive and rapid detection of Sr-90 which are necessary attributes for food tests during emergency events.

Keywords: strontium-90, rapid determination method, Sr resin

Tsutsumi T, Akiyama H, Demizu Y, Uchiyama N, Masada S, Tsuji G, Arai R, Abe Y, Hakamatsuka T, Izutsu K, Goda Y, Okuda H: Analysis of an Impurity, N-Nitrosodimethylamine, in Valsartan Drug Substances and Associated Products Using GC-MS.
Biol. Pharm. Bull. 2019;42:547-551

Valsartan products, commonly used to treat high blood pressure and heart failure, have been recalled in many countries due to the presence of an impurity, *N*-nitrosodimethylamine (NDMA), in the recalled products. We present and evaluate a GC-MS-based analytical method for the determination of NDMA levels and attempt an investigation of NDMA concentrations in valsartan drug substances and associated products. The limit of detection and limit of quantification for the method were estimated to be 0.1 and 0.5 μg/g, respectively, when testing a 0.5-g sample. A good trueness (99%) with a small relative standard deviation (1.9%) was obtained for a valsartan product spiked with NDMA at a concentration of 1.0 μg/g. Additionally, a valsartan drug substance and the associated product, which were previously determined to have NDMA contamination, were analyzed by the method. The NDMA content by our method was very close to previously determined values. Finally, six samples, including valsartan drug substances and associated, commercially available products in Japan, all of which were derived from the company implicated in the NDMA contamination, were analyzed by our method, revealing that none of these samples contained detectable concentrations of NDMA. Overall, the data indicate that the present method is reliable and useful for determination of NDMA in valsartan drug substances and associated products.
Keywords: *N*-nitrosodimethylamine, valsartan, GC-MS

田口貴章，山下涼香，成島純平，岸美紀*，赤星千絵*，岡部信彦*，穐山浩：食品テロ対策のためのLC-MS/MSによる血液・尿等人体試料中の有機リン系農薬の一斉分析法の検討.
日本食品化学学会誌 2020;27(1):33-39

The use of LC-MS/MS as a simultaneous analytical method for the determination of organophosphorus pesticides in human blood or urine in anti-food-terrorism measures was examined. Sample preparation required approximately 25 min, consisting of the addition of two volumes of methanol to blood or urine, vigorous shaking, cooling down, centrifugation and ultrafiltration. The simple reversed-phase LC-MS/MS condition required only 15 min per injection, being able to detect 47 pesticides in the blood and 46 pesticides in the urine. The average recoveries (n = 5) from the blood or the urine spiked at 50 ng/mL were 44.2-163.0% or 55.6-110.4%, respectively. The analytical method presented in this report is simple and could be applicable for any public health institution in anti-food-terrorism measures.
Keywords：食品テロ対策，血液試料，有機リン系農薬

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Hashimoto M*[1], Taguchi T, Ishikawa K*[1], Mori R*[1], Hotta A*[1], Watari S*[1], Katakawa K*[1], Kumamoto T*[2], Okamoto S*[3], Ichinose K*[1]: Unveiling Two Consecutive Hydroxylations: Mechanisms of Aromatic Hydroxylations Catalyzed by Flavin-Dependent Monooxygenases for the Biosynthesis of Actinorhodin and Related Antibiotics.
ChemBioChem 2020;21(5):623-627

Filling the gap: 6-Deoxy-dihydrokalafungin (DDHK) is a previously uncharacterized intermediate in actinorhodin (ACT) biosynthesis. A semisynthetic preparation of DDHK was established, followed by its use for in vitro enzymatic reactions with the aid of a Flavin-dependent monooxygenase, ActVA-ORF5, and a flavin reductase, ActVB. DDHK was hydroxylated stepwise at C-6 and C-8, its intermediacy in ACT biosynthesis thus being established.
Keywords: actinorhodin, aromatic hydroxylation, flavin-dependent monooxygenases

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Akiyama H, Nose M*[1], Takiguchi H, Sugiyama K, Tsutsui R*[1], Hisaka S*[1], Fuchino H*[2], Inui T*[2], Kawano N*[2], Taguchi T, Kudo T*[3], Kawahara N*[2], Yoshimatsu K*[2]: Mutagenetic and anti-allergic studies for evaluation of extracts of Coptis Rhizome produced by an artificial hydroponic system.
J. Nat. Med. 2019;73:608-613

As a part of the investigation of the safety and efficacy of the cultivated Coptis japonica rhizome extracts using an artificial hydroponic cultivation system, the mutagenetic and anti-allergic activities were evaluated. Some extracts of commercial crude drugs of Coptis sp. were also evaluated for the comparison. None of the extracts showed a significant mutagenicity in Salmonella typhimurium TA102 by the Ames tests, but all the extracts showed in S. typhimurium TA98. The extracts of the hydroponically cultivated rhizomes showed anti-allergic activities against contact hypersensitivity as well as those of commercial crude drugs of Coptis sp. These results suggested the potential of the hydroponically cultivated rhizomes as one of the alternative sources for the medicinal usage.

Keywords: Berberine, Coptis japonica, Hydroponic cultivation

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Nose M*1, Tsutsui R*1, Hisaka S*1, Akiyama H, Inui T*2,3, Kawano N*2, Hayashi S*3,4, Hishida A*4, Fuchino H*2, Kudo T*5, Kawahara N*2, Yoshimatsu K*2:Evaluation of the safety and efficacy of Glycyrrhiza uralensis root extracts produced using artificial hydroponic and artificial hydroponic-field hybrid cultivation systems III.: Anti-allergic effects of hot water extracts on IgE-mediated immediate hypersensitivity in mice.

J. Nat. Med. 2020;74:463-466

To evaluate the safety and efficacy of Glycyrrhiza uralensis root extracts produced using artificial hydroponic and artificial hydroponic-field hybrid cultivation systems, we investigated anti-allergic action in mice using IgE-mediated immediate hypersensitivity. Hot water extracts obtained from the roots of Glycyrrhiza uralensis cultivated using two systems were orally administered at a dose of 100 mg/kg as glycyrrhizin (GL) and compared with the commercial crude drug, Glycyrrhizae Radix. Both the artificial hydroponic and artificial hydroponic-field hybrid cultivated root extracts showed anti-allergic effects on IgE-mediated immediate hypersensitivity in mice, as did the commercial crude drugs. These results highlight the potential for artificially cultivated roots of Glycyrrhiza uralensis to be used as an alternative medicinal source.

Keywords: Anti-allergic property, Artificial hydroponic and hydroponic-filed hybrid cultivation, Glycyrrhiza uralensis

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高木彩*1，穐山浩，杉浦淳吉*2，竹村和久*3，吉川肇子*4，織朱實*5：参加型リスクコミュニケーション手法の有効性に影響を与える個人差要因の検討.

日本食品化学学会誌 2019;26:119-124

Risk communication is the interactive process of exchanging information and opinions on risk among stakeholders, and is a component of risk analysis ensuring food safety. This study examined individual differences in the effectiveness of the participatory risk communication method concerning food additives. The five individual differences which may influence the participatory method, measured at baseline, were trust in the government, perception of governmental procedural fairness, high expectation to participate in policy discussions, prior risk perception of food additives, and attitude. The results showed that most participants reported the enhanced understanding of food additives and more interest in food safety after the participatory risk communication. The tendency was higher for those who have a greater expectation to participate in political discussions. We also found that those with low trust in the government tended

to have a lower understanding of food additives. The results confirmed that the participatory risk communication method is more effective and suitable for public meetings where it is assumed that gathered those have a high expectation to participate in political discussions, and also suggested that the importance of establishing trust in advance.

Keywords: risk communication, food additives, food safety

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穐山浩，鈴木美成，浅井麻弓，佐藤惣一郎*1，井上小夕貴*1，佐藤清*2，高木彩*3，杉浦淳吉*4：残留農薬のリスクコミュニケーション手法の検討と評価に関する研究.

日本食品化学学会誌 2019;26:141-146

The risk communication method for pesticide residues was examined. The examined method was evaluated by analysis of a questionnaire conducted on participants before and after the risk communication event using Fisher's exact test and logistic regression analysis. The risk communication method consisted of a 65-min lecture with a 10-min break in the middle followed by a 15-min question-and-answer session. The lecture material consisted of the effectiveness of pesticides in food production, risk assessment of pesticides, and risk management of pesticide residues. Analysis of the questionnaires revealed that this risk communication method significantly promoted participants' understanding of pesticide residues. This method also decreased the number of participants with a negative image of pesticides and increased the number of participants with a positive image.

Keywords: risk communication, pesticide residue, safety

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鈴木美成，中島涼太*：喫煙室の排気に含まれる金属濃度のリアルタイム分析と階層ベイズモデルによる各銘柄によるニッケル排出寄与の推定　島根大学における事例.

環境化学 2019;29:41-49

A hyphenated analytical system with a gas exchange device (GED) and an inductively coupled plasma mass spectrometer (ICP-MS) enabled us to measure the concentration of metals in atmospheric particulate matter in real time. In this study, with a special focus on passive smoking whereby non-smokers inhale cigarette smoke in the environment, the concentration of metals contained in smoking booth exhaust was directly measured by GED-ICP-MS. Furthermore, we used a generalized linear mixture model (GLMM) and hierarchical Bayesian model (HBM) to explain the analytical results. To compute the statistical models, the number of smokers of each brand was used as an explanatory variable, and the emission contribution of each brand to the metal concentration in the exhaust was estimated. GLMM and HBM analyses were carried out based on random effects such as observation errors and errors among smokers. It became clear that the number of smokers of brand C contributes to the increase in the concentration of Mn, Fe, and Ni. As for Ni, where we also introduced a hierarchical Bayesian model to compare brands, the probability of the regression coefficient of brand C being larger than 0 was 1.0. Moreover, the probability that the regression coefficient of brand C is larger than those of other brands was 0.97 or more. Furthermore, in the scenario where four smokers smoke brand C near the exhaust port at the same time, the Ni concentration in the smoke exhaust was estimated to be increased by 0.05 ng/m^3, and if it was assumed that this exhaust is exposed for lifetime, the carcinogenic risk was estimated to increase by a maximum of 1.2×10^{-8}.

Keywords: tobacco, Ni, hierarchical Bayesian model

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Suzuki Y, Ogra Y*1, Machida N*2, Watanabe I*3: Changes in copper, zinc and cadmium distributions in the liver of Formosan squirrels with characteristic high copper accumulation.

Metallomics, 2019;11:1753-1758

We discovered previously that Formosan squirrels (Callosciurus erythraeus) accumulate copper (Cu)

in their livers at levels averaging 1700 μg per dry g (approximately 420 μg per wet g). In the current study, we investigated the relationship between Cu accumulation and hepatic injury, and we determined the distribution and chemical form of Cu in the liver supernatant. In particular, we explored the role of metallothionein in the liver supernatant. We observed no significant differences in hepatic Cu concentration between squirrels that showed pathological changes in the liver and those that did not. Serum alanine aminotransferase activity did not increase with increasing hepatic Cu concentration. These results suggest that abnormal Cu accumulation in the livers of Formosan squirrels does not induce severe hepatic injury. We found that 26.7% of the Cu in the liver was distributed to the supernatant, and only 11.0% of the Cu in the liver was bound to metallothionein, suggesting that metallothionein in the hepatic supernatant does not contribute to detoxification of excess Cu in Formosan squirrels.

Keywords: Formosan squirrels, Copper accumulation, metallothionein

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彌勒地義治*，齊藤憲二*，岸本一宏*，高木成典*，土屋一行*，鈴木紀生*，満月眞寿*，和田善行*，渡邊武俊*，阿部国広*，佐野恵右*，笠原陽子*，東仲隆治*，久能靖*，佐藤恭子：JECFA規格と我が国における食品香料化合物実測値の調査研究（第1報）．
日本食品化学学会誌 2019;26:1-10.

The survey of the contents and properties of food flavoring substances to verify JECFA specifications was conducted. Two hundred food flavoring substances which had no official specifications in Japan and had measured data of 3 or more lots in commerce were selected from the survey in descending order of its use volume in 2010 in Japan. The JECFA's specification values, ie. the content (Assay min %), acid value, melting point, congealing point, refractive index, or the specific gravity of these flavoring substances were compared with the corresponding measured values of the products in commerce. The results revealed that 69 flavoring substances are not in line with the JECFA specifications regarding one or more specific items. In addition, forty substances have some inappropriate specifications that the measured value is specified at the upper or the lower limit of the specifications, or the specifications of the melting points in terms of the property of the substances are selected under the room temperature. We made the guidelines to set the specifications and drafted specifications based on the established guideline and the measured values for the 109 substances. We concluded that the established draft of specifications appropriately enables to control the quality of flavoring substances based on the actual condition.

Keywords：JECFA規格，食品香料化合物，実測値

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増本直子，西﨑雄三，石附京子，中島馨，杉本直樹，多田敦子，曺永晩，小川久美子，佐藤恭子：香料2,4-ジメチル-4-フェニルテトラヒドロフランの異性体存在比の決定．
日本食品化学学会誌 2019;26:63-7.

2,4-dimethyl-4-phenyltetrahydrofuran is a flavoring agent that is permitted for use in food in Japan. To ensure the safety of this compound, its toxicity has been surveyed. For the safety assessment of chemicals, it is important to elucidate the abundance ratio of isomers, if they exist, because isomers can have different biological activities. For this compound, there are four potential isomers; however, no information on the abundance ratio of these isomers currently exists for the commercially available flavoring agent. In this study, the isomer abundance ratio in the commercial product was determined by quantitative ^{1}H-NMR and GC/MS equipped with a chiral column. All four isomers existed in almost equal amounts in the commercially available product.

Keywords：香料，異性体存在比，定量NMR

Masumoto N, Nishizaki Y, Maruyama T*1, Igarashi Y*1, Nakajima K, Yamazaki T*2, Kuroe M*2, Numata M*2, Ihara T*2, Sugimoto N, Sato K: Determination of perillaldehyde in perilla herbs using relative molar sensitivity to single - reference diphenyl sulfone.

J. Nat. Med., 2019;73:566-76.

Perillaldehyde (PRL) is one of the essential oil components derived from perilla plants (*Perilla frutescens* Britton) and is a characteristic compound of the traditional medicine "perilla herb (蘇葉)" listed in the *The Japanese Pharmacopoeia, 17th edition* (JP17). HPLC using an analytical standard of PRL has been used to quantitatively determine the PRL content in perilla herb. However, PRL reagents have been reported to decompose easily. In this study, we utilized an alternative quantitative method using on a single reference with relative molar sensitivity (*RMS*) based on the results of experiments performed in two laboratories. It was possible to calculate the exact *RMS* using an offline combination of ^{1}H-quantitative NMR spectroscopy (^{1}H-qNMR) and an HPLC/photodiode array (PDA) detector (or an HPLC/variable-wavelength detector [VWD]). Using the *RMS* of PRL to the single-reference compound diphenyl sulfone (DFS), which is an inexpensive and stable compound, the PRL content in the perilla herb could be determined using HPLC/PDA or HPLC/VWD without the need for the analytical standard of PRL. There was no significant difference between the PRL contents of perilla herb determined using the method employing the single-reference DFS with *RMS* and using the JP17 assay, the calibration curve of which was generated using the analytical standard of PRL with adjusted purity measured by ^{1}H-qNMR. These results demonstrate that our proposed method using a single reference with RMS is suitable for quantitative assays of perilla herb and can be an alternative method for the current assay method defined in the JP17.

Keywords: relative molar sensitivity, HPLC/PDA, ^{1}H-qNMR

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Suwannarach N*1, Kumla J*1, Nishizaki Y, Sugimoto N, Meerak J*1, Matsui K*2, Lumyong S*1: Optimization and characterization of red pigment production from an endophytic fungus, *Nigrospora aurantiaca* CMU-ZY2045, and its potential source of natural dye for use in textile dyeing.

Appl. Microbiol. Biotechnol., 2019;103:6973-87.

Some of the most important natural pigments have been produced from fungi and used for coloring in food, cosmetics, textiles, and pharmaceutical products. Forty-seven isolates of endophytic fungi were isolated from *Cinnamomum zeylanicum* in northern Thailand. Only one isolate, CMU-ZY2045, produced an extracellularly red pigment. This isolate was identified as *Nigrospora aurantiaca* based on morphological characteristics and the molecular phylogenetic analysis of a combined four loci (large subunit and internal transcribed spacer of ribosomal DNA, β-tubulin, and translation elongation factor 1-alpha genes). The optimum conditions for red pigment production from this fungus were investigated. The results indicated that the highest red pigment yield was observed in the liquid medium containing glucose as a carbon source and yeast extract as a nitrogen source, at a pH value of 5.0 and at 27℃ with shaking for 5 days. The crude red pigment revealed the highest level of solubility in methanol. A fungal red pigment was found to have high stability at temperatures ranging from 20 to 50℃ and pH values at a range of 5.0-6.0. Based on liquid chromatography-mass spectrometry analyses, the red pigment was characterized as bostrycin. The extracted pigment was used for the textile dyeing process. Crude fungal red pigment revealed the highest staining ability in cotton fabrics and displayed excellent fastness to washing, which showing negative cytotoxicity at the concentrations used to cell culture. This is the first report on bostrycin production from *N. aurantiaca*.

Keywords: fungal pigment, bostrycin, textile dye

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水本俊行*，中野扶佐子*，西﨑雄三，増本直子，杉本直樹：相対モル感度を利用したヒハツ抽出物中のピペリン類のHPLC定量分析.

食品衛生学雑誌 2019;60:134-44.

われわれは^{1}H核定量NMR（^{1}H-qNMR）で純度決定された化合物のHPLCピーク強度に基づく相対モル感度（RMS）を利用したヒハツ抽出物（LPE）中のpiperine類の定量法を考案した．Piperineを単一基準物質とした場合のpiperanine，chavicine，isopiperine，isochavicineのRMSは0.3693，1.138，0.9164，1.277であっ

た．RMSを利用したHPLC/UV（RMS法）によるLPE中のpiperine類の定量値と，^{1}H-qNMRや絶対検量線法との定量値の相対誤差は2.01%以下であった．RMS法を用い，piperine異性体の合計定量値を光異性化前後で比較した相対誤差は2.84%であった．RMS法によるLPE錠剤中のpiperine類の定量値は606 μg/gで，各成分の2施設間差は0.600～4.00 μg/gであった．

Keywords：ヒハツ，ピペリン類，相対モル感度

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阿部裕，木嶋麻乃*，山口未来，伊藤裕才*，六鹿元雄，穐山浩，佐藤恭子：ポリ塩化ビニル製おもちゃに使用される可塑剤の実態の変化．

日本食品衛生学雑誌 2019;60:38–44.

国内の市販ポリ塩化ビニル（PVC）製おもちゃに使用されている可塑剤の実態を明らかにするため，2014年度に購入したPVC製おもちゃ約500検体の可塑剤を調査した．その結果，テレフタル酸ジ（2-エチルヘキシル）（DEHTP）など15種類の可塑剤が検出された．その種類は2009年度に購入した試料の調査と大きく変わらなかった．おもちゃからの検出率はDEHTPが最も高く，指定おもちゃでは60.3%，指定おもちゃ以外では73.7%であり，2009年度の調査と比べいずれも20ポイント以上高い値であった．指定おもちゃにおいて使用が禁止されている6種類のフタル酸エステル類（PAEs）は引き続き使用されていなかった．一方，指定おもちゃ以外からは6種類のうち4種類が検出され，検出率は2.8～15.5%であったが，2009年度の調査と比べ10～26ポイント低い値であった．一方，試料あたりの可塑剤総含有量の平均値は2009年度の調査に比べて低い値であった．このように，現在国内で流通するPVC製おもちゃに使用されている主な可塑剤はDEHTPであり，可塑剤の使用量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，おもちゃ，可塑剤

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Abe Y, Ackerman LK*, Mutsuga M, Sato K, Begley TH*: Rapid identification of polyamides using direct analysis in real-time mass spectrometry.

Rapid Communications in Mass Spectrometry 2019. [Epub ahead of print] doi: 10.1002/rcm.8707

Polyamide (PA) is the generic name of polymers synthesized by linking monomers via amide bonds, and various types of PAs with different monomer compositions are known. Distinguishing PA polymers is useful in directing monomer residual testing, product testing, and reverse engineering, but is analytically challenging and cumbersome. To simplify this, we explored the applicability of direct analysis in real time mass spectrometry (DART-MS) for screening PA polymers. DART ion source coupled to a quadrupole Orbitrap (high-resolution (HR) mass spectrometer) was employed for this study. Ten types of PA polymers and four retail samples were evaluated. The DART-HRMS data for these samples, as well as the DART-MS/MS (MS^2) data for PA6 and PA66, were obtained, and their repeatability was assessed across days/calibrations, operators, and equipments. Ions corresponding to the cyclic or linear monomers and oligomers of each PA polymer were detected in each DART-HR mass spectrum. Although similar DART-HR mass spectra were obtained for PA6, PA66, and PA6/PA66 (polymer blends of PA6 and PA66), their DART tandem mass spectra were completely different. The analysis was repeatable, and nearly identical DART tandem mass spectra were obtained on different days, by different operators, and with different equipment. This technique was successfully applied to commercially available samples. Ten types of PA polymers were distinguished using DART-HRMS and DART-MS^2, and their identification using these techniques was straightforward as the characteristic ions for each PA polymer were identified and detected. Furthermore, the spectra were obtained rapidly. Therefore, DART-HRMS can be considered an efficient screening technique for the rapid identification and differentiation of PA polymers.

Keywords: Direct Analysis in Real Time-mass spectrometer (DART-MS), material differentiation, polyamide

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片岡洋平，渡邉敬浩，林恭子，高橋洋武*，滝澤和宏*，穐山浩：ミネラルウォーター類製品におけるクロロ酢酸類の実態調査．

日本食品化学学会誌 2019;26:112–8.

ミネラルウォーター類（MW）製品中のクロロ酢酸類（モノクロロ酢酸，ジクロロ酢酸，トリクロロ酢酸）分析法を構築し，妥当性を確認した．構築した分析法を用いて2016年に国内市場より入手したMW 150製品におけ

るクロロ酢酸類濃度の実態を調査した．実態調査試料と併行して分析した添加試料の分析結果は，3化合物を通じた回収率が90–110%の範囲であり，構築した分析法の適用性が高いことが示された．全150製品中，モノクロロ酢酸は3製品，ジクロロ酢酸は7製品，トリクロロ酢酸は1製品から定量下限値を上回る値で検出され，検出率はそれぞれ2.0%，4.7%，0.7%であった．WHO 飲料水水質ガイドラインの規格値（モノクロ酢酸：0.02 mg/L，ジクロロ酢酸：0.05 mg/L，トリクロロ酢酸：0.2 mg/L）を超過する濃度で検出された製品はなかった．

Keywords：クロロ酢酸類，ミネラルウォーター，実態調査

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河村葉子，和田岳成*，山口未来，六鹿元雄：油脂および脂肪性食品用合成樹脂製器具・容器包装の蒸発残留物試験に関する考察．

食品衛生学雑誌 2019;60:82-7.

食品衛生法では，油脂および脂肪性食品に使用される合成樹脂製器具・容器包装に，溶出試験として蒸発残留物を規定している．合成樹脂製品について，蒸発残留物試験と欧州標準規格EN1186-2によるオリブ油への総溶出物試験を実施し，両者の比較から蒸発残留物試験の溶出条件と規格値について考察した．食品衛生法に従い，ヘプタンを用いて25℃ 1時間溶出後の蒸発残留物量を測定したところ，多くの試料で規格値の30 μg/mL以下であった．また，耐衝撃性ポリスチレン，ポリメチルペンテン，ポリ塩化ビニル（PVC）では30 μg/mLを超えていたが，いずれも緩和された規格値（240，120，150 μg/mL）以下であった．一方，オリブ油への総溶出物量を測定したところ，60℃30分間ではPVCを除いていずれも定量限界以下であった．しかし，95および121℃30分間ではポリエチレン，ポリプロピレン，PVCにおいて30 μg/mLを超える溶出が見られ，蒸発残留物量より高かった．すなわち，一般の使用では現行の溶出条件で対応できているが，高温使用の場合は溶出条件や規格値の緩和が適切ではない可能性が示唆された．

Keywords：合成樹脂製器具・容器包装，蒸発残留物油脂および脂肪性食品

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尾崎麻子，岸映里，大嶋智子，角谷直哉，阿部裕，六鹿元雄，山野哲夫：ヘッドスペース-GC/MSによる食品用ラミネートフィルム中の残留有機溶剤の分析．

食品衛生学雑誌 2019;60:73-81.

ラミネートフィルムは，接着剤や印刷用インキなどに由来する有機溶剤を含有することがある．そこで，ヘッドスペース-GC/MSを用いて，これらの溶剤として使用される可能性のあるトルエン，キシレン，アセトン，メチルエチルケトン，酢酸エチル，酢酸ブチル，メタノール，エタノールなど30物質の有機溶剤の一斉分析法を確立した．本法は，試料に内標準物質を含むN,N-ジメチルホルムアミド溶液を加えて室温で一晩静置後，気相をGC/MSにより測定する方法であり，さまざまな材質からなるラミネートフィルムに適用が可能であった．本法を用いて，市販のラミネートフィルム製の食品包装袋42試料について測定した結果，6試料からトルエン，酢酸エチルやヘプタンなどが検出された．

Keywords：ラミネートフィルム，残留溶剤，ヘッドスペース-GC/MS

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Kishi E*, Ozaki A*, Ooshima T*, Abe Y, Mutsuga M, Yamaguchi Y*, Yamano T*: Determination of various constituent elements of polyethylene terephthalate bottles used for beverages in Japan.

Packaging Technology and Science 2020;33:183-93.

Polyethylene terephthalate（PET）bottles are some of the most commonly used containers for beverages. During the manufacturing process of PET resin in Japan, metallic catalysts such as Sb and Ge are widely used, with other metals or metallic compounds also being employed to improve the quality of PET bottles. However, few reports into the contents of such elements exist. Thus, we herein report the concentrations of 34 elements（ie, Li, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Zr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, W, Pb, and U）in 16 samples of unused virgin PET bottles for beverages. The measurement was performed by inductively coupled plasma optical emission spectrometry（ICP-OES）and inductively coupled plasma mass spectrometry（ICP-MS）, and these bottles were found to contain five main elements（ie, <0.5 to 50 mg/kg Ge, <1 to 26 mg/kg Ti, <0.1 to 279 mg/kg Sb, <10 to 48 mg/kg P, and <0.5 to 53 mg/kg Co）that were used as polymerisation catalysts, stabilisers, oxidation catalysts, and bluing agents. Furthermore, when these residual element concentrations in 21 commercial mineral water PET bottles were determined, there was no significant difference from unused bottles.

Keywords: polyethylene terephthalate (PET), multi element analysis, ICP

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Asakura H, Sakata J[*1], Nakamura H[*1], Yamamoto S, Murakami S[*2]: Phylogenetic diversity and antimicrobial resistance of *Campylobacter coli* from humans and animals in Japan.

Microbes and Environments. 2019;34:146-154

The phylogenetic diversity and antimicrobial resistance (AMR) of *Campylobacter coli* from humans and animals in Japan between 2008 and 2014 were investigated. 119 *C. coli* strains were examined by multilocus sequence typing (MLST), which assigned 36 sequence types (STs). The predominant human ST was ST-860 (18/42), followed by ST-1068 (8/42); these STs were also predominant in poultry (ST-860, 9/25) and cattle (ST-1068, 18/25). ST-1562 was only predominant in swine (11/25, 44.0%). Most swine strains showed resistance to erythromycin (EM). All EM-resistant swine strains (n=15) exhibited a common point mutation in the 23S rRNA sequence (A2085G), and the *tetO* gene was detected in 22 of the 23 TET-resistant swine strains. Four representative swine ST-1562 strains harboured AMR-associated gene clusters in their genomes, suggesting horizontal gene transfer events during host adaptation. This is the first study to demonstrate the phylogenetic diversity and AMR profiles of *C. coli* in Japan. The present results suggest that poultry and cattle are major reservoirs, improving our knowledge on the epidemiological and ecological traits of this pathogen.

Keywords: *Campylobacter coli*, ST-1562, antimicrobial resistance (AMR)

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Sugita-Konishi Y[*1], Kobayashi N[*1], Takasaki K[*2], Kanno T[*1], Itoh M[*1], Riztyan[*2], Futo S[*2], Asakura H, Taira K[*1], Kawakami Y[*1]: Detection of *Sarcocystis* spp. and Shiga toxin-producing *Escherichia coli* in Japanese sika deer meat using a loop-mediated isothermal amplification-lateral flow strip.

Journal of Veterinary Medical Science. 2019;81:586-592

Game meat potentially harbors a number of parasitic and bacterial pathogens that cause foodborne disease. It is thus important to monitor the prevalence of such pathogens in game meats before retail and consumption to ensure consumer safety. In particular, *Sarcocystis* spp. and Shiga toxin-producing *Escherichia coli* (STEC) have been reported to be causative agents of food poisoning associated with deer meat consumption. To examine the prevalence of these microbiological agents on-site at a slaughterhouse, the rapid, simple and sensitive detection method known as the "DNA strip" has been developed, a novel tool combining loop-mediated isothermal amplification and a lateral flow strip. This assay has achieved higher sensitivity and faster than conventional PCR and is suitable for on-site inspection.

Keywords: deer meat, DNA strip, loop-mediated isothermal amplification

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Asakura H, Makino SI[*1], Watanabe K[*2], Tuchida Y[*3], Kawabe M[*3], Sakurai D[*3]: Kuma bamboo grass (*Sasa veitchii*) extracts exhibit protective effects against atypical *Aeromonas salmonicida* infection in goldfish (*Carassius auratus*).

Biocontrol Science. 2019;24:145-154

Atypical *Aeromonas salmonicida* are one of the major opportunistic pathogens that cause ulcer diseases in a variety of fishes, of particular high-priced ornamental fishes. Here we reported that the kuma bamboo grass (*Sasa veitchii*) extracts (KBGE) that contained a variety of fatty acids, exhibited antibacterial activity against 5 atypical *A. salmonicida* strains. Experimental challenges with atypical *A. salmonicida* strains revealed that supplementation with 375 to 750 μg/ml of the KBGE restored the survival of goldfish in coincidence of inhibition of both bacterial replication and superoxide dismutase (SOD) activity upon infection, compared with those of untreated control. Together, our data proposes its possible implication for prevention of *Aeromonas* infection in the ornamental fish.

Keywords: *Aeromonas salmonicida*, antibacterial activity, kuma bamboo grass extracts

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Takahashi T*1, Kabeya H*1, Sato S*1, Yamazaki A*2, Kamata Y*3, Taira K*4, Asakura H, Sugiyama H*5, Takai S*6, Maruyama S*1: Prevalence of *Yersinia* among wild sika deer (*Cervus nippon*) and boars (*Sus scrofa*) in Japan.

Journal of Wildlife Diseases. 2020;56:270-277

We examined the prevalence of *Yersinia*, including pathogenic species such as *Yersinia enterocolitica* and *Y. pseudotuberculosis* among wild sika deer (*Cervus nippon*) and boars (*Sus scrofa*) captured in Japan. The prevalence of *Yersinia* in the wild deer was 75% (207/277) and in the boars was 74% (40/54). A total of 417 isolates of nine *Yersinia* species were isolated from the animals examined: the largest number of isolates (200/417) were *Y. enterocolitica* biotype 1A. Pathogenic *Y. enterocolitica* 1B/O:8 were also isolated from two deer, and *Y. pseudotuberculosis* serogroups 3 and 4 were isolated from two boars and a deer, respectively. The pathogenic *Y. enterocolitica* 1B/O:8 isolates carried four virulence genes (*ail, ystA, yadA,* and *virF*), and *Y. pseudotuberculosis* serogroups 3 and 4 isolates carried three virulence genes (*inv, yadA,* and *lcrF*). Although the *Y. enterocolitica* 1B/O:8 and *Y. pseudotuberculosis* isolates were sensitive to almost all the antimicrobials tested, two *Y. enterocolitica* 1B/O:8 isolates were resistant to azithromycin and ampicillin, and the three *Y. pseudotuberculosis* isolates were resistant only to azithromycin. These findings suggested that wild deer and boars might be important reservoirs for the agent causing human yersiniosis.

Keywords: *Stenotrophomonas maltophilia*, poultry meat

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Yamamoto S, Nakayama T, Asakura H: Draft genome sequence of *Stenotrophomonas maltophilia* CRB139-1, isolated from poultry meat in Japan.

Microbiology Resource Announcements. 2020; 9: e00075-20. doi:10.1128/MRA.00075-20

We report a draft genome sequence of *Stenotrophomonas maltophilia* strain CRB139-1 isolated from poultry meat in Japan. The genome size was 4,619,918 bp at 90× coverage.

Keywords: *Stenotrophomonas maltophilia*, poultry meat

Ikehara T*1, Kuniyoshi K, Yamaguchi H*2, Tanabe Y*2, Sano T*2, Yoshimoto M*3, Oshiro N, Nakashima S*4, Yasumoto-Hirose M*5: First report of *Microcystis* strains producing MC-FR and -WR toxins in Japan.

Toxins. 2019;11(9):521. doi:10.3390/toxins11090521

We surveyed and collected microcystin (MC)-producing cyanobacteria from environmental sources of water in Okinawa, Japan. Using a dual assay (LC-MS analysis and PP2A inhibition assay), we identified and isolated *Microcystis* strains producing five MC variants (MC-LR, -RR, -LA, -FR and -WR). MC-WR and -FR toxins were purified from the laboratory culture of the *Microcystis* isolate NIES-4344. Phylogenetic analysis revealed that NIES-4344 belongs to the identified groups in *Microcystis aeruginosa*. This is the first report of *Microcystis* strains producing mainly MC-WR and -FR toxins in Japan.

Keywords: cyanobacteria, *Microcystis*, microcystin

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Iritani N*, Yamamoto S*, Abe N*, Kanbayashi D*, Kubo H*, Uema M, Noda M, Kaida A*: GII.17 Norovirus infections in outbreaks of acute nonbacterial gastroenteritis in Osaka city, Japan during two decades.

Journal of Medical Virology. 2019;91(12):2101-2107

NoV molecular surveillance in Osaka City, Japan, has revealed that NoV GII.17 was detected for the first time in February 2001 and that NoV GII.17-associated outbreaks remarkably increased during the 2014 to 2015 season, with higher incidence recorded in January to March 2015.

Keywords: Norovirus, GII.P17-GII.17, molecular surveillance

* Osaka Institute of Public Health

Hoa TTT*1, Nakayama T, Huyen HM*1, Harada K*2, Hinenoya A*3, Phuong NT*1, Yamamoto Y*4: Extended-spectrum beta-lactamase-producing *Escherichia coli* harbouring *sul* and *mcr-1* genes isolates from fish gut contents in the Mekong Delta, Vietnam.

Letters in Applied Microbiology. 2019. doi:10.1111/lam.13222

A total of 88 ESBL-producing *E. coli* isolates were analysed for the presence of the ESBLs, *sul* (1, 2, 3) and *mcr* (1–3) genes by PCR. Antimicrobial resistance phenotypes of isolates were determined by disc diffusion.

Keywords: antibiotic resistance, ESBL-producing *E. coli*, fish

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Nakayama T, Kumeda Y*1, Kawahara R*2, Yamamoto Y*3: Quantification and long-term carriage study of human extended-spectrum / AmpC beta-lactamase-producing *Escherichia coli* after international travel to Vietnam

Journal of Global Antimicrobial Resistance. 2020;21:229-234

In total, 19 travellers and 34 travel events were investigated. After confirming that travellers were not colonised with CTX-resistant *E. coli* before travel, 15 travellers and 20 travel events were studied to quantify travellers harbouring CTX-resistant *E. coli* after travel. Isolated colonies from a stool sample was identified, genotyped and further verified by PFGE.

Keywords: extended-spectrum beta-lactamase, AmpC beta-lactamase, *Escherichia coli*

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佐々木貴正，米満研三，上間匡，五十君靜信*，朝倉宏：採卵養鶏場のサルモネラ汚染実態と有効なサルモネラ汚染低減対策の推定

鶏病研究会報 2019;55:159-163

2017年10月～2019年11月の間に延べ56養鶏場の112鶏群（各養鶏場２鶏群）から新鮮盲腸便を採取しサルモネラ検査を実施したところ，７養鶏場の９鶏群（8.0%）から分離された．サルモネラ分離率はサルモネラ不活化ワクチン接種鶏群（５/95，5.3%）の方が未接種鶏群（４/17，23.5%）よりも有意に低かった．以上の結果から，サルモネラ不活化ワクチンは採卵養鶏場のサルモネラ汚染低減策における有用資材の１つであると考えられた．

Keywords：採卵養鶏場，サルモネラ，サルモネラ不活化ワクチン

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鎌田洋一*1，藤田和弘*2，福沢栄太*2，佐藤信彦*3，佐野勇気*3，橘田規*3，高橋洋武*3，大城直雅，岡田由美子，五十君靜信*4，白藤由紀子*5，山崎朗子*5，梶田弘子*6，上田成子*7，奈賀俊人*8：LC-MS/MSによる米飯およびチャーハン中のセレウス菌嘔吐毒，セレウリド試験法．

日本防菌防黴学会誌 2020;48(2):49-56

LC-MS/MSによるセレウリド試験法について検討した．開発した試験法は，嘔吐型セレウス菌食中毒の原因食品におけるセレウリドの検出，また，一般市販米飯やチャーハンのセレウリド汚染の調査とその安全性を検証する方法として，今後用いることができると考えられた．

Keywords: cereulide, cooked rice, LC-MS/MS

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*8 東洋食品工業短期大学

佐々木美江*1，小泉光*2，生島詩織*1,3，菅原直子*4，植木洋*1，畠山敬*1，上間匡：宮城県における野生動物，ブタおよび流入下水におけるE型肝炎ウイルスの侵淫状況．

日本食品微生物学雑誌 2019;36(4):159-164

2015-2017年に宮城県において野生動物，ブタ，下水流入水からのHEV調査を実施し，イノシシ８/84

（9.5%），シカ 0 /76（0 %），ブタ 9 /156（5.8%），下水 7 /91（7.7%）からHEV遺伝子を検出した.
Keywords: hepatitis E virus, animal, sewage

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Kobayashi N*1, Sakurai K*1, Nakarai R*1, Shigaki K*1, Horikawa K*2, Honda M*3, Sugiura Y*1, Watanabe M, Takino M*4, Sugita-Konishi Y*1: Microflora of mycotoxigenic fungi in rice grains in kyushu region of japan and their changes during storage under non-controlled conditions.
Biocontrol Science. 2019;24:161-166

Contamination of agricultural crops by mycotoxins has increased because of the expansion of mycotoxin-producing fungi along with global warming. In this study, the fungal microflora of brown rice grains cultivated in Kyushu region in the southern part of Japan was investigated. A total of 75% of rice samples examined in this study showed less than 30% of fungal contamination rates with a median rate of 12.5%. Some isolates of *Aspergillus flavus* showed the ability to produce aflatoxins (AFs) (AFB1 production was 62.5-70.4 ng/mL). Furthermore, AF-producing *A. flavus* survived during storage and *Aspergillus creber*, which produced sterigmatocystin, was detected in a stored rice sample. Although AFs or sterigmatocystin-contamination was not detected in any rice samples, these mycotoxin-producing fungi are distributed and can survive during storage under the natural conditions in Japan. Employing suitable storage conditions is important for preventing mycotoxin contamination of brown rice grains.
Keywords: Kyushu region of Japan, microflora change, rice

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Ksieniewicz-Woźniak E*1, Bryła M*1, Waśkiewicz A*2, Yoshinari T, Szymczyk K*1: Selected trichothecenes in barley malt and beer from Poland and an assessment of dietary risks associated with their consumption.
Toxins (*Basel*). 2019;11:E715

Eighty-seven samples of malt from several Polish malting plants and 157 beer samples from the beer available on the Polish market (in 2018) were tested for *Fusarium* mycotoxins. DON and its metabolite, DON-3G, were found the most, among the samples analyzed; DON and DON-3G were present in 90% and 91% of malt samples, and in 97% and 99% of beer samples, respectively. NIV was found in 24% of malt samples and in 64% of beer samples, and NIV-3G was found in 48% of malt samples and 39% of beer samples. The risk of exposure to the tested mycotoxins, following the consumption of beer in Poland, was assessed. The corresponding probable daily intakes (PDIs) remained a small fraction of the tolerable daily intake (TDI). However, in the improbable worst-case scenario, in which every beer bottle consumed would be contaminated with mycotoxins present at the highest level observed among the analyzed beer samples, the PDI would exceed the TDI for DON and its metabolite after the consumption of a single bottle (0.5 L) of beer.
Keywords: *Fusarium* toxin, beer, modified mycotoxin

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*2 Poznan University of Life Sciences

Mori T*, Nagao S, Kishino K*, Namba T*, Hara-Kudo Y: DNA extraction for sensitive detection of Shiga toxin-producing *Escherichia coli* in food by real-time PCR assays.
Food Hygiene and Safety Science (*Shokuhin Eiseigaku Zasshi*). 2019;60:183-186

Alkali-heat DNA extraction, a rapid and economical method, was evaluated for use in the detection of Shiga toxin-producing *Escherichia coli* in food using real-time PCR assays. Alkali-heat DNA extracts led to highly sensitive detection (102–104 CFU/mL) of *stx* and O-antigen genes in beef liver, ground beef, sliced pork, cheese, lettuce, radish sprouts, tomato, and spinach, equivalent to the sensitivity obtained using a commercial DNA extraction kit that utilizes proteinase K lysis, and silica membrane purification. Although there were differences in DNA concentration

and purity between DNA extraction methods, the sensitivity of real-time PCR assays was similar. These results indicate that alkali-heat DNA extraction is a viable method when testing food products with real-time PCR assays for the presence of stx and O-antigen genes.

Keywords: alkali-heat DNA extraction, Shiga toxin-producing *Escherichia coli*, real-time PCR

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Nakajima K*1, Ito Y*1, Kikuchi S*1, Okano H*1, Takashima K*1, Woo GH*2, Yoshida T*1, Yoshinari T, Sugita-Konishi Y*3, Shibutani M*1: Developmental exposure to diacetoxyscirpenol reversibly disrupts hippocampal by inducing oxidative cellular injury and suppressed differentiation of granule cell lineages mice.

Food Chem Toxicol. 2020;136:111046

To investigate the developmental exposure effect of diacetoxyscirpenol (DAS) on postnatal hippocampal neurogenesis, pregnant ICR mice were provided a diet containing DAS at 0, 0.6, 2.0, or 6.0 ppm from gestational day 6 to day 21 on weaning after delivery. Offspring were maintained through postnatal day (PND) 77 without DAS exposure. On PND 21, neural stem cells (NSCs) and all subpopulations of proliferating progenitor cells were suggested to decrease in number in the subgranular zone (SGZ) at ≥ 2.0 ppm. At 6.0 ppm, increases of SGZ cells showing TUNEL+, metallothionein-I/II+, γ-H2AX+ or malondialdehyde+, and transcript downregulation of Ogg1, Parp1 and Kit without changing the level of double-stranded DNA break-related genes were observed in the dentate gyrus. This suggested induction of oxidative DNA damage of NSCs and early-stage progenitor cells, which led to their apoptosis. Cdkn2a, Rb1 and Trp53 downregulated transcripts, which suggested an increased vulnerability to DNA damage. Hilar PVALB+ GABAergic interneurons decreased and Grin2a and Chrna7 were downregulated, which suggested suppression of type-2progenitor cell differentiation. On PND 77, hilar RELN+ interneurons increased at ≥ 2.0 ppm; at 6.0 ppm, RELN-related Itsn1 transcripts were upregulated and ARC+ granule cells decreased. Increased RELN signals may ameliorate the response to the decreases of NSCs and ARC-mediated synaptic plasticity. These results suggest that DAS reversibly disrupts hippocampal neurogenesis by inducing oxidative cellular injury and suppressed differentiation of granule cell lineages. The noobserved-adverse-effect level of DAS for offspring neurogenesis was determined to be 0.6 ppm (0.09-0.29 mg/kg body weight/day).

Keywords: diacetoxyscirpenol, hippocampal neurogenesis, oxidative stress

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*3 Azabu University

Onami J*1, Kobayashi N*2, Watanabe M, Yamada O*3, Mizutani O*4, Yokoyama K*5, Haruo T, Chibana H*5, Kamata Y*6: An updated data portal for fungal allergens with curated information.

Bioinformation. 2019;1115:820-823

Allergens originating from fungal components abundantly exist in and around human life. We constructed a data portal specific for fungal allergens that includes genomic data from four Aspergillus species used by beverage industries. The fungal database contains the information of nucleotide sequences, which are similar to the coding region of already known allergens in the public database. The database will accelerate allergen identification and prediction in the fungal research field.

Keywords: fungal allergens, data portal

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Ohtsuka K*1, Hoshino K*1, Kadowaki N*1, Ohsaka M*1, Konishi N*2, Obata H*2, Kai A*2, Terajima J*3, Hara-Kudo Y: Selective media and real-time PCR assays for the effective detection of enterotoxigenic *Escherichia coli* in vegetables.

LWT- Food Science and Technology. 2019;114:108409

Enterotoxigenic *Escherichia coli* (ETEC) is a major foodborne pathogen. Along with water, vegetables are one of the major food sources related to infections. Effective detection methods for ETEC in food, however, have not yet been established. This study aimed to evaluate ETEC detection methods focusing on the major serogroups (O6, O25, O27, O148, O153, O159, and O169) with steps of enrichment, isolation, and real-time PCR targeting genes encoding the heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). ETEC strains (n = 20) were grown to 7.0-8.9 log CFU/mL in modified *E. coli* broth (mEC) at 42℃ for 18 h. The strains formed colonies typically representing *E. coli* on sorbitol MacConkey agar and Shiga toxin-producing *E. coli* on CHROMagar STEC base agar. The minimum detection levels for real-time PCR assays targeting LT and ST genes were 1.9-3.1 log CFU/mL of vegetable culture. Vegetables inoculated with 2.0 log CFU/g ETEC were cultured in mEC, and then ST and LT genes were detected in the culture by real-time PCR assays at low threshold cycle (Ct) values; further, ETEC in the culture was isolated by plating on agars. This study thus demonstrated effective detection methods for ETEC in vegetables.

Keywords: enterotoxigenic *Escherichia coli*, selective media, real-time PCR

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*3 Iwate University

Okano T*1, Kobayashi N*1, Izawa K*2, Yoshinari T, Sugita-Konishi Y*1: Whole genome analysis revealed the genes responsible for citreoviridin biosynthesis in *Penicillium citreonigrum*.
Toxins (*Basel*). 2020;12:E125

Citreoviridin (CTV) is a mycotoxin that is produced by *Aspergillus terreus, Eupenicillium ochrosalmoneum* and *Penicillium citreonigrum*, and CTV has been detected in a wide range of cereal grains throughout the world. In the present study, we determined the draft genome of the *P. citreonigrum* strain IMI92228 and revealed the presence of all four genes that form a gene cluster and that are homologous to the CTV biosynthesis genes of *A. terreus*. The expression of these four homologous genes were highly correlated with CTV production, suggesting that they may play an important role in CTV biosynthesis in *P. citreonigrum*. We concluded that the gene cluster is a CTV biosynthesis cluster of *P. citreonigrum*.

Keywords: *Penicillium citreonigrum*, biosynthesis gene cluster, citreoviridin

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Ooka T*1, Seto K*2, Ogura Y*3, Nakamura K*3, Iguchi A*4, Gotoh Y*3, Honda M*5, Etoh Y*6, Ikeda T*7, Sugitani W*8, Konno T*9, Kawano K*10, Imuta N*1, Yoshiie K*1, Hara-Kudo Y, Murakami K*11, Hayashi T*3, Nishi J*1: O-antigen biosynthesis gene clusters of *Escherichia albertii*: their diversity, similarity to *E. coli* gene clusters, and the development of an O-genotyping method.
Microbial Genomics. 2019;5:e000314

Escherichia albertii is a recently recognized human enteropathogen that is closely related to *Escherichia coli*. In many Gram-negative bacteria, including *E. coli*, O-antigen variation has long been used for the serotyping of strains. In *E. albertii*, while eight O-serotypes unique to this species have been identified, some strains have been shown to exhibit genetic or serological similarity to known *E. coli/Shigella* O-serotypes. However, the diversity of O-serotypes and O-antigen biosynthesis gene clusters (O-AGCs) of *E. albertii* remains to be systematically investigated. Here, we analysed the O-AGCs of 65 *E. albertii* strains and identified 40 *E. albertii* O-genotypes (EAOgs) (named EAOg1-EAOg40). Analyses of the 40 EAOgs revealed that as many as 20 EAOgs exhibited significant genetic and serological similarity to the O-AGCs of known *E. coli/Shigella* O-serotypes, and provided evidence for the inter-species horizontal gene transfer of O-AGCs between *E. albertii* and *E. coli*. Based on the sequence variation in the wzx gene among the 40 EAOgs, we developed a multiplex PCR-based O-genotyping system for *E. albertii* (EAO-genotyping PCR) and verified its usefulness by genotyping 278 *E. albertii* strains from various sources. Although 225 (80.9%) of the 278 strains could be genotyped, 51 were not assigned to any of the 40 EAOgs, indicating that further analyses are required to better understand the diversity of O-AGCs in *E. albertii* and improve the EAO-genotyping PCR method. A phylogenetic view of

E. albertii strains sequenced so far is also presented with the distribution of the 40 EAOgs, which provided multiple examples for the intra-species horizontal transfer of O-AGCs in *E. albertii*.

Keywords: *Escherichia albertii*, O-antigen gene cluster, genotyping

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Yoshinari T, Takeuchi H*1, Kosugi M*2, Taniguchi M*3, Waki M*4, Hashiguchi S*5, Fujiyoshi T*6, Shichinohe Y*7, Nakajima M*3, Ohnishi T, Hara-Kudo Y, Sugita-Konishi Y*8: Determination of sterigmatocystin in foods in Japan: method validation and occurrence data.

Food Addit Contam Part A. 2019;36:1404-1410

A survey of the contamination of foods by sterigmatocystin (STC) was performed by an analytical method based on LC-MS/MS. A total of 583 samples were analysed between 2016 and 2018, and STC was detected in 19.9% of all samples at >0.05 μg/kg (limit of quantification). The foods that were contaminated by STC were wheat flour, Job's tears products, rye flour, rice, buckwheat flour, white sorghum, barley products, azuki bean and corn flour. STC was not found in beer or wine. The highest mean concentrations were obtained for Job's tears products (0.3 μg/kg) and rye flour (0.3 μg/kg). The maximum contamination level was present in a sample of rye flour (7.1 μg/kg). Although the contamination levels were low, these results indicate that STC frequently contaminates Japanese retail foods. A continuous survey is required to assess exposure to STC in Japan.

Keywords: surveillance, sterigmatocystin, LC-MS/MS

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*7 Japan Food Inspection Corporation
*8 Azabu University

山本薫*1，前島圭*1，中田純子*1，奥田祐亮*1，和田安彦*1，寺杣文男*2，工藤由起子，大西貴弘：サルコシスティス属が寄生していた鹿肉を生で喫食したことによる食中毒事例.

日本獣医師会雑誌 2020;73:111-115

和歌山県で発生したサルコシスティス属が関与していると考えられる食中毒事例を紹介した.

Keywords：寄生虫，食中毒

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大西貴弘：寄生虫性食中毒の衛生管理と冷凍処理技術

フードケミカル 2020;36:25-28

最近の寄生虫性食中毒の事例紹介と，寄生虫性食中毒予防に応用可能な冷凍処理技術について紹介した.

Keywords：寄生虫，食中毒

渡辺麻衣子，横瀬英里子*1，小沼ルミ*2，入倉大祐*3，小林直樹*4，角泰人*5，原田奈穂子*6，大橋博樹*7，小西良子*4，工藤由起子，高鳥浩介*8，矢内勝*9，鎌田洋一*10，林健太郎*11, 12：東日本大震災被災地における避難施設内真菌叢に関する研究.

日本防菌防黴学会誌 2020;48: 3 - 9

東日本大震災被災地の4避難施設において，真菌叢調査を行った．その結果，室内空気中の浮遊真菌数は，真菌汚染の基準とされる1,000 CFU/m^3以上となった施設は無く，ヒト危害性の高い菌種の検出濃度も低かったことから，直接的な健康被害の原因となる真菌の異常発育は確認されなかった．しかし，*Aspergillus*属菌の菌数および菌種は，一般家屋室内と比較して異常な状態を示し，注意が必要と考えられた．また，避難施設の清掃ボランティアチームの衛生活動と施設内の真菌叢変動との関連性について検討したところ，本活動は，避難施設内の真菌叢正常化に対して一定の効果をもたらしたことが

示された．しかし，効果の継続性はなく，定期的な清掃が必要不可欠であることが明らかとなった．避難施設は真菌叢が変化しやすい環境であることを認識し，衛生状態に留意しなくてはならない．

Keywords：真菌叢，避難施設，公衆衛生

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*6 宮崎大学医学部
*7（医）家族の森多摩ファミリークリニック
*8（NPO）カビ相談センター
*9 石巻赤十字病院
*10 甲子園大学
*11 Barefoot Doctors Group
*12 国立保健医療科学院

林克彦*1，渡辺愛弓*2，門脇成武*2，湯之前雄太*3，中川香奈子，豊田淑江*4，鈴木俊宏*2，清水則夫*3，工藤由起子，菊池裕：マイコプラズマ否定試験に用いるマイコプラズマ参照品に関する研究（第1報）*Mycoplasma arginini* NBRC 111899株の核酸増幅法（NAT）への適用と維持管理に関する研究．
医薬品医療機器レギュラトリーサイエンス 2019;50:550-559

第十七改正日本薬局方（日局17）参考情報に収載のマイコプラズマ否定試験において，代替法として核酸増幅法（NAT）を用いるには，従来法の培養法及び指標細胞を用いたDNA染色法と検出感度を比較し，目的の結果が得られることを確認することが求められる．日局17には，NATの陽性対照のマイコプラズマ種として7菌種が収載され，NATではこれらを培養して調製した参照品を陽性対照とする．日局17未収載の*Mycoplasma arginini* NBRC 111899の参照品への適用について検討した．調製した参照品に対し，市販のNATキットMycoTOOL PCRで検出を行うと，F2，F3及びF4継代株からなる参照品を9.3 CFU/mLの検出感度で検出できた．培養法の検出感度基準10 CFU/mLを満たしたことから，*M. arginini* NBRC 111899を日局17収載のマイコプラズマ種と同等に扱えることが示された．

Keywords: mycoplasma testing, nucleic acid amplification test (NAT), validation

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*4 日本薬科大学

Shoda T, Ohoka N, Tsuji G, Fujisato T, Inoue H*1, Demizu Y, Naito M, Kurihara M*2: Targeted protein degradation by chimeric compounds using hydrophobic E3 ligands and adamantane moiety.
Pharmaceuticals, **2020**, *13*, 34.

Targeted protein degradation using small chimeric molecules, such as proteolysis-targeting chimeras (PROTACs) and specific and nongenetic inhibitors of apoptosis protein [IAP]-dependent protein erasers (SNIPERs), is a promising technology in drug discovery. We recently developed a novel class of chimeric compounds that recruit the aryl hydrocarbon receptor (AhR) E3 ligase complex and induce the AhR-dependent degradation of target proteins. However, these chimeras contain a hydrophobic AhR E3 ligand, and thus, degrade target proteins even in cells that do not express AhR. In this study, we synthesized new compounds in which the AhR ligands were replaced with a hydrophobic adamantane moiety to investigate the mechanisms of AhR-independent degradation. Our results showed that the compounds, 2, 3, and 16 induced significant degradation of some target proteins in cells that do not express AhR, similar to the chimeras containing AhR ligands. However, in cells expressing AhR, 2, 3, and 16 did not induce the degradation of other target proteins, in contrast with their response to chimeras containing AhR ligands. Overall, it was suggested that target proteins susceptible to the hydrophobic tagging system are degraded by chimeras containing hydrophobic AhR ligands even without AhR.

Keywords: protein-knockdown, PROTAC, SNIPER, hydrophobic tag

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Tsuchiya K*1, Umeno T*2, Tsuji G, Yokoo H, Tanaka M*2, Fukuhara K*1, Demizu Y, Misawa T: Development of photoswitchable estrogen receptor ligands.
Chem. Pharm. Bull. **2020**, *68*, 398-402.

Photopharmacology has attracted attention as an approach for the development of novel therapeutics

because it allows regulation of the bioactivity of compounds based on their conformational change by photo-irradiation. Previously, we have reported several types of selective estrogen receptor (ER) modulators based on diphenylmethane skeleton. To develop novel photopharmacological reagents, we designed and synthesized a set of ER ligands based on azobenzene skeleton, which can switch its conformation following UV irradiation. Our results showed that after UV irradiation, the Z-form of the synthesized compound 9 interacted with ER α, with a KD value of 2.5 μM, whereas the E-form of compound 9 did not bind ability to ER α at 10 μM.

Keywords: photopharmacology, Estrogen receptor, azobenzene

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Naganuma M*, Yokoo H, Misawa T, Matsuno K*, Tsuji G, Demizu Y: Design and Synthesis of 4-(2-Pyrrolyl)-4-Phenylheptane Derivatives as Estrogen Receptor Antagonists.

Heterocycles **2020**, *101*, 429-434.

The estrogen receptor (ER) has been recognized as a potential target for the treatment of breast cancer, which is the most common malignancy found in woman. In this study, a series of 4-(2-pyrrolyl) -4-phenylheptane derivatives as ER antagonists were designed and synthesized. The ER antagonistic activity of these compounds was evaluated to study their structure-activity relationships.

Keywords: Estrogen receptor, antagonist, structure-activity relationship

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* Kougakuin University

Goto C*1, Hirano M*2, Hayashi K, Kikuchi Y*3, Hara-Kudo Y, Misawa T, Demizu, Y: Development of amphipathic antimicrobial peptide foldamers based on magainin 2 sequence.

ChemMedChem **2019**, *14*, 1911-1916.

Magainin 2 (Mag 2), which is isolated from the skin of frogs, is a representative antimicrobial peptide (AMP), exerts its antimicro- bial activity via microbial membrane disruption. It has been reported that both the amphipathicity and helical structure of Mag 2 play an important role in its antimicrobial activity. In this study, we revealed that the sequence of 17 amino acid residues in Mag 2 (peptide 7) is required to exert sufficient activity. We also designed a set of Mag 2 derivatives, based on enhancement of helicity and/or amphipathicity, by incorpora- tion of α,α-disubstituted amino acid residues into the Mag 2 fragment, and evaluated their preferred secondary structures and their antimicrobial activities against both Gram-positive and Gram-negative bacteria. As a result, peptide 11 formed a stable helical structure in solution, and possessed potent antimicrobial activities against both Gram-positive and Gram- negative bacteria without significant cytotoxicity.

Keywords: Antimicrobial peptides, α,α-disubstituted amino acids, Helicity, Amphipathicity, Hemolysis

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Mizuno M*, Mori K*, Misawa T, Demizu Y, Shibanuma M*, Fukuhara K*: Inhibition of β-amyloid-induced neurotoxicity by planar analogues of procyanidin B3.

Bioorg. Med. Chem. Lett. **2019**, *29*, 2659-2663.

Reactive oxygen species (ROS) are known to be produced during the amyloid beta (Aβ) aggregation process. Both ROS production and Aβ fibril formation can result in nerve cell injury. Proanthocyanidins are oligomers of catechin that can act as inhibitors of Aβ aggregation. Procyanidin B3 (Cat-Cat), the dimer of (+)-catechin, can easily cross the blood-brain barrier. Previously, we synthesized two derivatives of Cat-Cat, namely Cat-PCat and PCat-PCat, in which the geometry of one or both catechin molecules in Cat-Cat was constrained to be planar. The antioxidative activities of Cat-PCat and PCat-PCat were found to be stronger than that of Cat-Cat, with PCat-PC at exhibiting the most potent activity. These compounds are predicted to protect against Aβ-induced neurotoxicity via inhibition of Aβ aggregation as well as by antioxidative effects toward Aβ-induced intracellular ROS generation. PCat-PCat exhibited the most potent neuroprotective effects against Aβ-induced

cytotoxicity, which resulted from inhibition of β-sheet structure formation during the Aβ aggregation process. PCat-PCat may be a promising lead compound for the treatment of Alzheimer's disease.
Keywords: Alzheimer's disease, Amyloid beta, Catechin, Proanthocyanidin, Procyanidin, Reactive oxygen species

* Showa University

Sakai-Kato K*[1], Yoshida K*[1], Ohgita T*[2], Takechi-Haraya Y, Demizu Y, Saito H*[2]: Refining calibration procedures of circular dichroism spectrometer to improve usability.
Anal. Sci. **2019**, *35*, 1275-1278.

Circular dichroism (CD) is a technique used for conformational studies of peptides and proteins. We studied the specific calibration procedures of CD spectrometers based on procedures specified in the European Pharmacopoeia. We aimed to develop procedures to improve the usability of CD, in addition to reducing adverse effects on users' health. The use of ethanol instead of 1,4-dioxane as the solvent for isoandrosterone was examined. Both solvents yielded the same maximum value of +3.3 for molar CD. We also studied a two-point calibration method using (1S)-(+)-ammonium 10-camphorsulfonate instead of (1S)-(+)-10-camphorsulfonic acid, which is a hygroscopic compound. Both compounds yielded similar results and the values for (1S)-(+)-ammonium 10-camphorsulfonate of 2.39 ± 0.04 and –4.92 ± 0.06 at 290.5 and 192.5 nm, respectively, were within the criteria defined in the European Pharmacopoeia. The inter-laboratory repeatability was also acceptable. These studies provide specific procedures for calibrating CD spectrometers for drug development.
Keywords: Circular dichroism, Calibration, Usability

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*[2] Kyoto Pharmaceutical University

Kuriyama M*, Yano G*, Kiba H*, Morimoto T*, Yamamoto K*, Demizu Y, Onomura O*: Palladium-catalyzed synthesis of deuterated alkenes through deuterodechlorination of alkenyl chlorides.
Org. Process Res. Dev. **2019**, *23*, 1552-1557.

The palladium-catalyzed deuterodechlorination of alkenyl chlorides has been developed, and a variety of deuterated alkenes were synthesized with precise control of the deuterium incorporation. This catalytic process tolerates heterocyclic moieties and frameworks derived from bioactive agents. In addition to the double incorporation of deuterium, the gram-scale synthesis of a deuterated iminostilbene unit including a core substructure of carbamazepine was achieved in a high yield with an excellent degree of deuteration.
Keywords: Deuteration, alkene, Palladium, *N*-heterocyclic carbene

* Nagasaki University Graduate School of Biomedical Sciences

Misawa T, Ohoka N, Oba M*, Yamashita H, Tanaka M*, Naito M, Demizu Y: Development of 2-aminoisobutyric acid (Aib)-rich cell-penetrating peptide foldamers for efficient siRNA delivery.
Chem. Commun. **2019**, *55*, 7792-7795.

We have designed and synthesized a set of cell-penetrating foldamers (CPFs), Blocks 1–8, composed of the common amino acids Leu, Arg, and Gly, as well as the helicogenic amino acid 2-aminoisobutyric acid. The findings showed that Block 3 could deliver siRNA into cells without significant cytotoxicity. We also demonstrated that Block 3 could be applied to selectively kill the oncogene-driven cancer cells.
Keywords: Cell penetrating foldmers, siRNA delivery, 2-aminoisobutyric acid

* Nagasaki University Graduate School of Biomedical Sciences

Misawa T, Goto C*, Shibata N, Hirano M*, Kikuchi Y, Naito M, Demizu Y: Rational design of novel amphipathic antimicrobial peptides focused on distribution of cationic amino acid residues.
MedChemComm **2019**, *10*, 896-900.

Antimicrobial peptides (AMPs) have garnered much attention as novel therapeutic agents against infectious diseases. They exhibit antimicrobial activity through microbial membrane disruption based on their amphipathic properties. In this study, we rationally designed and synthesized a series of novel AMPs Block, Stripe, and Random, and revealed that Stripe

exhibits potent antimicrobial activity against Gram-positive and Gram-negative microbes. Moreover, we also demonstrated that Stripe disrupts both Gram-positive and Gram-negative mimetic bacterial membranes. Finally, we investigated the hemolytic activity and cytotoxicity in human blood cells and human cell lines, and found that Stripe exhibited neither. These data indicated that Stripe is a promising antimicrobial reagent that does not display significant cytotoxicity.

Keywords: Antimicrobial peptides, Helix, Gram-positive and Gram-negative microbes

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Onizuka K*, Hazemi EM*, Sato N*, Tsuji G, Ishikawa S*, Ozawa M*, Tanno K*, Yamada K*, Nagatsugi F*: Reactive OFF-ON type alkylating agents for higher-ordered structures of nucleic acids.
Nucleic Acids Res. **2019**, *47*, 6578-6589.

Higher-ordered structure motifs of nucleic acids, such as the G-quadruplex (G-4), mismatched and bulge structures, are significant research targets because these structures are involved in genetic control and diseases. Selective alkylation of these higher-order structures is challenging due to the chemical instability of the alkylating agent and side-reactions with the single- or double-strand DNA and RNA. We now report the reactive OFF-ON type alkylating agents, vinyl-quinazolinone (VQ) precursors with a sulfoxide, thiophenyl or thiomethyl group for the OFF-ON control of the vinyl reactivity. The stable VQ precursors conjugated with aminoacridine, which bind to the G-4 DNA, selectively reacted with a T base on the G-4 DNA in contrast to the single- and double-strand DNA. Additionally, the VQ precursor reacted with the T or U base in the AP-site, G-4 RNA and T-T mismatch structures. These VQ precursors would be a new candidate for the T or U specific alkylation in the higher-ordered structures of nucleic acids.

Keywords: Nucleic acid, higher structure, alkylating agent, G-quadruplex

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大庭誠*1，梅澤直樹*2，出水庸介：フォルダマーの魅力－設計・構造・機能－.
Yakugaku-Zasshi **2019**, *139*, 579-580.

低分子を並べてオリゴマーにすると一定の二次構造をとる“フォルダマー（Foldamer）”は，1996年にGellman教授により使われた比較的新しい言葉である．ペプチド・タンパク質や核酸などの分野において，その考え方は古くからあったが，言葉として具現化された効果は非常に大きかった．Gellman教授の発表から約20年が経過し，そのユニークな構造特性や機能創出のための材料となりうることから，近年，精力的に研究が行われている．フォルダマーは低分子のように化学合成でき，一方で低分子化合物では困難な多点による分子認識が可能である．すなわち，抗体に代表される高分子化合物のような機能も有している．このような特性からフォルダマーは，ケミカルバイオロジーのツールとしてだけでなく，医薬品候補としても期待されている．本シンポジウムではこのフォルダマーのもつ魅力について，薬学部のみならず様々な学部に所属するフォルダマー研究者による講演を通して，議論した．

Keywords: Foldamer, Design, Structure, Function

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Yamamoto K*, Kikuchi N*, Hamamizu T*, Yoshimatsu H*, Kuriyama M*, Demizu Y, Onomura O*: Facile synthesis of α-*exo*-methylene ketones from α,α-disubstituted allyl alcohols by electrochemical oxidative migration.
ChemElectroChem **2019**, *16*, 4169-4172.

Oxidative migration of α,α-disubstituted allyl alcohols to α-exo-methylene ketones was accomplished through an electrochemical method by using CaX_2 or MgX_2 (X = Cl, Br) as a halogen mediator. Cyclic and acyclic α,α-disubstituted allyl alcohols were successfully employed in the present reaction, affording the corresponding migration products in good-to-excellent yields. α-exo-Methylene ketones bearing an aliphatic group on the α position of the carbonyl group were obtained by using a two-step procedure, that is, electrochemical oxidative migration followed by base-mediated dehydrohalogenation in a one-pot manner.

Keywords: Oxidative migration, α-*exo*-Methylene ketones, Electrosynthesis, Anodic oxidation, Halogen mediator

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Yamada T*1, Soga K, Hachinohe M*2: Hachisuka A. Performance evaluation of the equipment for measuring radioactivity in whole foodstuffs without destructive sample preparation developed after the Fukushima NPP accident.

Radiation Protection Dosimetry, 2019;184:355-358.

Recently, several types of instruments for measuring radioactivity in whole foodstuff were developed by manufacturers, in which any sample preparation technique such as machining was avoided, and such types of instruments are employed by agricultural producers or municipality radioactivity testing stations in Fukushima. In this study, radioactivity in various kinds of 91 samples collected by residents were measured by use of instruments for radioactivity measurement in whole samples, and the activity in each sample was also measured by use of the conventional gamma-ray spectrometry technique using calibrated Ge detectors after the sample machining procedure. The results obtained by instruments for measurement in whole samples were roughly proportional to the result obtained by a conventional technique, although large differences or unexpected variations were found in some specimens.

Keywords: radioactivity, food, gamma-ray spectrometry

*1 Kindai University
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Soga K, Nakamura K, Ishigaki T, Kimata S, Ohmori K*1, Kishine M*2, Mano J*2, Takabatake R*2, Kitta*2 K, Nagoya*3 H, Kondo K：Development of a novel method for specific detection of genetically modified Atlantic salmon, AquAdvantage, using real-time polymerase chain reaction.

Food Chem. 2020;305:125426.

Genetically modified (GM) Atlantic salmon, AquAdvantage (AquAd), was the first GM animal approved officially for human consumption. Many countries monitor the use of this product under their GM regulations, but a pragmatic system for AquAd-specific detection is needed. Here, we developed a real-time polymerase chain reaction method with high sensitivity for detection of AquAd in foods. This method showed high specificity for the AquAd transgene and the detection limit was 12.5-25 targeted DNA copies per test reaction. An inter-laboratory study using the method developed demonstrated reproducibility at >0.1% (w/w) AquAd content.

Keywords: AquAdvantage, detection method, genetically modified

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*3 Fisheries Research and Education Agency

Soga K, Nakamura K, Ishigaki T, Kimata S, Ohmori K*1, Kishine M*2, Mano J*2, Takabatake R*2, Kitta*2 K, Nagoya*3 H, Kondo K：Data representing applicability of developed growth hormone 1 (GH1) gene detection method for detecting Atlantic salmon (*Salmo salar*) at high specificity to processed salmon commodities.

Data Brief, 2019;104695.

Applicability of the developed growth hormone 1 (GH1) and 18S ribosomal DNA (18S rDNA) detection methods using real-time polymerase chain reaction (PCR) for detecting Atlantic salmon (*Salmo salar*) to processed food commodities was examined. DNAs extracted and purified from 24 commodities labelled to include salmon as an ingredient were used as template. Yield and purity of DNAs obtained and Cq values from real-time PCR analyses were provided.

Keywords: Atlantic salmon, detection method, specificity

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Ku WL*, Nakamura K, Gao W*, Cui K*, Hu G*, Tang Q*, Ni B*, Zhao K*: Single-cell chromatin immunocleavage sequencing (scChIC-Seq) to profile histone modification.

Nat Methods. 2019;16:323-325.

Developed method for analyzing histone modifications, scChIC-seq (single-cell chromatin immunocleavage sequencing), involves targeting of the micrococcal nuclease (MNase) to a histone mark of choice by

tethering to a specific antibody. Cleaved target sites are then selectively PCR amplified. We show that scChIC-seq reliably detects H3K4me3 and H3K27me3 target sites in single human white blood cells. The resulting data are used for clustering of blood cell types.

Keywords: histone modificaion, single-cell scChIC-seq

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Tamehiro N, Nishida K*1, Sugita Y*1, Hayakawa K*1, Oda H*1, Nitta T*1, Nakano M*1, Nishioka A*2, Yanobu-Takanashi R*1, Goto M*1, Okamura T*1, Adachi R, Kondo K, Morita A*2, Suzuki H*1: Ras homolog gene family H (RhoH) deficiency induces psoriasis-like chronic dermatitis by promoting T_H17 cell polarization.

J Allergy Clin Immunol. 2019;143:1878-1891.

Background: Ras homolog gene family H (RhoH) is a membrane-bound adaptor protein involved in proximal T-cell receptor signaling. Therefore RhoH plays critical roles in the differentiation of T cells; however, the function of RhoH in the effecter phase of the T-cell response has not been fully characterized.

Objective: We sought to explore the role of RhoH in inflammatory immune responses and investigated the involvement of RhoH in the pathogenesis of psoriasis.

Methods: We analyzed effector T-cell and systemic inflammation in wild-type and RhoH-null mice. RhoH expression in T cells in human PBMCs was quantified by using RT-PCR.

Results: RhoH deficiency in mice induced TH17 polarization during effector T-cell differentiation, thereby inducing psoriasis-like chronic dermatitis. Ubiquitin protein ligase E3 component N-recognin 5 (Ubr5) and nuclear receptor subfamily 2 group F member 6 (Nr2f6) expression levels decreased in RhoH-deficient T cells, resulting in increased protein levels and DNA binding activity of retinoic acid-related orphan receptor γt. The consequential increase in IL-17 and IL-22 production induced T cells to differentiate into TH17 cells. Furthermore, IL-22 binding protein/Fc chimeric protein reduced psoriatic inflammation in RhoH-deficient mice. Expression of RhoH in T cells was lower in patients with psoriasis with very severe symptoms.

Conclusion: Our results indicate that RhoH inhibits TH17 differentiation and thereby plays a role in the pathogenesis of psoriasis. Additionally, IL-22 binding protein has therapeutic potential for the treatment of psoriasis.

Keywords: Psoriasis, RhoH, TH17 cell

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Miyazaki A*1, Watanabe S*1, Ogata K*2, Nagatomi Y*2, Kokutani R*3, Minegishi Y*3, Tamehiro N, Sakai S, Adachi R, Hirao T*1: Real-time PCR Detection Methods for Food Allergens (Wheat, Buckwheat, and Peanuts) Using Reference Plasmids.

J Agric Food Chem. 2019;67:5680-5686.

Specific and sensitive real-time qualitative polymerase chain reaction (PCR) methods for the detection of food allergens including wheat, buckwheat, and peanuts were developed that could cancel between instrument effects and avoid risks of false-positives and false-negatives. In these real-time PCR analysis, the cutoff for determination of positive samples was set in every PCR run by using reference plasmids containing known copies of the target sequences. The copy numbers of the plasmids were used to detect the allergenic ingredients corresponding to 10 ppm (w/w) protein in highly processed foods (cooked for more than 30 min at 122℃). Reference plasmid analysis for each real-time PCR run helped to minimize variability between runs and instruments (7900HT Real-Time PCR systems and Light Cycler Nano). It also helped to avoid false positives due to trace levels of contaminants from the laboratory environment or agricultural products. The specificity of the real-time PCR method was verified using 79 commonly used food materials and some of their relatives. The method was sensitive enough to detect those allergenic ingredients corresponding to 10 ppm (w/w) in seven types of incurred samples. The current official Japanese method was not able to detect the allergens in some of the incurred samples. The developed method can avoid false negatives due to lack of sensitivity and is useful to confirm positive ELISA screening tests.

Keywords: food allergen, polymerase chain reaction (PCR), positive/negative threshold

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Fukutomi Y*1, Teruuchi Y*2, Nakatani E*3, Minami T*1, Sasagawa Y*2, Fukushima M*2, Kamide Y*1, Sekiya K*1, Saito H*4, Teshima R*5, Adachi R, Taniguchi M*1: Allergen-specific IgG4 over time: Observation among adults with hydrolyzed wheat protein allergy.
Allergy. 2019;74:1584-1587.

In Japan, we have experienced epidemics of immediate-type wheat allergy (IWA) caused by a specific hydrolyzed wheat protein (HWP-IWA). More than 2000 patients (mostly adults) developed IWA (mostly, wheat-dependent exercise-induced anaphylaxis) after skin and/or rhinoconjunctival sensitization to an HWP termed Glupearl 19S contained in facial soap (Cha-no-Shizuku). We consider that observation of the clinical course of this disease might contribute to our understanding of the pathogenesis and prognosis of other disease entities of adulthood-onset food allergy induced by extra-gut sensitization to food-related allergens. The aim of this study was to clarify the change in the levels of allergen-specific IgG4 to HWP after restarting wheat consumption and clarify its association with disease prognosis. In conclusion, this observational study in adults with hydrolyzed wheat protein allergy showed an increase in allergen-specific IgG4 after restarting consumption of wheat, which had been eliminated. Unexpectedly, this increase was associated with a poor disease prognosis, although we did not determine the causality. Considering the recent finding of increased IgG4 levels in eosinophilic esophagitis, more research is needed to examine the significance of allergen-specific IgG4 for various entities of allergic diseases.
Keywords: hydrolyzed wheat protein, extra-gut sensitization, allergen-specific IgG4

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Teno N*1, Yamashita Y*1, Masuda A*1, Iguchi Y*1, Oda K*1, Fujimori K*2, Hiramoto T*1, Nishimaki-Mogami T, Une M*1, Gohda K*3: Identification of potent farnesoid X receptor (FXR) antagonist showing favorable PK profile and distribution toward target tissues: Comprehensive understanding of structure-activity relationship of FXR antagonists.
Bioorg Med Chem. 2019;27:2220-2227.

Antagonizing transcriptional activity of farnesoid X receptor (FXR) in the intestine has been reported as an effective means for the treatment of nonalcoholic fatty liver disease, type 2 diabetes and obesity. We describe herein that the building blocks necessary to maintain the antagonism of our chemotype were investigated in order to modulate in vivo pharmacokinetic behavior and the tissue distribution without blunting the activity against FXR. A comprehensive understanding of the structure-activity relationship led to analog 30, which is superior to 12 in terms of its pharmacokinetic profiles by oral administration and its tissue distribution toward target tissues (liver and ileum) in rats while preserving the in vitro activity of 12 against FXR. Thus, 30 should be a candidate compound to investigate the effects of inhibiting FXR activity while simultaneously improving the outcome of nonalcoholic fatty liver disease, type 2 diabetes and obesity.
Keywords: Antagonists, FXR

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Kondo K, Sakata K, Kato R, Sugano Y*, Takeuchi S*, Sato M*: Qualitative Real-Time PCR Method for Poisonous *Entoloma rhodopolium*-Related Species in Japan: Real-Time PCR Method for *Entoloma Mushroom*s.
Food Hyg Safety Sci. 2019;60:144-150.

Qualitative real-time PCR method for three poisonous *Entoloma rhodopolium*-related species in Japan was established using specific primers and FAM, VIC, Texas Red, Cy5-labeled probes. The use of multicolor probes can extend the method to simultaneous detection of different targets. Standard plasmids were constructed as reference materials. Designed primers and probes in the method detect only a target species,

and the detection limit was 12.5 copies or below. This indicates it is highly specific and sensitive enough to detect the poisonous mushrooms in food residues. Next, we applied the method to four food residue samples obtained from food poisoning cases. The real-time PCR method did identify all of four samples as E. subrhodopolium and *E. pseudorhodopolium*, whereas PCR-RFLP did not. The method established here revealed *Entoloma rhodopolium*-related species in Hokkaido were different species such as *E. eminens* and unknown species.

Keywords: *Entoloma rhodopolium*, Real-time PCR, mushrooms

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Kakimoto S*, Yoshimitsu M*, Akutsu K*, Kiyota K*, Fujiwara T*, Watanabe T, Kajimura K*, Yamano T*: Concentrations of Total Mercury and Methylmercury in Red Snow Crabs (*Chionoecetes Japonicus*) Caught Off the Coast of Japan.

Mar. Pollut. Bull. 2019;145:1-4

The total mercury (T-Hg) and methylmercury (MeHg) concentrations in red snow crabs (*Chionoecetes japonicus*) caught off the coast of Japan were analyzed. The T-Hg concentration ranged from 0.03 to 0.56 mg/kg (mean: 0.21 mg/kg) in the raw muscle, and 0.02 to 0.74 mg/kg (mean: 0.27 mg/kg) in the boiled muscle. The MeHg concentration ranged from 0.04 to 0.54 mg/kg (mean: 0.20 mg/kg) in the raw muscle. The mean ratio of MeHg to T-Hg was 0.88. The crab body weight was found to significantly correlate with the concentrations of T-Hg ($r = 0.488$) and MeHg ($r = 0.490$) ($p \leq 0.01$). For the general population in Japan, the intake of MeHg from eating red snow crab was estimated to be lower than 0.013 mg/week, which was less than one-sixth of the tolerable MeHg intake (0.08 mg/week).

Keywords: Methylmercury, Red snow crab, Total mercury

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渡邉敬浩，片岡洋平，荒川史博*，松田りえ子，畝山智香子：食事を介した摂取量の推定を目的とする元素類一斉分析法の妥当性確認手法.

食品衛生学雑誌 2020;61:7-16

トータルダイエットスタディ（TDS）は，食事を介した化学物質の摂取量推定に有効な方法論であり，有害物質の摂取量推定にも用いられる．TDSにおける試料の分析には，摂取量推定の目的に合致した方法を選択すると同時に，その妥当性を確認することが勧告されている．しかし，妥当性確認に必要な具体的な考え方や方法論は示されていない．そこで本研究では，まず摂取量推定の目的で使用される分析法の性能を評価可能な試料（Samples to estimate methods performance; SEMPs）を開発した．次いでヒ素やカドミウム，鉛を含む元素類の摂取量推定の目的で使用する一斉分析法の妥当性を確認するために，SEMPsにおける各元素濃度を明らかにした．さらに，明らかにした各元素濃度を考慮した添加量を決定し，添加試料と未添加試料のそれぞれを5併行分析した結果から真度と併行精度を推定する，分析法の性能評価方法を確立した．性能評価によって推定した真度と併行精度をCodex委員会のProcedural Manualに収載されているガイドラインに基づき設定した性能規準と比較した結果，検討した一斉分析法が対象とする14元素と14食品群の組合せの多くで性能規準の値を満たしたことから妥当性を確認した．

Keywords：摂取量推定，トータルダイエットスタディ，妥当性確認

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Saito K, Tanaka N*, Ikari J*, Suzuki M*, Anazawa R*, Abe M*, Saito Y, Tatsumi K*: Comprehensive lipid profiling of bleomycin-induced lung injury.

J Appl Toxicol. 2019;39:658-671.

Drug-induced lung injury is an adverse effect of drug treatment that can result in respiratory failure. Because lipid profiling could provide cutting-edge understanding of the pathophysiology of toxicological responses, we performed lipidomic analyses of drug-induced lung injury. We used a mouse model of bleomycin-induced lung injury and followed the physiological responses at the acute inflammatory (day 2), inflammatory-to-fibrosis (day 7) and fibrosis (day 21) phases. The overall lipid profiles of plasma, lung and bronchoalveolar lavage fluid (BALF) revealed that drastic changes in lipids occurred in the lung and BALF, but not in the plasma, after 7 and 21 days of bleomycin treatment. In the lung, the levels of ether-type phosphatidylethanolamines decreased, while those of phosphatidylcholines, bismonophosphatidic acids and cholesterol esters increased on days 7 and

21. In BALF, the global lipid levels increased on days 7 and 21, but only those of some lipids, such as phosphatidylglycerols/bismonophosphatidic acids and phosphatidylinositols, increased from day 2. The lung levels of prostaglandins, such as prostaglandin D2, were elevated on day 2, and those of 5- and 15-lipoxygenase metabolites of docosahexaenoic acid were elevated on day 7. In BALF, the levels of 12-lipoxygenase metabolites of polyunsaturated fatty acids were elevated on day 7. Our comprehensive lipidomics approach suggested anti-inflammatory responses in the inflammatory phase, phospholipidosis and anti-inflammatory responses in the inflammatory-to-fibrosis phase, and increased oxidative stress and/or cell phenotypic transitions in the fibrosis phase. Understanding these molecular changes and potential mechanisms will help develop novel drugs to prevent or treat drug-induced lung injury.

Keywords: bleomycin, bronchoalveolar lavage fluid, fibrosis

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Sai K, Yoshida A*1, Hanatani T, Imatoh T, Takeuchi M*1, Narukawa M*1, Watanabe H*2,3, Uyama Y*4, Saito Y: Population/regional differences in efficacy of 3 drug categories (antidiabetic, respiratory and psychotropic agents) among East Asians: A retrospective study based on multiregional clinical trials.

Br J Clin Pharmacol. 2019;85:1270-1282.

This study aimed to identify population/regional differences in drug efficacy and the influencing factors among East Asians to be considered when planning multi-regional clinical trials (MRCTs) to facilitate rapid drug approval in Asians.

A retrospective analysis of efficacy among East Asian populations for antidiabetic, respiratory, and psychotropic agents was conducted in collaboration with pharmaceutical companies using their MRCT data. Among 17 endpoints for eight pharmaceutical products from three drug categories, no substantial population/regional differences were detected in the three drug categories examined, except for HbA1c change between Japan and Korea for an antidiabetic drug, insulin glulisine (p=0.0068). Variability in disease severity at baseline and concomitant drugs were determined to be potential influencing factors for regional differences. This study suggests that the regional variability in efficacy of these three drug categories is not large among East Asians, and reveals the importance of considering background factors when planning MRCTs. Further studies are needed to evaluate regional variability in the efficacy of other drug categories and clarify the factors leading to regional differences in East Asians.

Keywords: population difference, East Asian, multiregional clinical trial

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Sun Y, Saito K, Iiji R, Saito Y: Application of ion chromatography coupled with mass spectrometry for human serum and urine metabolomics.

SALS Discovery. 2019;24:778-786.

Biomarkers that indicate the presence or severity of organ damage caused by diseases and toxicities are useful diagnostic tools. Metabolomics platforms using chromatography coupled with mass spectrometry (MS) have been widely used for biomarker screening. In this study, we aimed to establish a novel metabolomics platform using ion chromatography coupled with MS (IC-MS) for human biofluids. We found that ethylenediaminetetraacetic acid (EDTA) plasma is not suitable for IC-MS metabolomics platforms because of the desensitization of MS. IC-MS enabled detection of 131 polar metabolites in human serum and urine from healthy volunteers. Pathway analysis demonstrated that the metabolites detectable using our platform were composed of a broad spectrum of organic acids with carboxylic moieties. These metabolites were significantly associated with pathways such as the tricarboxylic acid (TCA) cycle; glyoxylate and dicarboxylate metabolism; alanine, aspartate, and glutamate metabolism; butanoate metabolism; and the pentose phosphate pathway. Moreover, comparison of serum and urine samples showed that four metabolites (4-hydroxybutyric acid, aspartic acid, lactic acid, and γ-glutamyl glutamine) were abundant in serum, whereas 62 metabolites, including phosphoric acid, vanillylmandelic acid, and

N-tiglylglycine, were abundant in urine. In addition, allantoin and uric acid were abundant in male serum, whereas no gender-associated differences were found for polar metabolites in urine. Our results demonstrate that the present established IC-MS metabolomics platform can be applied for analysis of human serum and urine as well as detection of a broad spectrum of polar metabolites in human biofluids.

Keywords: ion chromatography-mass spectrometry, nontargeted metabolomics, polar metabolite

Imatoh T, Sai K, Takeyama M[*1], Segawa K, Yamashita T[*2], Nakashima N[*2], Kataoka Y[*3], Yokoi H[*3], Hiramatsu T[*4], Ohe K[*4], Kimura M[*5], Hori K[*5], Kawakami J[*5], Saito Y: Evaluating the impact of regulatory action on denosumab-induced hypocalcaemia in Japan.

J Clin Pharm Ther. 2019;44(5):788-795.

Since its introduction in April 2012, denosumab has been administered to approximately 7,300 patients as of August 2012, and 32 cases of serious hypocalcaemia after denosumab administration, including two deaths, have been reported in Japan. A Dear Healthcare Professional Letter of Rapid Safety Communication ('Blue letter') was released to warn about the risks of hypocalcaemia associated with denosumab. The goal of this study therefore was to measure the impact of regulatory action on denosumab-induced hypocalcaemia in Japan by using an electronic medical information database (MID).We used two different aggregated data sets based on MIDs (data sets one and two). The patients studied were those who were newly prescribed denosumab or zoledronic acid between April 2012 and September 2014. We assessed four indicators: (a) the proportion of patients with calcium supplementation at the initial denosumab treatment, (b) the proportion of patients who underwent a serum calcium test, (c) the average number of serum calcium tests performed and (d) the prevalence of hypocalcaemia. All indices were aggregated by every 3 months. To evaluate the impact of regulatory action, we used difference in difference (DID) analysis. The proportion of patients with calcium supplementation at the initial denosumab treatment increased year by year in both data sets. The average number of serum calcium tests increased year by year in data set two. There was a significant difference in the prevalence of hypocalcaemia in data set two. This suggests that the estimate of impact of the regulatory action may vary according to the database. In DID analysis, however, significant influences of the regulatory action on combination use with a calcium supplement were detected in both data sets. There was a significant influence on combination use of denosumab with vitamin D and/or calcium supplement in both data sets. That there was no apparent increase in the prevalence of denosumab-induced hypocalcaemia, suggests that the regulatory action had an impact in the clinical setting studied. Such regulatory actions may play an important role in the promotion of drug safety.

Keywords: denosumab, hypocalcaemia, medical information database

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Ueta M[*1], Nakamura R, Saito Y, Tokunaga K[*2,3], Sotozono C[*1], Yabe T[*4], Aihara M[*5], Matsunaga K[*6], Kinoshita S[*1]: Association of HLA class I and II gene polymorphisms with acetaminophen-related Stevens-Johnson syndrome with severe ocular complications in Japanese individuals.

Hum Genome Var. 2019;6:50. doi: 10.1038/s41439-019-0082-6.

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are acute-onset mucocutaneous diseases induced by infectious agents and/or inciting drugs. We have reported that the main causative drugs for SJS/TEN with severe ocular complications (SOC) were cold medicines, including multi-ingredient cold medications and nonsteroidal anti-inflammatory drugs (NSAIDs). Moreover, we also reported that acetaminophen is the most frequent causative drug in various cold medicines. In this study, we focused on acetaminophen-related SJS/TEN with SOC and analyzed *HLA-class II* (*HLA-DRB1, DQB1*) in addition to *HLA-class I* (*HLA-A, B, C*). We studied the histocompatibility antigen genes *HLA-DRB1* and *DQB1* in addition to *HLA-A, B*, and *C* in 80 Japanese patients with acetaminophen-related

SJS/TEN with SOC. We performed polymerase chain reaction amplification followed by hybridization with sequence-specific oligonucleotide probes (PCR-SSO) using commercial bead-based typing kits. We also used genotyped data from 113 healthy volunteers for *HLA-DRB1* and *DQB1*, and 639 healthy volunteers for *HLA-A, B*, and *C*. *HLA-DRB1*08:03* and *DRB1*12:02* were associated with acetaminophen-related SJS/TEN with SOC, although the results ceased to be significant when we corrected the p-value for the number of alleles detected. *HLA-A*02:06* was strongly associated with acetaminophen-related SJS/TEN with SOC (carrier frequency: $p = 4.7 \times 10^{-12}$, $Pc = 6.6 \times 10^{-11}$, OR = 6.0; gene frequency: $p = 8.0 \times 10^{-13}$, $Pc = 1.1 \times 10^{-11}$, OR = 4.9). *HLA-B*13:01* (carrier frequency: $p = 2.0 \times 10^{-3}$, Pc = 0.042, OR = 4.1; gene frequency: $p = 2.2 \times 10^{-3}$, Pc = 0.047, OR = 3.9), *HLA-B*44:03* (carrier frequency: $p = 2.1 \times 10^{-3}$, Pc = 0.045, OR = 2.4) and *HLA-C*14:03* (carrier frequency: $p = 3.4 \times 10^{-3}$, Pc = 0.045, OR = 2.3) were also significantly associated, while *HLA-A*24:02* was inversely associated (gene frequency: $p = 6.3 \times 10^{-4}$, $Pc = 8.8 \times 10^{-3}$, OR = 0.5). Acetaminophen-related SJS/TEN with SOC was not associated with *HLA-class II* (*HLA-DRB1, DQB1*). However, for acetaminophen-related SJS/TEN with SOC, we found an association with *HLA-B*13:01* and *HLA-C*14:03* in addition to *HLA-A*02:06* and *HLA-B*44:03*, which have been described previously.

Keywords: Immunological disorders, Predictive markers

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Saito K, Yagi H[*1], Maekawa K, Nishigori M[*1,2], Ishikawa M, Muto S[*2], Osaki T[*1], Iba Y[*2], Minatoya K[*2], Ikeda Y[*2], Ishibashi-Ueda H[*2], Ogino H[*2], Sasaki H[*2], Matsuda H[*2], Saito Y, Minamino N[*1,2]: Lipidomic signatures of aortic media from patients with atherosclerotic and nonatherosclerotic aneurysms.

Sci Rep. 2019;9:15472. doi: 10.1038/s41598-019-51885-4.

Aortic aneurysms are associated with fatal aortic rupture. Current therapeutic approaches are limited to implantation of aortic prostheses and stent-grafts; no effective drugs are available because the pathogenic mechanisms of aortic aneurysms remain unclear. Here, we aimed to elucidate the molecular mechanisms of the initiation and progression of aortic aneurysm by lipidomics. We performed lipidomics analyses of lipids in the aortic media of normal, border, and aneurysm areas from patients with thoracic atherosclerotic aortic aneurysm (N = 30), thoracic nonatherosclerotic aortic aneurysm (N = 19), and abdominal atherosclerotic aortic aneurysm (N = 11) and from controls (N = 8) using liquid chromatography and mass spectrometry. Significant alterations were observed in the lipid profiles of patients with atherosclerotic aortic aneurysms and to a lesser extent in those with nonatherosclerotic aneurysms. Increased triacylglycerols (TGs) and decreased ether-type phosphatidylethanolamines (ePEs) were observed throughout the normal, border, and aneurysm areas of thoracic and abdominal atherosclerotic aortic aneurysms. Prostaglandin D2 increased, but ePEs and TGs decreased in normal areas of thoracic atherosclerotic aortic aneurysms and thoracic nonatherosclerotic aortic aneurysms compared with the control tissues. These findings expand our knowledge of metabolic changes in aortic aneurysms and provide insights into the pathophysiology of aortic aneurysms.

Keywords: Aortic diseases, Aneurysm

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Izumi Y[*1], Matsuda F[*2], Hirayama A[*3], Ikeda K[*4], Kita Y[*5], Horie K[*6], Saigusa D[*7], Saito K, Sawada Y[*8], Nakanishi H[*9], Okahashi N[*2], Takahashi M[*1], Nakao M[*1], Hata K[*1], Hoshi Y[*10], Morihara M[*10], Tanabe K[*11], Bamba T[*1], Oda Y[*5]: Inter-Laboratory Comparison of Metabolite Measurements for Metabolomics Data Integration.

Metabolites. 2019;9(11):257. doi: 10.3390/metabo9110257.

BACKGROUND: One of the current problems in the field of metabolomics is the difficulty in integrating data collected using different equipment at different

facilities, because many metabolomic methods have been developed independently and are unique to each laboratory.

METHODS: In this study, we examined whether different analytical methods among 12 different laboratories provided comparable relative quantification data for certain metabolites. Identical samples extracted from two cell lines (HT-29 and AsPc-1) were distributed to each facility, and hydrophilic and hydrophobic metabolite analyses were performed using the daily routine protocols of each laboratory.

RESULTS: The results indicate that there was no difference in the relative quantitative data (HT-29/AsPc-1) for about half of the measured metabolites among the laboratories and assay methods. Data review also revealed that errors in relative quantification were derived from issues such as erroneous peak identification, insufficient peak separation, a difference in detection sensitivity, derivatization reactions, and extraction solvent interference.

CONCLUSION: The results indicated that relative quantification data obtained at different facilities and at different times would be integrated and compared by using a reference materials shared for data normalization.

Keywords: data integration, inter-laboratory comparison, metabolomics

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Sun Y, Saito K, Iiji R, Saito Y: Lipid Profile Characterization and Lipoprotein Comparison of Extracellular Vesicles from Human Plasma and Serum.

Metabolites. 2019;9(11):259. doi: 10.3390/metabo9110259.

Extracellular vesicles (EVs) consist of lipid bilayers, occur in various biofluids, and are invaluable in biomarker screening. Liquid chromatography coupled with high-resolution mass spectrometry (LC-MS) was recently used to study comprehensive EV lipid profiles in vitro. The aim of this study was to establish a lipidomics platform for human plasma and serum EVs for comprehensive characterization of their lipid profiles, and to compare them with those of other lipid-containing particles, such as high-density lipoproteins (HDL), and low/very low-density lipoproteins (LDL/VLDL). Isolation was validated by specific protein markers; CD9 and MHC class for EVs, apoA-I for HDL, and apoB-100 for LDL/VLDL. Lipidomics identified 264 lipids from isolated plasma EVs, HDL, and LDL/VLDL. The absolute lipid levels per unit protein content in the EVs were more than eight times lower than those of the lipoproteins. Moreover, the EVs had higher lysoglycerophospholipid levels than HDL or LDL/VLDL. Similar profiles were also determined for human serum. The present study found that the lipid profiles of EVs are unique and distinctly different from those of lipoproteins. The lipidomics platform applied to human plasma and serum EVs could generate important information for the exploration and qualification of biomarkers in disease diagnosis.

Keywords: extracellular vesicle, lipidomics, lipoprotein

Saito K, Ueno S*1, Nakayama A*1, Nitta SI*2, Arai K*2, Hasunuma T*3, Saito Y: Overall Similarities and a Possible Factor Affecting Plasma Metabolome Profiles Between Venous and Capillary Blood Samples From 20 Healthy Human Males.

J Pharm Sci. 2019;108:3737-3744.

Amino acids and lipids are biomarkers used to assess the presence and severity of disease, as well as the toxicological response to drugs. Although upper-extremity venipuncture is a well-used standard technique, fingertip capillary sampling is a more convenient procedure. Delineating the global differences in amino acid and lipid levels in capillary and venous blood samples is paramount for expanding the application of capillary blood tests in biomarker assays. We recruited 20 healthy male subjects and collected plasma obtained from both fingertip capillary and antecubital venous blood. The samples were analyzed to determine the overall profiles of amino acids and lipids and to test for differences

in their levels between both vessel types. The results demonstrated that the differences between capillary and venous blood had a lower impact than interindividual variations; however, trends of separation between them were observed for amino acids. The levels of 5 out of 28 amino acids scored fold changes over 30%, while 9 out of 498 lipids had a fold change over 30%. The time required for fingertip blood collection could be a factor for the differences in 3 metabolites. These findings provide useful information for the application of fingertip capillary blood sampling in biomarker assays.

Keywords: amino acids, fingertip, lipids

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Nishi T*1, Maeda T*2, Imatoh T, Babazono A*3: Comparison of regional with general anesthesia on mortality and perioperative length of stay in older patients after hip fracture surgery.
Int J Qual Health Care. 2019;31(9):669-675.

The aim of this study was to examine whether anesthetic technique is associated with 30- or 90-day mortality and perioperative length of stay (LOS). We used a retrospective cohort design using a healthcare insurance claims database. The Fukuoka Prefecture's claims database of older patients who underwent hip fracture surgery under general or regional (spinal or epidural) anesthesia from April 2012 to March 2016 was used for analyses. The database under analyses contained 16 125 participants of hip fracture surgery under general or regional anesthesia. We measured 30- and 90-day mortalities and perioperative LOS. In a propensity score-matched cohort, we found no significant differences in 30- and 90-day mortalities after adjusting for confounding factors. The reconverted perioperative LOS for the general and regional anesthesia groups was, respectively, 29.7 (29.1-30.4) and 28.0 (27.4-28.6) days in the matched cohort. Therefore, the perioperative LOS in the regional anesthesia group was significantly shorter by 1.7 days than in the general anesthesia group ($P < 0.001$). This study demonstrated that the use of regional anesthesia was not associated with 30- or 90-day mortality, but it was associated with slightly shorter perioperative LOS. Since Japan has much longer LOS than other countries, our findings have implications for more efficient healthcare resource utilization and quality assurance in geriatric care.

Keywords: general anesthesia, perioperative length of stay, mortality

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Akiyama H*1, Kawamata K*2, Fukutomi Y*3, Matsufuji H*2, Kai S*4, Miyazawa M*4, Nakamura R: Novel *in vitro* test for pollen-related vegetable/fruit allergy using the EXiLE method.
Allergol Int. 2020 Jan 18. pii: S1323-8930(20)30001-0. doi: 10.1016/j.alit.2019.12.007. [Epub ahead of print]

Pollen-associated food allergy syndrome (PFS), which is a common problem worldwide, develops when an individual who is sensitized to an inhaled pollen ingests fruits or vegetables that cross-react with the sensitizing pollen allergen. The *in vivo* prick-prick test using fresh fruits or vegetables is the most sensitive test for detecting IgE to food in PFS. However, test results vary depending on the overall condition of the patient, and the number of antigens that can be tested at one time is limited. We established a novel allergy test based on IgE crosslinking-induced luciferase expression (EXiLE) using a humanized cultured rat mast cell line, which expresses human Fc ε RI and the nuclear factor of activated T-cell-responsive luciferase reporter gene. Using preserved sera, preserved extracts, and a cultured mast cell line, the EXiLE method can detect cross-reactive IgE and allergen-specific IgE from several microliters of serum. The use of freshly prepared tomato or apple skin juice, which is a stimulating antigen, was also effective. Therefore, the EXiLE method can be used for the screening of potentially cross-reactive fruit and vegetable allergens in patients allergic to pollen. In addition to pollen allergen-specific IgE titers, this novel *in vitro* method may help clinicians in diagnosing PFS before performing the *in vivo* prick-prick test, which is burdensome.

Keywords: Pollen-associated food allergy syndrome, EXiLE method, prick-prick test

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Villazala-Merino S*1, Rodriguez-Dominguez A*1, Stanek V*1, Campion NJ*1, Gattinger P*1, Hofer G*2, Froeschl R*1, Fae I*1, Lupinek C*1, Vrtala S*1, Breiteneder H*1, Keller W*2, Perkmann T*1, Nakamura R, Pickl WF*1, Valenta R*1,3, Eckl-Dorna J*1, Niederberger V*1: Allergen-specific IgE levels and the ability of IgE-allergen complexes to cross-link determine the extent of CD23-mediated T-cell activation.

J Allergy Clin Immunol. 2020;145(3):958-967.

CD23 mediates IgE-facilitated allergen presentation and subsequent allergen-specific T-cell activation in allergic patients. We sought to investigate key factors regulating IgE-facilitated allergen presentation through CD23 and subsequent T-cell activation. To study T-cell activation by free allergens and different types of IgE-Bet v 1 complexes, we used a molecular model based on monoclonal human Bet v 1-specific IgE, monomeric and oligomeric Bet v 1 allergen, an MHC-matched CD23-expressing B-cell line, and a T-cell line expressing a human Bet v 1-specific T-cell receptor. The ability to cross-link Fc ε receptors of complexes consisting of either IgE and monomeric Bet v 1 or IgE and oligomeric Bet v 1 was studied in human Fc ε RI-expressing basophils. T-cell proliferation by monomeric or oligomeric Bet v 1, which cross-links Fc ε receptors to a different extent, was studied in allergic patients' PBMCs with and without CD23-expressing B cells. In our model non-cross-linking IgE-Bet v 1 monomer complexes, as well as cross-linking IgE-Bet v 1 oligomer complexes, induced T-cell activation, which was dependent on the concentration of specific IgE. However, T-cell activation by cross-linking IgE-Bet v 1 oligomer complexes was approximately 125-fold more efficient. Relevant T-cell proliferation occurred in allergic patients' PBMCs only in the presence of B cells, and its magnitude depended on the ability of IgE-Bet v 1 complexes to cross-link CD23. The extent of CD23-mediated T-cell activation depends on the concentration of allergen-specific IgE and the cross-linking ability of IgE-allergen complexes.

Keywords: CD23, IgE, T cell

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Maekawa K*1, Ri M*2, Nakajima M*3, Sekine A*4, Ueda R*5, Tohkin M*2, Miyata N*2, Saito Y, Iida S*2: Serum lipidomics for exploring biomarkers of bortezomib therapy in patients with multiple myeloma.

Cancer Sci. 2019;110:3267-3274.

Although the proteasome inhibitor bortezomib (BTZ) shows excellent efficacy in multiple myeloma (MM), a fraction of patients has a suboptimal or no response to this agent. In addition, BTZ-induced peripheral neuropathy (BiPN), a frequent side-effect of this therapy, limits its use in some patients. This study aimed to explore serum lipid biomarker candidates to predict the response to BTZ and the severity of BiPN. Fifty-nine serum samples were collected from patients with MM prior to receiving BTZ plus low-dose dexamethasone therapy. Serum levels of phospholipids, sphingolipids, neutral lipids, and polyunsaturated fatty acids and their oxidation products were measured by a comprehensive lipidomic study. Overall, 385 lipid metabolites were identified in patients' sera; lower levels of several glycerophospholipids, sphingolipids, and cholesteryl esters were associated with a poor treatment response. Metabolites related to platelet-activating factor biosynthesis and cholesterol metabolism appeared particularly relevant. Furthermore, several lysophosphatidylcholines, phosphatidylcholines, ceramides, neutral lipids, and oxidative fatty acids were significantly increased or decreased in patients with BiPN grades ranging from G0 to G3. Among these compounds, mediators reportedly inducing myelin breakdown and stimulating inflammatory responses were prominent. Although further study is necessary to validate these biomarker candidates, our results contribute to the development of predictive biomarkers for response to BTZ treatment, or ensuing severe BiPN, in patients with MM.

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Oka SI*1, Chin A*1, Park JY*2, Ikeda S*1, Mizushima W*1, Ralda G*1, Zhai P*1, Tong M*1, Byun J*1, Tang F*1, Einaga Y*1, Huang CY*1, Kashihara T*1, Zhao M*1, Nah J*1, Tian B*1, Hirabayashi Y, Yodoi J*3, Sadoshima J*1: Thioredoxin-1 maintains mitochondrial function via mTOR signaling in the heart.

Cardiovasc Res. 2019 Oct 4; doi: 10.1093/cvr/cvz251. Online ahead of print.

AIMS: Thioredoxin 1 (Trx1) is an evolutionarily conserved oxidoreductase that cleaves disulfide bonds in oxidized substrate proteins such as mechanistic target of rapamycin (mTOR) and maintains nuclear-encoded mitochondrial gene expression. The cardioprotective effect of Trx1 has been demonstrated via cardiac-specific overexpression of Trx1 and dominant negative Trx1. However, the pathophysiological role of endogenous Trx1 has not been defined with a loss-of-function model. To address this, we have generated cardiac-specific Trx1 knockout (Trx1cKO) mice. METHODS AND RESULTS: Trx1cKO mice were viable but died with a median survival age of 25.5 days. They developed heart failure, evidenced by contractile dysfunction, hypertrophy, and increased fibrosis and apoptotic cell death. Multiple markers consistently indicated increased oxidative stress and RNA-sequencing revealed downregulation of genes involved in energy production in Trx1cKO mice. Mitochondrial morphological abnormality was evident in these mice. Although heterozygous Trx1cKO mice did not show any significant baseline phenotype, pressure-overload-induced cardiac dysfunction and downregulation of metabolic genes were exacerbated in these mice. mTOR was more oxidized and phosphorylation of mTOR substrates such as S6K and 4EBP1 was impaired in Trx1cKO mice. In cultured cardiomyocytes, Trx1 knockdown inhibited mitochondrial respiration and metabolic gene promoter activity, suggesting that Trx1 maintains mitochondrial function in a cell autonomous manner. Importantly, mTOR-C1483F, an oxidation resistant mutation, prevented Trx1 knockdown-induced mTOR oxidation and inhibition and attenuated suppression of metabolic gene promoter activity. CONCLUSION (S): Endogenous Trx1 is essential for maintaining cardiac function and metabolism, partly through mTOR regulation via Cys1483. TRANSLATIONAL PERSPECTIVE: Although cell protective effects of Trx1 have been demonstrated previously, the in vivo function and the direct target of endogenous Trx1 remain to be elucidated. Using cardiac-specific Trx1 KO mice, this study demonstrates that endogenous Trx1 plays an essential role in maintaining cardiac function and redox homeostasis and confers stress resistance to the heart. The salutary effect of Trx1 in the heart is primarily mediated through reduction of mTOR in vivo.

Keywords: Heart, Redox, mechanistic target of rapamycin (mTOR)

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Kobayashi K*1, Kuze J*2, Abe S*3, Takehara S*3, Minegishi G*1, Igarashi K*4, Kitajima S, Kanno J*5, Yamamoto T*6, Oshimura M*3, Kazuki Y*3: CYP3A4 induction in the liver and intestine of pregnane X receptor/CYP3A-humanized mice: approaches by mass spectrometry imaging and portal blood analysis.

Mol Pharmacol. 2019;96(5):600-608. doi 10.1124/mol.119.117333

Induction of cytochrome P450 3A (CYP3A) in response to pregnane X receptor (PXR) activators shows species-specific differences. To study the induction of human CYP3A in response to human PXR activators, we generated a double humanized mouse model of PXR and CYP3A. CYP3A-humanized mice generated by using a mouse artificial chromosome (MAC) vector containing the entire genomic human CYP3A locus (hCYP3A-MAC mouse line) were bred with PXR-humanized mice in which the ligand binding domain of mouse PXR was replaced with that of human PXR, resulting in double humanized mice (hCYP3A-MAC/hPXR mouse line). Oral administration of the human PXR activator rifampicin increased hepatic expression of CYP3A4 mRNA and triazolam 1'- and 4-hydroxylation activities, CYP3A probe activities, in the liver and intestine microsomes of hCYP3A-MAC/hPXR mice. The plasma concentration of

triazolam after oral dosing was significantly decreased by rifampicin treatment in hCYP3A-MAC/hPXR mice but not in hCYP3A-MAC mice. In addition, mass spectrometry imaging analysis showed that rifampicin treatment increased the formation of hydroxytriazolam in the intestine of hCYP3A-MAC/hPXR mice after oral dosing of triazolam. The plasma concentration of 1'- and 4-hydroxytriazolam in portal blood was also increased by rifampicin treatment in hCYP3A-MAC/hPXR mice. These results suggest that the hCYP3A-MAC/hPXR mouse line may be a useful model to predict human PXR-dependent induction of metabolism of CYP3A4 substrates in the liver and intestine.

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Yokota S, Shirahata T*1, Yusa J*2, Sakurai Y*2, Ito H*2, Oshio S*1: Long-term dietary intake of excessive vitamin A impairs spermatogenesis in mice.
J Toxicol Sci. 2019;44(4):257-71. doi 10.2131/jts.44.257

Vitamin A and its derivatives contribute to many physiological processes, including vision, neural differentiation, and reproduction. Vitamin A deficiency causes early cessation of spermatogenesis, characterized by a marked depletion of germ cells. However, there has been no clear understanding about the role of chronic intake of vitamin A excess (VAE) in spermatogenesis. The objective of this study was to investigate whether chronic intake of VAE diet causes arrest of spermatogenesis. To examine the effects of VAE on spermatogenesis, we used ICR male mice fed with control (AIN-93G purified diet: 4 IU/g) diet or VAE (modified AIN-93G diet with VAE: 1,000 IU/g) diet for 7 weeks (from 3 to 10 weeks of age). At 10 weeks of age, the retinol concentration in the testes of VAE mice was significantly higher than that of control mice. Testicular cross sections from control mice contained a normal array of germ cells, while the seminiferous tubules from VAE mice exhibited varying degrees of testicular degeneration. Daily sperm production in VAE testes was dramatically decreased compared to that in control testes. Sperm viability, motility, and morphology were also impaired in VAE mice. Furthermore, we examined the effects of VAE on the expression of genes involved in retinoid signaling and spermatogenesis to determine the underlying molecular mechanisms. Therefore, we are the first to present results describing the long-term dietary intake of VAE impairs spermatogenesis using a mouse model.

Keywords: Spermatogenesis, Retinoid, Toxicology

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Nomura Y*, Ikuta S*, Yokota S, Mita J*, Oikawa M*, Matsushima H*, Amano A*, Shimonomura K*, Seya Y*, Koike C*: Evaluation of critical flicker-fusion frequency measurement methods using a touchscreen-based visual temporal discrimination task in the behaving mouse.
Neurosci Res. 2019;148:28-33. doi 10.1016/j.neures.2018.12.001

The critical flicker-fusion frequency (CFF), defined as the frequency at which a flickering light is indistinguishable from a continuous light, is a useful measure of visual temporal resolution. The mouse CFF has been studied by electrophysiological approaches such as recordings of the electroretinogram (ERG) and the visually evoked potential (VEP), but it has not been measured behaviorally. Here we estimated the mouse CFF by using a touchscreen based operant system. The test with ascending series of frequencies and that with randomized frequencies resulted in about 17 and 14 Hz, respectively, as the frequency which could not be distinguished from steady lights. Since the ascending method of limits tend to overestimate the threshold than the descending method, we estimated the mouse CFF to be about 14 Hz. Our results highlight usefulness of the operant conditioning method in measurement of the mouse visual temporal resolution.

Keywords: Visual contrast, Operant behavior, Critical flicker-fusion

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Goto M*, Saito H, Hiradate Y*, Hara K*, Tanemura K*: Differences in resistance against osmotic challenge among C57BL/6, DBA/2 and their hybrid mice MII stage oocytes.
Zygote. 2019;27(4):250-254. doi 10.1017/S0967199418000370

Oocytes of B6D2F1 (BDF1) mice are often used as recipients for intracytoplasmic sperm injection because of their cell membrane resistance against capillary penetration. It is assumed that oocytes of BDF1 mice have superior traits because of their hybrid vigour. However, the mechanisms of hybrid vigour are unclear. In this study, we focused on the membrane resistance of MII stage oocytes against changes in extracellular osmotic pressure. As a result, MII stage oocytes of inbred C57BL/6 and DBA/2 mice showed high tolerance in either a hypertonic or a hypotonic environment. Conversely, MII stage oocytes of hybrid BDF1 and D2B6F1 mice showed high tolerance in both hypertonic and hypotonic environments. Therefore, it is considered that MII stage oocytes of hybrid mice have superior traits than those of inbred mice. Our findings demonstrated that the hybrid vigour exists in the form of resistance to extracellular osmotic environment in hybrid MII stage oocytes.

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Saito H, Hara K*1, Tominaga T*2, Nakashima K*3, Tanemura K*1: Early-life exposure to low levels of permethrin exerts impairments in learning and memory with the effects on neuronal and glial population in adult male mice.
Journal of Applied Toxicology. 2019;39(12):1651-1662. doi 10.1002/jat.3882

Permethrin, a pyrethroid chemical, is widely used as a pesticide because of its rapid insecticidal activity. Although permethrin is considered to exert very low toxicity in mammals, the effects of early, low-level, chronic exposure on the adult central nervous system are unclear. In this study, we investigated the effects of low-level, chronic permethrin exposure in early life on the brain functions of adult mice, using environmentally relevant concentrations. We exposed mice to the acceptable daily intake level of permethrin (0.3 ppm) in drinking water during the prenatal and postnatal periods. We then examined the effects on the central nervous system in adult male offspring. In the permethrin group, we detected behavior that displayed incomplete adaptation to a novel environment, as well as an impairment in learning and memory. In addition, immunohistochemical analysis revealed an increase in doublecortin-(an immature neuron marker) positive cells in the hippocampal dentate gyrus in the permethrin exposure group compared with the control group. Additionally, in the permethrin exposure group there was a decrease in astrocyte number in the hilus of the dentate gyrus, and remaining astrocytes were often irregularly shaped. These results suggest that exposure to permethrin at low levels in early life affects the formation of the neural circuit base and behavior after maturation. Therefore, in the central nervous system of male mice, low - level, chronic permethrin exposure during the prenatal and postnatal periods has effects that were not expected based on the known effects of permethrin exposure in mature animals.

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Igarashi T, Suzuki H, Ushida K, Matsumoto M, Inoue K, Kanno T*1, Miwa Y*2, Ishii T*3, Nagase T*2, Katsumata Y*3, Hirose A: Initial hazard assessment of 1,4-dichlorobutane: Genotoxicity tests, 28-day repeated-dose toxicity test, and reproductive/developmental toxicity screening test in rats.
Regul Toxicol Pharmacol. 2020;112:104610. doi 10.1016/j.yrtph.2020.104610

1,4-Dichlorobutane (1,4-DCB) is used as raw materials for drugs, pesticides, fragrances, and chemical fibers, and being used as a solvent. Its toxicity data was insufficient for screening assessment under the Japanese Chemical Substances Control Law. We conducted toxicity tests and hazard classification for screening assessment. 1,4-DCB showed negative in the Ames test, positive in the in vitro chromosomal aberrations test with metabolic activation, and negative in the in vivo mouse bone-marrow micronucleus test.

The 28-day repeated-dose toxicity study, where male and female rats were administered 1,4-DCB by gavage at 0, 12, 60, and 300 mg/kg/day, showed significant effects on the liver and pancreas from 12 mg/kg/day and kidney at 300 mg/kg/day. Based on periportal hepatocellular hypertrophy and decreased zymogen granules in pancreas, the lowest observed adverse effect level (LOAEL) of 12 mg/kg/day was obtained. The reproductive/developmental toxicity screening study, in which male and female rats were administered 1,4-DCB by gavage at dose of 0, 2.4, 12, and 60 mg/kg/day for 42–46 days, showed that the delivery index was decreased at 60 mg/kg/day without maternal toxicity. Based on the general toxicity, we classified this chemical as hazard class 2, with a D-value (Derived No Effect Level) of 0.002 mg/kg/day.

Keywords: cas no. 110-56-5, chemical substances control law, D-value

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Abdelgied M*1, El-Gazzar AM*1, Alexander WT*1, Numano T*1 , Iigou M*1, Naiki-Ito A*1, Takase H*1 , Hirose A, Taquahashi Y, Kanno J*2, Abdelhamid M*1, Abdou KA*3, Takahashi S*1, Alexande DB*1, Tsuda H*1: Carcinogenic effect of potassium octatitanate (POT) fibers in the lung and pleura of male Fischer 344 rats after intrapulmonary administration.

Part Fibre Toxicol 2019 2;16(1):34. doi 10.1186/s12989-019-0316-2

Background: Potassium octatitanate fibers (K2O•8TiO2, POT fibers) are used as an asbestos substitute. Their physical characteristics suggest that respirable POT fibers are likely to be carcinogenic in the lung and pleura. However, previous 2-year inhalation studies reported that respired POT fibers had little or no carcinogenic potential. In the present study ten-week old male F344 rats were left untreated or were administered vehicle, 0.25 or 0.5 mg rutile-type nano TiO2 (r-nTiO2), 0.25 or 0.5 mg POT fibers, or 0.5 mg MWCNT-7 by intra-tracheal intra-pulmonary spraying (TIPS), and then observed for 2 years. Results: There were no differences between the r-nTiO2 and control groups. The incidence of bronchiolo-alveolar cell hyperplasia was significantly increased in the groups treated with 0.50 mg POT and 0.50 mg MWCNT-7. The overall incidence of lung tumors, however, was not increased in either the POT or MWCNT-7 treated groups. Notably, the carcinomas that developed in the POT and MWCNT-7 treated rats were accompanied by proliferative fibrous connective tissue while the carcinomas that developed in the untreated rats and the r-nTiO2 treated rats were not (carcinomas did not develop in the vehicle control rats). In addition, the carcinoma that developed in the rat treated with 0.25 mg POT was a squamous cell carcinoma, a tumor that develops spontaneously in about 1 per 1700 rats. The incidence of mesothelial cell hyperplasia was 4/17, 7/16, and 10/14 and the incidence of malignant mesothelioma was 3/17, 1/16, and 2/14 in the 0.25 mg POT, 0.5 mg POT, and MWCNT-7 treated groups, respectively. Neither mesothelial cell hyperplasia nor mesothelioma developed in control rats or the rats treated with r-nTiO2. Since the incidence of spontaneously occurring malignant mesothelioma in rats is extremely low, approximately 1 per 1000 animals (Japan Bioassay Research Center [JBRC] historical control data), the development of multiple malignant mesotheliomas in the POT and MWCNT-7 treated groups was biologically significant.

Conclusion: The incidence of pleural mesotheliomas in male F344 rats administered POT fibers and MWCNT-7 was significantly higher than the JBRC historical control data, indicating that the incidence of pleural mesothelioma in the groups administered POT fibers and MWCNT-7 fibers via the airway using TIPS was biologically significant. The incidence of type II epithelial cell hyperplasia and the histology of the carcinomas that developed in the POT treated rats also indicates that respirable POT fibers are highly likely to be carcinogenic in the lungs of male F344 rats.

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A[*1], Takase H[*1], Abdou KA[*2], Hirose A, Taquahashi Y, Kanno J[*3], Abdelhamid M[*2], Tsuda H[*1], Takahashi S[*1]: Pulmonary and pleural toxicity of potassium octatitanate fibers, rutile titanium dioxide nanoparticles, and MWCNT-7 in male Fischer 344 rats.

Arch Toxicol 2019;93(4):909-920. doi 10.1007/s00204-019-02410-z

Potassium octatitanate ($K_2O{\cdot}8TiO_2$, POT) fibers are used as an alternative to asbestos. Their shape and biopersistence suggest that they are possibly carcinogenic. However, inhalation studies have shown that respired POT fibers have little carcinogenic potential. We conducted a short-term study in which we administered POT fibers, and anatase and rutile titanium dioxide nanoparticles (a-nTiO_2, r-nTiO_2) to rats using intra-tracheal intra-pulmonary spraying (TIPS). We found that similarly to other materials, POT fibers were more toxic than non-fibrous nanoparticles of the same chemical composition, indicating that the titanium dioxide composition of POT fibers does not appear to account for their lack of carcinogenicity. The present report describes the results of the 3-week and 52-week interim killing of our current 2-year study of POT fibers, with MWCNT-7 as a positive control and r-nTiO_2 as a non-fibrous titanium dioxide control. Male F344 rats were administered 0.5 ml vehicle, 62.5 μg/ml and 125 μg/ml r-nTiO_2 and POT fibers, and 125 μg/ml MWCNT-7 by TIPS every other day for 2 weeks (eight doses: total doses of 0.25 and 0.50 mg/rat). At 1 year, POT and MWCNT-7 fibers induced significant increases in alveolar macrophage number, granulation tissue in the lung, bronchiolo-alveolar cell hyperplasia and thickening of the alveolar wall, and pulmonary 8-OHdG levels. The 0.5 mg POT- and the MWCNT-7-treated groups also had increased visceral and parietal pleura thickness, increased mesothelial cell PCNA labeling indices, and a few areas of visceral mesothelial cell hyperplasia. In contrast, in the r-nTiO_2-treated groups, none of the measured parameters were different from the controls.

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Kuwagata M, Muneoka K[*], Honda K[*], Miyazaki A[*]: Hypothalamic monoaminergic pathology in a neurodevelopmental rat model showing prenatal 5-bromo-2'-deoxyuridine treatment-induced hyperactivity and hyporeproductivity

Neuropsycology 2019 3;79:161-169. doi 10.1159/000504552

Objective: Prenatal treatment of rats with 5-bromo-2'-deoxyuridine (BrdU) is a neurodevelopmental model showing hyperactivity and impaired sexual activity. Human neurodevelopmental disorders, such as autism, exhibit sex-related pathology, but sex-related neurodevelopment has not been fully investigated in this model. We conducted this study to facilitate the understanding of the pathophysiology of neurodevelopmental disorders. Methods: Pregnant rats received 50 mg/kg BrdU on gestational days 9-15. The tissue content of dopamine (DA), serotonin (5-HT), and their metabolites dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid were measured in male and female offspring at 3 weeks (juveniles) and 10 weeks (adults) of age. Results: Prenatally BrdU-treated rats had reduced DA metabolism or DA content in the hypothalamus from the juvenile through the adult period without sex differences, but sex-specific striatal DA abnormalities emerged after maturation. A reduction in 5-HT metabolism was measured in the hypothalamus without sex differences throughout development. Developmental alterations in the striatal 5-HT states were sex-dependent. Temporal changes in DA or 5-HT metabolism were found in the frontal cortex and midbrain. Conclusion: The sex-specific influence of a genotoxic factor on the development of the DA and 5-HT systems was clarified in the hypothalamus and striatum. The results suggest that the observed sex dependence and region specificity are related to the pathology of social dysfunction in neurodevelopmental disorders.

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Fritsche E[*14], Clark R[*15], Shiota K[*11], Chahoud I[*3]: Update of the DevTox data database for harmonized risk assessment and alternative methodologies in developmental toxicology: Report of the 9th Berlin Workshop on developmental toxicity.

Reprod Toxicol 2019;89:124-129. doi 10.1016/j.reprotox.2019.07.003

Representatives of applied science (e.g. governmental organizations, academia, and industry) met to discuss the progress towards a harmonized human health risk assessment in developmental toxicology of plant protection products, biocidal products, and other environmental chemicals at the 9th Berlin Workshop on Developmental Toxicity held in September 2018. Within the focus of the scientific discussion were the future of in-vitro methods for developmental and reproductive toxicology, the potential relevance of alternative species in testing of de-velopmental effects, and risk and hazard assessment of developmental and endocrine effects.

Furthermore, the need for a harmonized terminology for classification of anomalies in laboratory animals in developmental toxicity studies aiming for human health risk assessment was determined. Here, the DevTox database was identified as an extremely valuable tool. Overall, the participants agreed that still one of the biggest challenges for testing developmental toxicity in the 21st century is the development of animal-free test strategies and alternatives to animal testing that could provide human-relevant information in a rapid, efficient, and mechanistically informative manner.

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Kumamoto T[*1], Senuma M[*2], Todoroki M[*2], Kumagai F[*2], Imai H[*1], Suzuki R[*1], Ogawa T[*3], Kuwagata M: 5-Fluorocytosine induces fetal skeletal malformations in rats by altering expression of Homeobox genes.

Fundam Toxicol Sci 2020;7(2):97-103. doi 10.2131/fts.7.97

5-Fluotocytosine (5-FC) is an antimycotic and teratogenic compound. Oral administration of 5-FC to pregnant rats on gestational day (GD)9 and 13 was shown to induce thoracolumbar supernumerary ribs (TSR, 14th rib) and abnormal digits, respectively, in fetuses. This study investigated the effects of 5-FC on homeobox genes, which control the anterior-posterior-axis. 5-FC (75 mg/kg) was administered orally on GD9 and GD13, and tissues collected from cranial and caudal regions of TSR sites were analyzed. Following 5-FC administration on GD9, the levels of expression of Hoxa10, which determine the position of the thoracic and lumbar vertebrae, were decreased at GD13. Analysis of hindlimbs 6 hours after administration on GD13 showed decreases in expression of Hoxa11, Hoxd12, and Hoxd13, the Hox genes responsible for limb formation from the proximal to distal, and from the anterior to posterior directions. The present findings showed that altered expression of Hox genes contributes to 5-FC teratogenicity.

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Izumi-Nakaseko H[*1], Fujiyoshi M[*2], Hagiwara-Nagasawa M[*1], Goto A[*1], Chiba K[*1], Kambayashi R[*1], Naito AT[*1], Ando K[*3], Kanda Y, Ishii I[*4], Sugiyama A[*1]: Dasatinib can Impair Left Ventricular Mechanical Function But May Lack Proarrhythmic Effect: A Proposal of Non-clinical Guidance for Predicting Clinical Cardiovascular Adverse Events of Tyrosine Kinase Inhibitors.

Cardiovasc Toxicology 2020;20:58-70

Tyrosine kinase inhibitors are known to clinically induce various types of cardiovascular adverse events; however, it is still difficult to predict them at preclinical stage. In order to explore how to better predict such drug-induced cardiovascular adverse events, we tried to develop a new protocol by assessing acute electrophysiological, cardiohemodynamic, and cytotoxic effects of dasatinib in vivo and in vitro. Dasatinib at 0.03 and 0.3 mg/kg was intravenously administered to the halothane-anesthetized dogs for 10 min with an interval of 20 min between the dosing (n = 4). Meanwhile, that at 0.1, 0.3, and 1 μM was cumulatively applied to the human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (n = 7). In the dogs, the low and high doses provided peak plasma concentrations of 40 ± 5 (0.08) and 615 ± 38 ng/mL (1.26 μM), respectively. The low dose decreased the heart rate, impaired the left ventricular mechanical function, and prolonged the ventricular effective refractory period. The high dose prolonged the repolarization period, induced hemorrhagic tendency, and increased plasma cardiac troponin I level in addition to enhancement of the changes observed after the low dose, whereas it neither affected the cardiac conduction nor induced ventricular arrhythmias. In the hiPSC-CMs, dasatinib prolonged the repolarization and refractory periods like in dogs, while it did not induce apoptotic or necrotic process, but that it increased the conduction speed. Clinically observed major cardiovascular adverse events of dasatinib were observed qualitatively by currently proposed assay protocol, which may become a useful guide for predicting the cardiotoxicity of new tyrosine kinase inhibitors.

Keywords: dasatinib, halothane-anesthetized dogs, human induced pluripotent stem cell-derived cardiomyocytes

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Goto A*, Hagiwara-Nagasawa M*, Chiba K*, Kambayashi R*, Nunoi Y*, Izumi-Nakaseko H*, Matsumoto A*, Kanda Y, Sugiyama A*: Pharmacological β-adrenoceptor blockade can augment torsadogenic action of IKr inhibitor: Comparison of proarrhythmic effects of d-sotalol and dl-sotalol in the chronic atrioventricular block dogs.

J Pharmacol Sci. 2019;141:86-9

Information is still limited whether β-blockade may augment or attenuate the onset of torsade de pointes in patients with I_{Kr} inhibitor-induced labile repolarization process. We compared the proarrhythmic effects of d-sotalol with those of dl-sotalol using the chronic atrioventricular block dogs, since d- and l-isomers share a similar blocking action on I_{Kr} but β-blocking activity resides only in l-isomer. dl-Sotalol (3 mg/kg, p.o.) induced torsade de pointes in 3 out of 4 animals, whereas d-sotalol (3 mg/kg, p.o.) induced it in only 1 out of 4 animals. Thus, β-blockade can augment torsadogenic action of IKr inhibitor.

Keywords: I_{Kr} inhibitor, β-blockade, torsade de pointes

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Yamada S*, Kanda Y: Retinoic acid promotes barrier functions in human iPSC-derived intestinal epithelial monolayers.

J Pharmacol Sci. 2019;140:337-44

Vitamin A (VA) is a fat-soluble micronutrient that plays essential roles in various biological processes, including cell growth, differentiation, and apoptosis. In the intestine, VA are known to promote mucosal homeostasis and immunity. However, the effect of VA in intestinal development has not been well elucidated. In the present study, we generated human intestine organoids from human induced pluripotent stem cells (iPSCs), and investigated the effect of the VA active metabolite all-trans retinoic acid (RA), on differentiation into intestinal organoids. As a result, RA increased the gene expression of a drug-metabolizing enzyme CYP3A4, as a functional molecule of intestinal mature development, in iPSC-derived intestinal organoids. In addition, RA increased transepithelial electrical resistance, an indicator of epithelial integrity, and decreased the permeability of monolayers to fluorescein isothiocyanate-labeled dextran in intestinal epithelial monolayers. Finally, RA increased the expression of ZO-1, a marker of tight junctions, which are essential for intestinal epithelial barrier function. Taken together, these results indicate that RA promotes barrier functions of iPSC-derived intestinal

epithelial monolayers by increasing ZO-1 expression.
Keywords: retinoic acid, induced pluripotent stem cells, intestinal organoids

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Kodama M*1, Furutani K*2,3, Kimura R*4, Ando T*4, Sakamoto K*5, Nagamori S*6, Ashihara T*7, Kurachi Y*2, Sekino Y*1, Furukawa T*4, Kanda Y, Kurokawa J*5: Systematic expression analysis of genes related to generation of action potentials in human iPS cell-derived cardiomyocytes.
J Pharmacol Sci. 2019;140:325-30

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a valuable tool to characterize the pharmacology and toxic effects of drugs on heart cells. In particular, hiPSC-CMs can be used to identify drugs that generate arrhythmias. However, it is unclear whether the expression of genes related to generation of CM action potentials differs between hiPSC-CM cell lines and the mature human heart. To address this, we obtained accurate gene expression profiles of commercially available hiPSC-CM cell lines with quantitative real time RT-PCR analysis. Expression analysis of ten cardiac proteins important for generation of action potentials and three cardiac proteins important for muscle contractility was performed using GAPDH for normalization. Comparison revealed large variations in expression levels among hiPSC-CM cell lines and between hiPSC-CMs and normal human heart. In general, gene expression in hiPSC-CM cell lines was more similar to an immature, stem-like cell than a mature cardiomyocyte from human heart samples. These results provide quantitative information about differences in gene expression between hiPSC-CM cell lines, essential for interpreting pharmacology experiments. Our approach can be used as an experimental guideline for future research on gene expression in hiPSC-CMs.
Keywords: iPS cells, quantitative real time-PCR, reference gene

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Sugiyama A*, Hagiwara-Nagasawa M*, Kambayashi R*, Goto A*, Chiba K*, Naito AT*, Kanda Y, Matsumoto A*, Izumi-Nakaseko H*: Analysis of electro-mechanical relationship in human iPS cell-derived cardiomyocytes sheets under proarrhythmic condition assessed by simultaneous field potential and motion vector recordings.
J Pharmacol Sci. 2019;140:317-20

We investigated the electro-mechanical relationship of human iPS cell-derived cardiomyocyte sheets under arrhythmic condition, which was induced by digitalis intoxication along with the electrical train stimulation (n = 4). We adopted motion vector analysis by high-speed video microscopy and extracellular field potential recording by 64-microelectrode array system. The motion vector analysis uncovered local contractile events at resting phase, at which the field potential analysis showed no deflection in any cell sheet. Thus, motion vector analysis may provide supplemental information over field potential recording in detecting Ca^{2+}-triggered arrhythmias, which may become a new strategy for assessing arrhythmic liability of test articles.
Keywords: electro-mechanical relationship, human iPS cell-derived cardiomyocytes, motion vector

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Goto A*, Hagiwara-Nagasawa M*, Kambayashi R*, Chiba K*, Izumi-Nakaseko H*, Naito AT*, Kanda Y, Sugiyama A*: Measurement of J-T_{peak}c along with QT-Interval Prolongation May Increase the Assay Sensitivity and Specificity for Predicting the Onset of Drug-Induced Torsade de Pointes: Experimental Evidences Based on Proarrhythmia Model Animals.
Cardiovasc Toxicol. 2019;19:357-64

dl-Sotalol which can block both K^+ channel and ß-adrenoceptor has been shown to prolong the J-T_{peak}c of electrocardiogram in beagle dogs but tended to shorten it in microminipigs, although the drug prolonged the QT interval in both animals under physiologically maintained experimental condition. In order to estimate how the changes in the J-T_{peak}c in

the normal hearts would be reflected in the pathologic hearts, we compared proarrhythmic effects of dl-sotalol by using proarrhythmia models of beagle dogs and microminipigs, of which atrioventricular node had been ablated > 2 months and 8-9 weeks before, respectively (n = 4 for each species). dl-Sotalol in an oral dose of 10 mg/kg induced torsade de pointes in three out of four beagle dogs, which degenerated into ventricular fibrillation. In microminipigs, the same dose did not trigger torsade de pointes at all, whereas intermittent ventricular pauses were observed in each animal after the drug treatment. These results indicate that assessment of the J-T_{peak}c along with the QT-interval prolongation in healthy subjects may provide reliable information of risk prediction for patients susceptible to the drug-induced torsade de pointes.

Keywords: beagle dogs, J–Tpeakc, microminipigs

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Nishimura A*[1,2], Shimoda K*[2,3], Tanaka T*[2,4], Toyama T*[2], Nishiyama K*[1], Shinkai Y*[2], Numaga-Tomita T*[2,3], Yamazaki D, Kanda Y, Akaike T*[5], Kumagai Y*[2], Nishida M*[1,2,3]: Depolysulfidation of Drp1 induced by low-dose methylmercury exposure increases cardiac vulnerability to hemodynamic overload.

Sci Signal. 2019;12(587):eaaw1920. doi: 10.1126/scisignal.aaw1920.

Chronic exposure to methylmercury (MeHg), an environmental electrophilic pollutant, reportedly increases the risk of human cardiac events. We report that exposure to a low, non-neurotoxic dose of MeHg precipitated heart failure induced by pressure overload in mice. Exposure to MeHg at 10 ppm did not induce weight loss typical of higher doses but caused mitochondrial hyperfission in myocardium through the activation of Drp1 by its guanine nucleotide exchange factor filamin-A. Treatment of neonatal rat cardiomyocytes with cilnidipine, an inhibitor of the interaction between Drp1 and filamin-A, suppressed mitochondrial hyperfission caused by low-dose MeHg exposure. Modification of cysteine residues in proteins with polysulfides is important for redox signaling and mitochondrial homeostasis in mammalian cells. We found that MeHg targeted rat Drp1 at Cys^{624}, a redox-sensitive residue whose SH side chain forms a bulky and nucleophilic polysulfide (Cys^{624}-$S_{(n)}H$). MeHg exposure induced the depolysulfidation of Cys^{624}-$S_{(n)}H$ in Drp1, which led to filamin-dependent activation of Drp1 and mitochondrial hyperfission. Treatment with NaHS, which acts as a donor for reactive polysulfides, reversed MeHg-evoked Drp1 depolysulfidation and vulnerability to mechanical load in rodent and human cardiomyocytes and mouse hearts. These results suggest that depolysulfidation of Drp1 at Cys^{624}-$S_{(n)}H$ by low-dose MeHg increases cardiac fragility to mechanical load through filamin-dependent mitochondrial hyperfission.

Keywords: human cardiomyocyte, mitochondria, Drp1

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Akiyama Y*, Shinose M*, Watanabe H*, Yamada S, Kanda Y: Cryoprotectant-free cryopreservation of mammalian cells by superflash freezing.

Proc Natl Acad Sci U S A. 2019;116:7738-43

Cryopreservation is widely used to maintain backups of cells as it enables the semipermanent storage of cells. During the freezing process, ice crystals that are generated inside and outside the cells can lethally damage the cells. All conventional cryopreservation methods use at least one cryoprotective agent (CPA) to render water inside and outside the cells vitreous or nanocrystallized (near-vitrification) without forming damaging ice crystals. However, CPAs should ideally be avoided due to their cytotoxicity and potential side effects on the cellular state. Herein, we demonstrate the CPA-free cryopreservation of mammalian cells by ultrarapid cooling using inkjet cell printing, which we named superflash freezing (SFF). The SFF cooling rate, which was estimated by a heat-transfer stimulation, is sufficient to nearly vitrify the cells. The experimental results of Raman spectroscopy measurements, and observations with an ultrahigh-speed video camera support the near-vitrification of the droplets under these conditions. Initially, the practical utility of SFF was demonstrated on mouse

fibroblast 3T3 cells, and the results were comparable to conventional CPA-assisted methods. Then, the general viability of this method was confirmed on mouse myoblast C2C12 cells and rat primary mesenchymal stem cells. In their entirety, the thus-obtained results unequivocally demonstrate that CPA-free cell cryopreservation is possible by SFF. Such a CPA-free cryopreservation method should be ideally suited for most cells and circumvent the problems typically associated with the addition of CPAs.

Keywords: cryopreservation, cryoprotectant agent-free, inkjet cell printing

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Front Pharmacol. 2019;10:934.

Contractility of the myocardium engines the pumping function of the heart and is enabled by the collective contractile activity of its muscle cells: cardiomyocytes. The effects of drugs on the contractility of human cardiomyocytes *in vitro* can provide mechanistic insight that can support the prediction of clinical cardiac drug effects early in drug development. Cardiomyocytes differentiated from human-induced pluripotent stem cells have high potential for overcoming the current limitations of contractility assays because they attach easily to extracellular materials and last long in culture, while having human- and patient-specific properties. Under these conditions, contractility measurements can be non-destructive and minimally invasive, which allow assaying sub-chronic effects of drugs. For this purpose, the function of cardiomyocytes *in vitro* must reflect physiological settings, which is not observed in cultured cardiomyocytes derived from induced pluripotent stem cells because of the fetal-like properties of their contractile machinery. Primary cardiomyocytes or tissues of human origin fully represent physiological cellular properties, but are not easily available, do not last long in culture, and do not attach easily to force sensors or mechanical actuators. Microengineered cellular systems with a more mature contractile function have been developed in the last 5 years to overcome this limitation of stem cell-derived cardiomyocytes, while simultaneously measuring contractile endpoints with integrated force sensors/actuators and image-based techniques. Known effects of engineered microenvironments on the maturity of cardiomyocyte contractility have also been discovered in the development of these systems. Based on these discoveries, we review here design criteria of microengineered platforms of cardiomyocytes derived from pluripotent stem cells for measuring contractility with higher physiological relevance. These criteria involve the use of electromechanical, chemical and morphological cues, co-culture of different cell types, and three-dimensional cellular microenvironments. We further discuss the use and the current challenges for developing and improving these novel technologies for predicting clinical effects of drugs based on contractility measurements with cardiomyocytes differentiated from induced pluripotent stem cells. Future research should establish contexts of use in drug development for novel contractility assays with stem cell-derived cardiomyocytes.

Keywords: cellular alignment, co-culture, electrical stimulation

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Guth BD[*1,2], Engwall M[*3], Eldridge S[*4], Foley CM[*], Guo L[*6], Gintant G[*5], Koerner J[*7], Parish ST[*8], Pierson JB[*8], Ribeiro AJS[*7], Zabka T[*9], Chaudhary KW[*10], Kanda Y, Berridge B[*11]: Considerations

for an In Vitro, Cell-Based Testing Platform for Detection of Adverse Drug-Induced Inotropic Effects in Early Drug Development. Part 1: General Considerations for Development of Novel Testing Platforms.

Front Pharmacol. 2019;10:884.

Drug-induced effects on cardiac contractility can be assessed through the measurement of the maximal rate of pressure increase in the left ventricle (LVdP/dt_{max}) in conscious animals, and such studies are often conducted at the late stage of preclinical drug development. Detection of such effects earlier in drug research using simpler, *in vitro* test systems would be a valuable addition to our strategies for identifying the best possible drug development candidates. Thus, testing platforms with reasonably high throughput, and affordable costs would be helpful for early screening purposes. There may also be utility for testing platforms that provide mechanistic information about how a given drug affects cardiac contractility. Finally, there could be *in vitro* testing platforms that could ultimately contribute to the regulatory safety package of a new drug. The characteristics needed for a successful cell or tissue-based testing platform for cardiac contractility will be dictated by its intended use. In this article, general considerations are presented with the intent of guiding the development of new testing platforms that will find utility in drug research and development. In the following article (part 2), specific aspects of using human-induced stem cell-derived cardiomyocytes for this purpose are addressed.

Keywords: cardiomyocyte, contractility, inotropic state

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Satsuka A, Kanda Y: Cardiotoxicity assessment of drugs using human iPS cell-derived cardiomyocytes: From proarrhythmia risk to cardiooncology.

Curr Pharm Biotechnol. 2019 doi: 10.2174/1389201020666190628143345.

Growing evidence suggests that human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) can be used as a new human cell-based platform to assess cardiac toxicity/safety during drug development. Cardiotoxicity assessment is highly challenging due to species differences and various toxicities, such as electrophysiological and contractile toxicities, which can result in proarrhythmia and heart failure. To explore proarrhythmia risk, the multi-electrode array (MEA) platform is widely used to assess QT-interval prolongation and the proarrhythmic potential of drug candidates using hiPSC-CMs. Several consortiums, including the Comprehensive in vitro Proarrhythmia Assay (CiPA) and the Japanese iPS Cardiac Safety Assessment (JiCSA) have demonstrated the applicability of hiPSC-CMs/MEA for assessing the torsadogenic potential of drug candidates. Additionally, contractility is a key safety issue in drug development, and efforts have been undertaken to measure contractility by a variety of imaging-based methods using iPS-CMs. Therefore, hiPSC-CMs might represent a standard testing tool for evaluating proarrhythmic and contractile potentials. This review provides new insights into the practical application of hiPSC-CMs in early or late-stage non-clinical testing during drug development.

Keywords: cardiac safety, contractility, human iPS cells

Hayashi MK*1,2, Nishioka T*3, Shimizu H, Takahashi K, Kakegawa W*2, Mikami T*4, Hirayama Y*5, Koizumi S*5, Yoshida S*4, Yuzaki M*2, Tammi M*6, Sekino Y*7, Kaibuchi K*5, Mogami Y, Yasui M*2, Sato K: Hyaluronan synthesis supports glutamate transporter activity.

J Neurochem. 2019;150:249-63

Hyaluronan is synthesized, secreted, and anchored by hyaluronan synthases (HAS) at the plasma membrane and comprises the backbone of perineuronal nets around neuronal soma and dendrites. However, the molecular targets of hyaluronan to regulate synaptic transmission in the central nervous system have not been fully identified. Here, we report that

hyaluronan is a negative regulator of excitatory signals. At excitatory synapses, glutamate is removed by glutamate transporters to turn off the signal and prevent excitotoxicity. Hyaluronan synthesized by HAS supports the activity of glial glutamate transporter 1 (GLT1). GLT1 also retracted from cellular processes of cultured astrocytes after hyaluronidase treatment and hyaluronan synthesis inhibition. A serial knockout study showed that all three HAS subtypes recruit GLT1 to cellular processes. Furthermore, hyaluronidase treatment activated neurons in a dissociated rat hippocampal culture and caused neuronal damage due to excitotoxicity. Our findings reveal that hyaluronan helps to turn off excitatory signals by supporting glutamate clearance.

Keywords: excitotoxicity, glutamate transporter, hyaluronan

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Mizoi K*1, Fukai Y*1, Matsumoto E*1, Koyama S*1, 2, Ishida S, Kojima H, Ogihara T*1: Usefulness and limitations of mRNA measurement in HepaRG cells for evaluation of cytochrome P450 induction.

Fundamental Toxicological Sciences. 2020;7:9-14

Cytochrome P450s (CYPs) are involved in the metabolism of various drugs, and may generate toxic metabolites or intermediates that result in drug-induced liver injury (DILI). Consequently, inducers of CYPs may promote DILI. In a draft test guideline, the Organisation for Economic Co-operation and Development (OECD) recommends measurement of the metabolic activity of CYP as an index for assessing CYP-inducing activity. However, change of mRNA level has also been used as a simple parameter to evaluate CYP induction. In this study, therefore, we examined the usefulness and limitations of mRNA expression measurement for evaluation of the CYP-genes induction in HepaRG cells. Our results indicate that mRNA measurements and metabolic activity measurements in HepaRG cells generally give comparable results for fold-induction of CYPs.

Keywords: CYP induction, HepaRG, OECD

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Takayama K*1, Negoro R*1, Yamashita T*1, Kawai K*1, Ichikawa M*1, Mori T*2, Nakatsu N*3, Harada K*1, Ito S*4, Yamada H*3, Yamaura Y*2, Hirata K*1, Ishida S, Mizuguchi H*1: Generation of human iPS cell-derived intestinal epithelial cell monolayers by CDX2 transduction.

Cell Mol Gastroenterol Hepatol. 2019. doi:10.1016/j.jcmgh.2019.06.004.

To develop an effective and safe orally administered drug, it is important to predict its intestinal absorption rate, intestinal first-pass effect, and drug-drug interactions of orally administered drugs. We generated almost-homogenous Villin- and zonula occludens-1 (ZO1)-positive intestinal epithelial cells by caudal-related homeobox transcription factor 2 (CDX2) transduction into human iPS cell-derived intestinal progenitor cells. As the results, we succeeded in generating the human iPS-IECM that can be applied to various intestinal pharmacokinetics and drug-response tests of orally administered drugs.

Keywords: iPS cell-derived intestinal epithelial cell, differentiation, human iPS cell

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Nakai S*1, Shibata I*1, Shitamichi T*1, Yamaguchi H*2, Takagi N*2, Inoue T*3, Nakagawa T*3, Kiyokawa J*3, Wakabayashi S*4, Miyoshi T*5, Higashi E*5, Ishida S, Shiraki N*1, Kume S*1: Collagen vitrigel promotes hepatocytic differentiation of induced pluripotent stem cells into functional hepatocyte-like cells.

Biol Open. 2019. doi: 10.1242/bio.042192.

Differentiation of stem cells to hepatocytes provides an unlimited supply of human hepatocytes and therefore has been vigorously studied. To obtain matured hepatocytes from stem cells, we tested the effect of culturing human-induced pluripotent stem

(hiPS) cell-derived endoderm cells on collagen vitrigel membrane and compared with our previous reported nanofiber matrix. We cultured hiPS cell-derived endoderm cells on a collagen vitrigel membrane and examined the expression profiles, and tested the activity of metabolic enzymes. The results indicated that the present approach identified that collagen vitrigel membrane provides a suitable environment for the generation of hepatocytes from hiPS cells that resemble many characteristics of primary human hepatocytes.

Keywords: iPS cell-derived endoderm cells, collagen vitrigel membrane, human iPS cell

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Irie T: Loose coupling between SK and P/Q-type Ca^{2+} channels in cartwheel cells of the dorsal cochlear nucleus.

J Neurophysiol 2019;12:1721-27

Small-conductance Ca^{2+}-activated K^+ (SK) and large conductance voltage- and Ca^{2+}-activated K^+ (BK) channels are Ca^{2+}-activated K^+ channels that control action potential firing in diverse neurons in the brain. In cartwheel cells of the dorsal cochlear nucleus, blockade of either channel type leads to excessive production of spike bursts. In the same cells, P/Q-type Ca^{2+} channels in plasma membrane and ryanodine receptors in endoplasmic reticulum supply Ca^{2+} to BK channels through Ca^{2+} nanodomain signaling. In this study, voltage-clamp experiments were performed in cartwheel cells in mouse brain slices to examine the Ca^{2+} signaling pathways underlying activation of SK channels. As with BK channels, SK channels required the activity of P/Q-type Ca^{2+} channels. However, this signaling occurred across Ca^{2+} micro- rather than nanodomain distances and was independent of Ca^{2+} release from endoplasmic reticulum. These differential modes of activation may lead to distinct time courses of the two K^+ currents and therefore control excitability of auditory neurons across different timescales.

Keywords: Ca^{2+} microdomains, P/Qtype Ca^{2+} channels

Sato C*1, Yamazaki D, Sato M*1, Takeshima H*1, Memtily N*1,3, Hatano Y*1, Tsukuba T*4, Sakai E*4: Calcium Phosphate Mineralization in Bone Tissues Directly Observed in Aqueous Liquid by Atmospheric SEM (ASEM) Without Staining: Microfluidics Crystallization Chamber and immuno-EM.

Sci Rep. 2019;9:7352

The malformation and disordered remodeling of bones induce various diseases, including osteoporosis. We have developed atmospheric SEM (ASEM) to directly observe aldehyde-fixed bone tissue immersed in radical scavenger buffer without thin sectioning. The short procedure realized the observation of bone mineralization surrounded by many cells and matrices in natural aqueous buffer, decreasing the risk of changes. In osteoblast primary cultures, mineralization was visible without staining. Correlative energy dispersive X-ray spectrometry indicated the formation of calcium phosphate mineral. Fixed bone was sectioned, and the section surface was inspected by ASEM. Mineralized trabeculae of talus spongy bone were directly visible. Associated large and small cells were revealed by phosphotungstic acid staining, suggesting remodeling by bone-absorbing osteoclasts and bone-rebuilding osteoblasts. In tibia, cortical bone layer including dense grains, was bordered by many cells with protrusions. Tissue immuno-EM performed in solution for the first time and anti-cathepsin-K antibody, successfully identified osteoclasts in femur spongy bone. A microfluidics chamber fabricated on the silicon nitride film window of an ASEM dish allowed mineralization to be monitored in vitro; calcium phosphate crystals as small as 50 nm were imaged. ASEM is expected to be widely applied to study bio-mineralization and bone-remodeling, and to help diagnose bone-related diseases.

Keywords: ASEM, X-ray, bone

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M[*2], Ma Z[*3], Qiu L[*3], Murayama T[*4], Zou X[*3], Takeshima H[*2], Zhou J[*5], Ma J[*1]: TRIC-A Channel Maintains Store Calcium Handling by Interacting With Type 2 Ryanodine Receptor in Cardiac Muscle.

Circ Res. 2020;126:417-35

Rationale: Trimeric intracellular cation (TRIC) -A and B are distributed to endoplasmic reticulum/sarcoplasmic reticulum intracellular Ca^{2+} stores. The crystal structure of TRIC has been determined, confirming the homotrimeric structure of a potassium channel. While the pore architectures of TRIC-A and TRIC-B are conserved, the carboxyl-terminal tail (CTT) domains of TRIC-A and TRIC-B are different from each other. Aside from its recognized role as a counterion channel that participates in excitation-contraction coupling of striated muscles, the physiological function of TRIC-A in heart physiology and disease has remained largely unexplored.

Objective: In cardiomyocytes, spontaneous Ca^{2+} waves, triggered by store overload-induced Ca^{2+} release mediated by the RyR2 (type 2 ryanodine receptor), develop extrasystolic contractions often associated with arrhythmic events. Here, we test the hypothesis that TRIC-A is a physiological component of RyR2-mediated Ca^{2+} release machinery that directly modulates store overload-induced Ca^{2+} release activity via CTT.

Methods and results: We show that cardiomyocytes derived from the TRIC-A-/- (TRIC-A knockout) mice display dysregulated Ca^{2+} movement across sarcoplasmic reticulum. Biochemical studies demonstrate a direct interaction between CTT-A and RyR2. Modeling and docking studies reveal potential sites on RyR2 that show differential interactions with CTT-A and CTT-B. In HEK293 (human embryonic kidney) cells with stable expression of RyR2, transient expression of TRIC-A, but not TRIC-B, leads to apparent suppression of spontaneous Ca^{2+} oscillations. Ca^{2+} measurements using the cytosolic indicator Fura-2 and the endoplasmic reticulum luminal store indicator D1ER suggest that TRIC-A enhances Ca^{2+} leak across the endoplasmic reticulum by directly targeting RyR2 to modulate store overload-induced Ca^{2+} release. Moreover, synthetic CTT-A peptide facilitates RyR2 activity in lipid bilayer reconstitution system, enhances Ca^{2+} sparks in permeabilized TRIC-A-/- cardiomyocytes, and induces intracellular Ca^{2+} release after microinjection into isolated cardiomyocytes, whereas such effects were not observed with the CTT-B peptide. In response to isoproterenol stimulation, the TRIC-A-/- mice display irregular ECG and develop more fibrosis than the WT (wild type) littermates.

Conclusions: In addition to the ion-conducting function, TRIC-A functions as an accessory protein of RyR2 to modulate sarcoplasmic reticulum Ca^{2+} handling in cardiac muscle.

Keywords: calcium signaling, mice, peptides

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Akagi J, Hashimoto K[*1], Suzuki K[*2], Yokoi M[*2,3], de Wind N[*4], Iwai S[*5], Ohmori H[*2], Moriya M[*1], Hanaoka F[*2,5,6]: Effect of sequence context on Pol ζ-dependent error-prone extension past (6-4) photoproducts.

DNA Repair. 2020;87:102771. doi: 10.1016/j.dnarep.2019.102771

The (6-4) pyrimidine-pyrimidone photoproduct [(6-4)PP] is a major DNA lesion induced by ultraviolet radiation. (6-4)PP induces complex mutations opposite its downstream bases, in addition to opposite 3' or 5' base, as has been observed through a site-specific translesion DNA synthesis (TLS) assay. The mechanism by which these mutations occur is not well understood. To elucidate the mechanisms underlying mutagenesis induced by (6-4)PP, we performed an intracellular TLS assay using a replicative vector with site-specific T(thymidine)-T (6-4)PP. *Rev3*$^{-/-}$ *p53*$^{-/-}$ mouse embryonic fibroblast (MEF) cells (defective in Polζ) were almost completely defective in bypassing T-T (6-4)PP, whereas both *Rev1*$^{-/-}$ and *Polh*$^{-/-}$ *Poli*$^{-/-}$ *Polk*$^{-/-}$ MEF cells (defective in Polη, Polι, and Polκ) presented bypassing activity comparable to that of wild-type cells, indicating that Y-family TLS polymerases are dispensable for bypassing activity, whereas Polζ plays an essential role, probably at the extension step. Among all cells tested, misincorporation occurred most frequently

just beyond the lesion (position +1), indicating that the Pol ζ-dependent extension step is crucial for (6-4)PP-induced mutagenesis. We then examined the effects of sequence context on T-T (6-4)PP bypass using a series of T-T (6-4)PP templates with different sequences at position +1 or -1 to the lesion, and found that the dependency of T-T (6-4)PP bypass on Pol ζ is not sequence specific. However, the misincorporation frequency at position +1 differed significantly among these templates. The misincorporation of A at position +1 occurred frequently when a purine base was located at position -1. These results indicate that Pol ζ-dependent extension plays a major role in inducing base substitutions in (6-4)PP-induced mutagenesis, and its fidelity is affected by sequence context surrounding a lesion.

Keywords: translesion synthesis, Pol ζ, sequence context

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Matsushita K, Toyoda T, Morikawa T, Ogawa K: A 13-week subchronic toxicity study of vanillin propylene glycol acetal in F344 rats.
Food Chem Toxicol. 2019;132:110643. doi: 10.1016/j.fct.2019.110643

Vanillin propylene glycol acetal (VPGA) has been used as a flavoring agent. Here, we performed a 13-week subchronic toxicity study of VPGA in F344 rats with oral administration by gavage at doses of 0, 100, 300, and 1000 mg/kg body weight (BW)/day. In the 1000 mg/kg BW group, loss of vigorous activity and listlessness immediately after administration were observed for both sexes throughout the experimental period. Reduction of body weight gain was noted in both sexes at 1000 mg/kg BW. Serum biochemistry demonstrated significant increases in total protein, albumin, total cholesterol, calcium, inorganic phosphorus, and γ-glutamyl transpeptidase in both sexes at 1000 mg/kg BW and increases in the albumin/globulin ratio and urea nitrogen in the male 1000 mg/kg BW group. A significant increase in relative liver weight was detected in both sexes at 1000 mg/kg BW. Histopathologically, centrilobular hepatocellular hypertrophy in the liver was observed in both sexes at 1000 mg/kg BW. In addition, the incidence of fatty changes in hepatocytes in the male 1000 mg/kg BW group was decreased compared with that in the control. Based on these results, the no-observed-adverse-effect level for VPGA was evaluated to be 300 mg/kg BW/day for both sexes in the current study.

Keywords: flavoring agent, subchronic toxicity, vanillin propylene glycol acetal

Ishii Y, Yokoo Y, Kijima A, Takasu S, Ogawa K, Umemura T: DNA modifications that do not cause gene mutations confer the potential for mutagenicity by combined treatment with food chemicals.
Food Chem Toxicol. 2019;129:144-52.

Cell proliferation plays a key role in fixing mutations induced by DNA damage. We clarified whether this phenomenon occurred after combined treatment with chemicals in food. The effects of antibiotic flumequine (FL), a residue of veterinary medicinal products in foodstuffs, on mutagenicity in the liver were examined in mice treated with estragole (ES), a natural food flavouring compound. *Gpt* delta mice were orally administered 10 or 100 mg/kg/day ES and simultaneously fed a diet containing 0.4% FL for 4 weeks. Proliferating cell nuclear antigen-positive cells and cell cycle-related genes were additively increased in the livers of combined treatment groups as compared with high-dose ES or FL groups. Mutant frequencies (MFs) in *gpt* after cotreatment with low-dose ES and FL were significantly increased, although treatment with ES alone increased MFs only in the high-dose group. Sult1a1 mRNA levels were unchanged after FL treatment. Liquid chromatography with tandem-mass spectrometry analysis showed that FL did not affect the amount of ES-specific DNA adducts in the livers, indicating that FL treatment did not influence metabolic pathways of ES. Thus, enhancement of the mutagenic potential of a chemical by chemical-induced cell proliferation may occur as a result of the combined effects of chemicals in food.

Keywords: cell proliferation, combined effect, *gpt* delta mouse

Sone M, Toyoda T, Cho YM, Akagi J, Matsushita K, Mizuta Y, Morikawa T, Nishikawa A, Ogawa K: Immunohistochemistry of γ-H2AX as a method of early detection of urinary bladder carcinogenicity in mice.

J Appl Toxicol. 2019;39:868-76.

Phosphorylated histone H2AX (γ-H2AX) has been demonstrated as a DNA damage marker both in vitro and in vivo. We previously reported the effects of genotoxic carcinogens in the urinary bladder of rats by immunohistochemical analysis of γ-H2AX using samples from 28-day repeated-dose tests. To evaluate the application of γ-H2AX as a biomarker of carcinogenicity in the bladder, we examined species differences in γ-H2AX formation in the urinary bladder of mice. Six-week-old male $B6C3F_1$ mice were treated orally with 12 chemicals for 4 weeks. Immunohistochemical analysis demonstrated that *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, *p*-cresidine, and 2-acetylaminofluorene (2-AAF), classified as genotoxic bladder carcinogens, induced significant increases in γ-H2AX levels in the bladder urothelium. In contrast, genotoxic (2-nitroanisole, glycidol, *N*-nitrosodiethylamine, and acrylamide) and non-genotoxic (dimethylarsinic acid and melamine) non-bladder carcinogens did not upregulate γ-H2AX. Importantly, 2-nitroanisole, a potent genotoxic bladder carcinogen in rats, significantly increased the proportion of γ-H2AX-positive cells in rats only, reflecting differences in carcinogenicity in the urinary bladder between rats and mice. Significant upregulation of γ-H2AX was also induced by uracil, a non-genotoxic bladder carcinogen that may be associated with cell proliferation, as demonstrated by increased Ki67 expression. 2-AAF caused γ-H2AX formation mainly in the superficial layer, together with reduced and disorganized expression of uroplakin III, unlike in rats, suggesting the mouse-specific cytotoxicity of 2-AAF in umbrella cells. These results suggest γ-H2AX to be a useful biomarker reflecting species differences in carcinogenicity in the urinary bladder.

Keywords: carcinogenicity, urinary bladder, γ-H2AX

Ishii Y, Kijima A, Takasu S, Ogawa K, Umemura T: Effects of inhibition of hepatic sulfotransferase activity on renal genotoxicity induced by lucidin-3-O-primeveroside.

J Appl Toxicol. 2019;39:650-7.

Sulfotransferase 1A (SULT1A) expression is lower in the liver of humans than that of rodents. Therefore, species differences should be taken into consideration when assessing the risk of rodent hepatocarcinogens metabolically activated by SULT1A in humans. Although some renal carcinogens require SULT1A-mediated activation, it is unclear how SULT1A activity in the liver affects renal carcinogens. To explore the effects of SULT1A activity in the liver on genotoxicity induced by SULT1A-activated renal carcinogens, $B6C3F_1$ mice or *gpt* delta mice of the same strain background were given lucidin-3-O-primeveroside (LuP), a hepatic and renal carcinogen of rodents, for 4 or 13 weeks, respectively, and pentachlorophenol (PCP) as a liver-specific SULT inhibitor, was given from 1 week before LuP treatment to the end of the experiment. A 4 week exposure of LuP induced lucidin-specific DNA adduct formation. The suppression of Sult1a expression was observed only in the liver but not in the kidneys of PCP-treated mice, but co-administration of PCP suppressed LuP-induced DNA adduct formation in both organs. Thirteen-week exposure of LuP increased mutation frequencies and cotreatment with PCP suppressed these increases in both organs. Given that intact levels of SULT activity in the liver were much higher than in the kidneys of rodents, SULT1A may predominantly activate LuP in the liver, consequently leading to genotoxicity not only in the liver but also in the kidney. Thus, species differences should be considered in human risk assessment of renal carcinogens activated by SULT1A as in the case of the corresponding liver carcinogens.

Keywords: DNA adduct, lucidin-3-O-primeveroside, sulfotransferase

Toyoda T, Cho YM, Matsushita K, Tachibana S*, Senuma M*, Akagi J, Ogawa K: A 13-week subchronic toxicity study of hexyl acetate in SD rats.

J Toxicol Pathol. 2019;32:205-12.

Hexyl acetate is a naturally occurring ester compound that has a fruity odor. Despite its frequent use as a nature identical flavoring agent, there are limited repeated dose toxicity data for hexyl acetate. Here we performed a 13-week subchronic toxicity

study of hexyl acetate in male and female Crl: CD(SD) rats under GLP regulations. Hexyl acetate was given orally by gavage at doses of 0, 100, 300, or 1000 mg/kg/day using corn oil as the vehicle. No significant toxicological changes in general condition, body weights, food intake, ophthalmology, hematology, organ weights, and histopathological findings were observed in any groups. Urinalysis revealed occult blood in two male animals treated with 1000 mg/kg/day hexyl acetate, and one showed red blood cells in the urine sediment. Furthermore, blood biochemistry showed a significant increase in inorganic phosphorus levels in males treated with 1000 mg/kg/day hexyl acetate. These results indicated that the no-observed-adverse-effect level of hexyl acetate was 300 mg/kg/day for males and more than 1000 mg/kg/day for females.

Keywords: hexyl acetate, food additive, subchronic toxicity

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Suzuki S*, Toyoda T, Kato H*, Naiki-Ito A*, Yamashita Y*, Akagi J, Cho YM, Ogawa K, Takahashi S*: Dimethylarsinic acid may promote prostate carcinogenesis in rats.

J Toxicol Pathol. 2019;32:73-7.

Arsenic is a known human carcinogen, inducing tumors of the lung, urinary bladder, skin, liver and prostate. However, there were no reports of prostate tumors induced by arsenicals in *in vivo* animal models. In a previous study, we found that HMGB2 expression was a predictive marker for prostate carcinogens in the rat 4-week repeated dose test. In this study, six-week-old male F344 rats were orally treated with a total of six chemicals (2-acetylaminofluorene (2-AAF), *p*-cresidine, dimethylarsinic acid (DMA), glycidol, *N*-nitrosodiethylamine and acrylamide) for four weeks. Animals were sacrificed at the end of the study and HMGB2 and Ki-67 immunohistochemistry was performed. The number of HMGB2 and Ki-67 positive cells in all prostate lobes was significantly increased by DMA, one of the arsenicals, compared with the controls. Meanwhile, the number of Ki-67 in lateral and dorsal prostate lobes was significantly decreased by 2-AAF with the reduction of body weight, but HMGB2 expression was not. The other chemicals did not change HMGB2 and Ki-67 expressions. These data indicate that DMA may have an ability to enhance prostate carcinogenesis.

Keywords: dimethylarsinic acid, prostate, carcinogenesis

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Hori H*1, Shimoyoshi S*2, Tanaka Y*1, Momonami A*2, Masumura K, Yamada M, Fujii W*1, Kitagawa Y*2, Hayashi M*3: Multiple-endpoint genotoxicity assay for colon carcinogen 1,2-dimethylhydrazine.

Mutat Res. 2020;849:503130. doi:10.1016/j.mrgentox.2019.503130

Human risk assessment of the toxic potency of chemicals typically includes genotoxicity assays for predicting carcinogenicity. Gene mutation frequency and chromosomal aberration are two major genotoxicity endpoints in standardized *in vitro* and *in vivo* assays. The weight-of-evidence approach in risk assessment is more focused on *in vivo* assay results; however, animal welfare considerations are aimed at the reduction, replacement, and refinement (3R's) of animal experiments, including a reduction in the number of experimental animals. Proposals to reduce experimental animals in genotoxicity testing include the incorporation of genotoxicity endpoint(s) into other toxicological studies and the combination of two or more assays detecting different genotoxicity endpoints in the same animals. In this study, we used 1,2-dimethylhydrazine as a model chemical of colon carcinogen to assess gene mutation frequency and chromosomal aberration *in vivo* simultaneously. Specifically, a gene mutation frequency assay was combined with a multiple-organ micronucleus test (peripheral blood, bone marrow, liver, and colon) in F344 *gpt* delta transgenic rats. Both *gpt* mutant frequency and micronucleated cell frequency significantly increased in colon and liver but not in bone marrow. Interestingly, we found that the colon carcinogen induced both gene mutations and micronuclei in the targeted colon tissue. Thus, we demonstrated that the mechanism of a carcinogen could be derived from an animal experiment using a lower number of experimental animals as currently recommended. Moreover, a significant increase in mutant frequency in colon and liver was already observed on the first day after treatment completion, as well as on the third day, which is the guideline-

recommended period. Thus, this endpoint is compatible with other genotoxicity assays. We confirmed that performing the micronucleus assay in combination with a gene mutation assay in F344 *gpt* delta transgenic rats is useful to evaluate different genotoxic endpoints simultaneously in the same animals, which reduces the number of experimental animals.

Keywords: *gpt* delta transgenic rat, micronucleus, DMH

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Aoki Y*1, Taniguchi Y*2, Matsumoto M*1, Matsumoto M*1, Ohno M*2, Masumura K, Sasaki S*2, Tsuzuki T*2, Yamamoto M*3, Nohmi T: Oxidative-stress-driven mutagenesis in the small intestine of the *gpt* delta mouse induced by oral administration of potassium bromate.

Mutat Res. 2020;850-851:503136. doi:10.1016/j.mrgentox.2020.503136

Tumorigenesis induced by oxidative stress is thought to be initiated by mutagenesis, but via an indirect mechanism. The dose-response curves for agents that act by this route usually show a threshold, for unknown reasons. To gain insight into these phenomena, we have analyzed the dose response for mutagenesis induced by the oral administration of potassium bromate, a typical oxidative-stress-generating agent, to *gpt* delta mice. The agent was given orally for 90 d to either *Nrf2*+ or *Nrf2*-knockout (KO) mice and mutants induced in the small intestine were analyzed. In *Nrf2*+mice, the mutant frequency was significantly greater than in the vehicle controls at a dose of 0.6 g/L but not at 0.2 g/L, indicating that a practical threshold for mutagenesis lies between these doses. At 0.6 g/L, the frequencies of G-to-T transversions (landmark mutations for oxidative stress) and G-to-A transitions were significantly elevated. In *Nrf2*-KO mice, too, the total mutant frequency was increased only at 0.6 g/L. G-to-T transversions are likely to have driven tumorigenesis in the small intestine. A site-specific G-to-T transversion at guanine (nucleotide 406) in a 5'-TGAA-3' sequence in *gpt*, and our primer extension reaction showed that formation of the oxidative DNA base modification 8-oxo-deoxyguanosine (8-oxo-dG) at nucleotide 406 was significantly increased at doses of 0.6 and 2 g/L in the *gpt* delta mice. In the *Apc* oncogene, guanine residues in the same or similar sequences (TGAA or AGAA) are highly substituted by thymine (G-to-T transversions) in potassium bromate-induced tumors. We propose that formation of 8-oxo-dG in the T(A)GAA sequence is an initiating event in tumor formation in the small intestine in response to oxidative stress.

Keywords: oxidative stress, threshold, *Nrf2*-knockout

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Grúz P, Sugiyama K, Honma M, Nohmi T: Purification and Interactions of the MucA' and MucB Proteins Constituting the DNA Polymerase RI.

Genes Environ. 2019;41:10. doi:10.1186/s41021-019-0125-8

Background: The MucA' and MucB proteins comprise the core of DNA polymerase RI which is a strong mutator utilized in mutagenicity assays such as the standard Ames test. A close relative DNA polymerase V, composed of the homologous UmuD' and UmuC proteins, is considered to be an ortholog of the mammalian DNA polymerase η. The catalytic subunits of these polymerases belong to the Y-family which specializes in the translesion DNA synthesis across various DNA adducts to rescue stalled chromosomal replication at the expense of mutations. Based on genetic evidence, DNA polymerase RI possesses the greatest ability to induce various types of mutations among all so far characterized members of the Y-superfamily. The exceptionally high mutagenic potential of MucA'B has been taken advantage of in numerous bacterial mutagenicity assays incorporating the conjugative plasmid pKM101 carrying the *mucAB* operon such as the Ames Test.

Results: We established new procedures for the purification of MucB protein as well as its accessory protein MucA' using the refolding techniques. The purified MucA' protein behaved as a molecular dimer which was fully stable in solution. The soluble monomeric form of MucB protein was obtained after refolding on a gel-filtration column and remained

stable in a nondenaturing buffer containing protein aggregation inhibitors. Using the surface plasmon resonance technique, we demonstrated that the purified MucA' and MucB proteins interacted and that MucB protein preferentially bound to single-stranded DNA. In addition, we revealed that MucB protein interacted with the β-subunit of DNA polymerase III holoenzyme of *E. coli*.

Conclusion: The MucA' and MucB proteins can be isolated from inclusion bodies and solubilized *in vitro*. The refolded MucB protein interacts with its MucA' partner as well as with DNA what suggests it retains biological activity. The interaction of MucB with the processivity subunit of DNA polymerase III may imply the role of the subunit as an accessory protein to MucB during the translesion DNA synthesis.

Keywords: pKM101, BIAcore, mucAB

Sassa A[*1], Fukuda T[*2], Ukai A, Nakamura M[*2], Takabe M[*2], Takamura-Enya T[*3], Honma M, Yasui M: Comparative study of cytotoxic effects induced by environmental genotoxins using XPC- and CSB-deficient human lymphoblastoid TK6 cells.

Genes Environ. 2019;41:15. doi:10.1186/s41021-019-0130-y

Background: The human genome is constantly exposed to numerous environmental genotoxicants. To prevent the detrimental consequences induced by the expansion of damaged cells, cellular protective systems such as nucleotide excision repair (NER) exist and serve as a primary pathway for repairing the various helix-distorting DNA adducts induced by genotoxic agents. NER is further divided into two sub-pathways, namely, global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). Both NER sub-pathways are reportedly involved in the damage response elicited by exposure to genotoxins. However, how disruption of these sub-pathways impacts the toxicity of different types of environmental mutagens in human cells is not well understood.

Results: To evaluate the role of NER sub-pathways on the cytotoxic effects of mutagens, we disrupted *XPC* and *CSB* to selectively inactivate GG-NER and TC-NER, respectively, in human lymphoblastoid TK6 cells, a standard cell line used in genotoxicity studies. Using these cells, we then comparatively assessed their respective sensitivities to representative genotoxic agents, including ultraviolet C (UVC) light, benzo [a] pyrene (B(a)P), 2-amino-3,8-dimethylimidazo [4,5-f] quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), γ-ray, and 2-acetylaminofluorene (2-AAF). $CSB^{-/-}$ cells exhibited a hyper-sensitivity to UVC, B(a)P, and MeIQx. On the other hand, $XPC^{-/-}$ cells were highly sensitive to UVC, but not to B(a)P and MeIQx, compared with wild-type cells. In contrast with other genotoxins, the sensitivity of $XPC^{-/-}$ cells against PhIP was significantly higher than $CSB^{-/-}$ cells. The toxicity of γ-ray and 2-AAF was not enhanced by disruption of either *XPC* or *CSB* in the cells.

Conclusions: Based on our findings, genetically modified TK6 cells appear to be a useful tool for elucidating the detailed roles of the various repair factors that exist to combat genotoxic agents, and should contribute to the improved risk assessment of environmental chemical contaminants.

Keywords: CSB, XPC, environmental mutagen

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Grúz P, Shimizu M[*1], Sugiyama K, Yamada M[*2], Honma M: Effect of episomally encoded DNA polymerases on chemically induced mutagenesis at the *hisG46* target in Ames test.

Genes Environ. 2020;42:14. doi:10.1186/s41021-020-00154-2

Background: The standard Ames test strains owe their high sensitivity to chemical and physical mutagens to the episomal Y-family DNA polymerase RI encoded by the *mucAB* operon. The S. typhimurium test strains carry also another related *samAB* operon on a 60-kDa cryptic plasmid. In contrast to the chromosomally encoded Y-family DNA polymerases V and IV, these plasmid born polymerase genes have no direct counterpart in mammalian cells. By replicating damaged templates, DNA polymerases play a central role in mutagenesis and genome stability. It is therefore imperative to investigate their specificity to understand differences in mutagenesis between the prokaryotic versus eukaryotic (mammalian) systems. To this end we have isolated and separately expressed the DNA polymerase subunits encoded by the *mucAB*

and *samAB* operons. After demonstrating how these enzymes control chemical and UV mutagenesis at the standard *hisD3052* and *hisG428* Ames test targets, we are now adding the third Ames test target *hisG46* to the trilogy.

Results: Four new Ames tester strains based on the *hisG46* target have been constructed expressing the activated DNA polymerase MucA' and SamA' accessory subunits combined with the MucB and SamB catalytical subunits under the control of lac promoter. These polymerase assemblies were substituted for the endogenous PolRI, PolV and SamAB polymerases present in the standard TA100 strain and tested for their abilities to promote chemically induced mutagenesis. SamA'+SamB has been able to promote mutagenesis induced by AF-2 and 1,8-DNP to higher extent than SamA'+MucB. The MucA'+MucB (PolRI*) more efficiently promoted MMS as well as spontaneous mutagenesis than its wild type counterpart but was less efficient for other mutagens including AFB1. Strikingly azide mutagenesis was inhibited by PolRI and also SamA'B.

Conclusion: A new system for SOS-independent overexpression of the activated DNA polymerases RI and SamA'B and their chimeras in the *hisG46* Ames test background has been established and validated with several representative mutagens. Overall, the TA100 strain showed the highest sensitivity towards most tested mutagens. The observed inhibition of azide mutagenesis by PolRI* suggests that this type of Y-family DNA polymerases can perform also "corrective" error free replication on a damaged DNA.

Keywords: Ames test, DNA polymerase, azide

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Sassa A*1, Tada H*2, Takeishi A*1, Harada K*1, Suzuki M*1, Tsuda M*3, Sasanuma H*4, Takeda S*4, Sugasawa K*2, Yasui M, Honma M, Ura K*1: Processing of a single ribonucleotide embedded into DNA by human nucleotide excision repair and DNA polymerase η.

Scientific Reports. 2019;9:13910. doi:10.1038/s41598-019-50421-8

DNA polymerases often incorporate non-canonical nucleotide, i.e., ribonucleoside triphosphates into the genomic DNA. Aberrant accumulation of ribonucleotides in the genome causes various cellular abnormalities. Here, we show the possible role of human nucleotide excision repair (NER) and DNA polymerase η (Pol η) in processing of a single ribonucleotide embedded into DNA. We found that the reconstituted NER system can excise the oxidized ribonucleotide on the plasmid DNA. Taken together with the evidence that Pol η accurately bypasses a ribonucleotide, i.e., riboguanosine (rG) or its oxidized derivative (8-oxo-rG) *in vitro*, we further assessed the mutagenic potential of the embedded ribonucleotide in human cells lacking NER or Pol η. A single rG on the *sup*F reporter gene predominantly induced large deletion mutations. An embedded 8-oxo-rG caused base substitution mutations at the 3'-neighboring base rather than large deletions in wild-type cells. The disruption of XPA, an essential factor for NER, or Pol η leads to the increased mutant frequency of 8-oxo-rG. Furthermore, the frequency of 8-oxo-rG-mediated large deletions was increased by the loss of Pol η, but not XPA. Collectively, our results suggest that base oxidation of the embedded ribonucleotide enables processing of the ribonucleotide via alternative DNA repair and damage tolerance pathways.

Keywords: ribonucleotide, translesion DNA synthesis, nucleotide excision repair

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Sugiyama K, Furusawa H, Grúz P, Kinoshita M, Honma M: Inhibitory effect of ochratoxin A on DNMT-mediated flocculation of yeast.

Mutagenesis. 2019;34:173-180

The mycotoxin ochratoxin A (OTA) is considered to be a human carcinogen. However, the mode of its carcinogenetic action has not been elucidated. Recently, it has become evident that epigenetic changes influence the risk of developing cancer. Since it has been revealed that the yeast flocculation displayed by the strains transformed with human DNA methyltransferases (DNMT) can be regulated by epigenetic mechanisms, we examined the effect

of OTA on the transcription level of *FLO1*, which mediates the flocculation phenotype. OTA but not a non-carcinogenetic mycotoxin deoxynivalenol (DON) inhibited the intensity of GFP fluorescence under the transcriptional regulation of *FLO1* promoter in a dose-dependent manner. At the same time, OTA had no effect on the reporter activity under the control of modified *FLO1* promoter with reduced CpG motifs. In addition, it was confirmed that the flocculation and *FLO1* mRNA of *DNMT* gene-transformed yeast (*DNMT* yeast) were decreased by OTA. *In vitro* methylation assay using a bacterial DNMT revealed an inhibitory effect of OTA on the DNMT activity, and OTA treatment reduced the frequency of abnormally shaped nuclei which were often observed in *DNMT* yeast. These results suggest that the carcinogenicity of OTA may involve inhibition of DNMT-mediated epigenetic regulation.

Keywords: ochratoxin A, yeast *FLO1* promoter, DNMT

Gi M[*1], Fujioka M[*1], Totsuka Y[*2], Matsumoto M[*3], Masumura K, Kakehashi A[*1], Yamaguchi T[*1], Fukushima S[*3,4], Wanibuchi H[*1]: Quantitative analysis of mutagenicity and carcinogenicity of 2-amino-3-methylimidazo [4,5-*f*] quinoline in F344 *gpt* delta transgenic rats.

Mutagenesis. 2019;34:279-287

Quantitative analysis of the mutagenicity and carcinogenicity of the low doses of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the mutagenicity and carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ). Male F344 *gpt* delta transgenic rats were fed diets supplemented with 0, 0.1, 1, 10 or 100 ppm IQ for 4 weeks. The frequencies of *gpt* transgene mutations in the liver were significantly increased in the 10 and 100 ppm groups. In addition, the mutation spectra was altered in the 1, 10 and 100 ppm groups: frequencies of G:C to T:A transversion were significantly increased in groups administered 1, 10 and 100 ppm IQ in a dose-dependent manner, and the frequencies of G:C to A:T transitions, A:T to T:A transversions and A:T to C:G transversions were significantly increased in the 100 ppm group. Increased frequencies of single base pair deletions and Spi^- mutants in the liver, and an increase in glutathione S-transferase placental form (GST-P)-positive foci, a preneoplastic lesion of the liver in rats, was also observed in the 100 ppm group. In contrast, neither mutations nor mutation spectra or GST-P-positive foci were statistically altered by administration of IQ at 0.1 ppm. We estimated the point of departure for the mutagenicity and carcinogenicity of IQ using the no-observed-effect level approach and the Benchmark dose approach to characterise the dose-response relationship of low doses of IQ. Our findings demonstrate the existence of no effect levels of IQ for both *in vivo* mutagenicity and hepatocarcinogenicity. The findings of the present study will facilitate an understanding of the carcinogenic effects of low doses of IQ and help to determine a margin of exposure that may be useful for practical human risk assessment.

Keywords: IQ, benchmark dose, *gpt* delta transgenic rat

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Sugiyama K, Furusawa H, Honma M: Detection of Epigenetic Effects of Citrinin Using a Yeast-Based Bioassay.

Mycotoxin Res. 2019;35:363-368

The present study investigated the effects of citrinin (CIT) on a yeast-transformed human DNA methyltransferase (DNMT) associated with flocculation that can be inhibited by epigenetic mutagens. CIT (0.5-2 μmol/L) inhibited the flocculation levels of yeast transfected with *DNMT*-genes (*DNMT* yeast) and the reporter gene activity of *FLO1*, which has been associated with flocculation. In contrast, the same concentrations of CIT had little effect on reporter activity under the control of a less methylation-sensitive *FLO1* promoter. It was also shown that bacterial DNMT activity could be inhibited in the presence of CIT (4 and 40 μmol/L). These results show that CIT has inhibitory activity of DNMT, suggesting that the cytotoxicity of CIT may be involved in epigenetic mutagenicity.

Keywords: citrinin, yeast *FLO1* promoter, epigenetic

mutagen

Petkov PI[*1], Kuseva C[*1], Kotov S[*1], Honma M, Kitazawa A, Kulkarni S[*2], Schultz TW[*3], Mekenyan OG[*1]: Procedure for toxicological predictions based on mechanistic weight of evidences: Application to Ames mutagenicity.

Computational Toxicology 2019;12:100009. doi:10.1016/j.comtox.2017.02.004

Typically, (Q)SAR models are deemed decision-making rather than decision-supporting computational methods. In some (Q)SARs, the relation between chemicals structures and biological activity is described statistically. In other models, this relation is based on mechanistic-related events which contribute to the apical effect. Whether the definitive decision is based on a single model or series of models, usually predictions are not supported by mechanistic justification. The lack of mechanistic justification often limits the use of (Q)SAR predictions, especially for regulatory decisions. With this in mind, a workflow based on combining mechanistic (Q)SAR, read-across analysis and expert knowledge is used examine four different scenarios where the workflow provides enough weight-of-evidence, to allow users to make a transparent decision as to the ultimate prediction. When the OASIS TIMES Ames model and read-across analysis based on well-selected analogues within the OECD Toolbox show consistent predictions, expert input may not be needed to make a final decision. Nonetheless, expert input may be useful by adding weight-of-evidence by expanding the set of read-across analogues from literature sources and/or for providing rational of the endpoint-specific similarity between source analogue(s) and target chemical. In cases where there is inconsistency between TIMES Ames model and read-across predictions, expert input is critical for assigning the ultimate effect. Specifically, expert evaluation assists in assessing the correctness/validity of used experimental data, as well as assessing their recentness, presence of S9 metabolic activation accordance to guideline protocols, cytotoxicity, etc. In the latter cases, after evaluating all relevant evidences, expert knowledge provides transparency for the conclusion regarding the ultimate effect.

Keywords: QSAR, mechanistic workflow, Ames test

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Shemansky JM[*1], McDaniel LP[*1], Klimas C[*2], Dertinger SD[*3], Dobrovolsky VN[*1], Kimoto T[*4], Horibata K, Polli JE[*2], Heflich RH[*1]: *Pig-a* gene mutation database.

Environ Mol Mutagen. 2019;60:759-762

Mutations in the X-linked phosphatidylinositol glycan, class A gene (*Pig-a*) lead to loss of glycosylphosphatidylinositol (GPI) anchors and GPI-anchored proteins from the surface of erythrocytes and other mammalian cells. The *Pig-a* gene mutation assay quantifies *in vivo* gene mutation by immunofluorescent labeling and flow cytometry to detect the loss of GPI-anchored proteins on peripheral blood erythrocytes. As part of the regulatory acceptance of the assay, a public database has been created that provides detailed information on *Pig-a* gene mutation assays conducted in rats and mice. A searchable version of the database is available through a website designed and hosted by the University of Maryland School of Pharmacy. Currently, the database contains only mouse and rat data, but it is anticipated that it will expand to include data from other species, including humans. A major purpose in developing the database was to aid in the preparation of a Retrospective Performance Analysis and Detailed Review Paper required for Organisation for Economic Co-operation and Development Test Guideline acceptance. We anticipate, however, that it also will be useful for accessing and comparing *Pig-a* data to data from other assays and for conducting quantitative assessments of *Pig-a* gene mutation responses.

Keywords: *Pig-a* gene mutation assay, *in vivo* gene mutation, test guideline

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Jojima K, Yamada T, Hirose A: Development of a hepatotoxicity prediction model using in vitro assay data of key molecular events.

Fundamental Toxicological Sciences. 2019;6:327-332

In this study, we developed screening-level hepatotoxicity prediction models using test data on *in vitro* assays, which measure key events at molecular levels that are possibly linked to hepatotoxicity. Hepatotoxic chemicals were retrieved from repeated-dose toxicity databases of the Hazard Evaluation Support System Integrated Platform and the Toxicogenomics Project. *In vitro* assay data with specified protein targets likely leading to hepatotoxicity were selected using the hepatotoxic chemicals. In total, 47 *in vitro* assays were selected for constructing the hepatotoxicity prediction models. Then, two predictive models were constructed. Model A returns "Hepatotoxic" if the query chemical is tested, and the test result is "Active" in any of the selected *in vitro* assays. Model B returns "Hepatotoxic" if an analog of the query chemical is tested, and the test result is "Active" in any of the selected *in vitro* assays. External validation of the two models was performed using repeated-dose toxicity test data from the Toxicity Reference Database. Model A and Model B had sensitivity values of 0.67 and 0.72 and specificity values of 0.74 and 0.72, respectively. Our models could predict the hepatotoxic chemicals underlying the toxic mechanisms that are not established by the existing knowledge base model. On the other hand, false negatives were found to involve mechanisms requiring metabolic activation. Because our hepatotoxicity prediction model is based on the biological activity of key molecular events leading to the toxicity endpoint, scientific justification would be more acceptable as adverse outcome pathway information becomes more available.

Keywords: hepatotoxicity, *in silico* model, *in vitro* assay data

Yamada T, Matsumoto M, Miura M, Hirose A: Case study on the use of integrated approaches to testing and assessment for testicular toxicity of ethylene glycol methyl ether (EGME)-related chemicals.

OECD, Series on Testing & Assessment. 2019;308:1-75

This case study was developed to demonstrate how read-across can be applied to fill data gaps in reproductive toxicity endpoints for screening assessments under the Japanese Chemical Substances Control Law (CSCL). A category approach was used to assess the testicular toxicity of ethylene glycol methyl ether (EGME)-related chemicals. Based on toxicity information for EGME and related chemicals and possible adverse outcome pathway information on the testicular toxicity of EGME, the category members were defined as chemicals that are metabolised to methoxy- or ethoxyacetic acid, which are responsible for testicular toxicity. A Japanese chemical inventory was screened using the metabolism simulator of the Hazard Evaluation Support System (HESS) to obtain metabolism information for EGME-related chemicals. This resulted in 15 chemicals being shortlisted for the category. Published data show that chemicals that produce methoxy- or ethoxyacetic acid as metabolites possess testicular toxicity, suggesting that untested chemicals that are predicted to produce these toxic metabolites will also have this effect. Although the overall uncertainty of the case study was low, some of the original compounds are structurally diverse, and metabolic hydrolysis or dealkylation could produce additional toxic compounds that need to be explicitly considered. However, a database search for toxicity and metabolism information suggested that these possible metabolites do not affect the toxicity levels through different mechanisms of action.

Keywords: ethylene glycol methyl ether, testicular toxicity, category approach

Patlewicz G[*1], Lizarraga LE[*2], Rua D[*3], Allen DG[*4], Daniel AB[*4], Fitzpatrick SC[*5], Garcia-Reyero N[*6], Gordon J[*7], Hakkinen P[*8], Howard AS[*4], Karmaus A[*4], Matheson J[*7], Mumtaz M[*9], Richarz A[*10], Ruiz P[*9], Scarano L[*11], Yamada T, Kleinstreuer N[*12]: Exploring current read-across applications and needs among selected U.S. Federal Agencies.

Regulatory Toxicology and Pharmacology. 2019;106:197-209

Read-across is a well-established data gap-filling technique applied for regulatory purposes. In US Environmental Protection Agency's New Chemicals Program under TSCA, read-across has been used extensively for decades, however the extent of application and acceptance of read-across among U.S.

federal agencies is less clear. In an effort to build read-across capacity, raise awareness of the state of the science, and work towards a harmonization of read-across approaches across U.S. agencies, a new read-across workgroup was established under the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). This is one of several ad hoc groups ICCVAM has convened to implement the ICCVAM Strategic Roadmap. In this article, we outline the charge and scope of the workgroup and summarize the current applications, tools used, and needs of the agencies represented on the workgroup for read-across. Of the agencies surveyed, the Environmental Protection Agency had the greatest experience in using read-across whereas other agencies indicated that they would benefit from gaining a perspective of the landscape of the tools and available guidance. Two practical case studies are also described to illustrate how the read-across approaches applied by two agencies vary on account of decision context.

Keywords: ICCVAM, read-across, regulatory purpose

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Matsumoto M, Hirata-Koizumi M, Kawamura T, Sakuratani S, Ono A*, Hirose A: Validation of the statistical parameters and model selection criteria of the benchmark dose methods for the evaluation of various endpoints in repeated-dose toxicity studies.
Fundamental Toxicological Sciences. 2019;6:125-136

The benchmark dose (BMD) approach is one of the important techniques in dose-response assessment for the risk assessment of chemicals and adapted by various international organizations. We investigated the appropriateness of the statistical parameters and model selection criteria for BMD lower bound (BMDL) estimation by BMD software (BMDS) (developed by the US Environmental Protection Agency) and PROAST (developed by the National Institute for Public Health and the Environment of the Netherlands). Publicly available repeated-dose toxicity study data (226 dichotomous datasets and 151 continuous datasets) were used for the investigation. Our findings were applied to establish BMD technical guidance for BMDS for the evaluation of various endpoints in repeated-dose toxicity studies. Under the Japan Chemical Substance Control Law (CSCL), the DRA-BMDS guidance (i.e., Division of Risk Assessment-BMDS guidance) is used for the evaluation of a "Priority Assessment Chemical Substance." Namely, selecting of an extra risk of 10% (dichotomous data) or a level change of 1SD (continuous data) as a default benchmark response. Running all the models without or with parameter constraints. Selecting the model that calculated the lowest BMDL but excluding the one that estimated a BMD/BMDL ratio ≥ 10 or lowest dose/BMDL ratio ≥ 10. We believe that the DRA-BMDS guidance can assist risk assessors in the selection of the BMD model.

Keywords: benchmark dose, benchmark response, BMD

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Kobayashi-Tsukumo H*1, Oiji K*2, Xie D*1, Sawada Y*1, Yamashita K*3, Ogata S*4, Kojima H, Itagaki H*1: Eliminating the contribution of lipopolysaccharide to protein allergenicity in the human cell-line activation test (h-CLAT).
J Toxicol Sci. 2019;44:283-297

We previously developed a test for detecting naturally occurring protein-induced skin sensitization

based on the markers and criteria of the human cell-line activation test (h-CLAT) and showed that the h-CLAT was useful for assessing the allergenic potency of proteins. However, test proteins were contaminated with varying amounts of lipopolysaccharide (LPS), which might have contributed to the stimulation of CD86 and CD54 expression. In this study, we developed a method to exclude the effects of LPS in the assessment of skin sensitization by naturally occurring proteins. We tested two inhibitors [the caspase-1 inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk; hereafter referred to as YVAD), which can mitigate the LPS-induced increases in CD54 expression, and polymyxin B (PMB), which suppresses the effect of LPS by binding to its lipid moiety (i.e., the toxic component of LPS)]. After a 24 hr exposure, YVAD and PMB reduced LPS-induced CD86 and CD54 expression. In particular, the effect of PMB was dependent upon pre-incubation time and temperature, with the most potent effect observed following pre-incubation at 37℃ for 24 hr. Moreover, only pre-incubation with cell-culture medium (CCM) at 37℃ for 24 hr showed an inhibitory effect similar to that of PMB, with this result possibly caused by components of CCM binding to LPS. Similar effects were observed in the presence of ovalbumin (with 1070 EU/mg LPS) and ovomucoid, and lysozyme (with 2.82 and 0.234 EU/mg LPS, respectively) in CCM. These results indicated that PMB and CCM effectively eliminated the effects of LPS during assessment of protein allergenicity, thereby allowing a more accurate evaluation of the potential of proteins to induce skin sensitization.

Keywords: lipopolysaccharide, skin sensitization, h-CLAT

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Fujita M[*1], Yamamoto Y[*1], Watanabe S[*2], Sugawara T[*2], Wakabayashi K[*3], Tahara Y[*3], Horie N[*4], Fujimoto K[*4], Kusakari K[*5], Kurokawa Y[*5], Kawakami T, Kojima K[*6], Sozu T[*7], Nakayama T[*7], Kusao T[*7], Richmond J[*8], Nicole K[*9], Kim BH[*10], Kojima H, Kasahara T[*1], Ono A[*11]: The within- and between-laboratory reproducibility and predictive capacity of the *in chemico* amino acid derivative reactivity assay: Results of validation study implemented in four participating laboratories.

J Appl Toxicol. 2019;39:1492-1505

The amino acid derivative reactivity assay (ADRA) is an *in chemico* alternative method that focuses on protein binding as the molecular initiating event for skin sensitization. It is a simple and versatile method that has successfully solved some of the problems of the direct peptide reactivity assay (DPRA). The transferability and within- and between-laboratory reproducibility of ADRA were evaluated and confirmed as part of a validation study conducted at four participating laboratories. The transfer of ADRA technology from the lead laboratory to the four participating laboratories was completed successfully during a two-step training program, after which the skin sensitization potentials of 40 coded chemicals were predicted based on the results of ADRA testing. Within-laboratories reproducibility was 100% (10 of 10), 100% (10 of 10), 100% (7 of 7) and 90% (9 of 10), or an average of 97.3% (36 of 37); between-laboratory reproducibility as calculated on the results of three laboratories at the time was 91.9%. The overall predictive capacity comprised an accuracy of 86.9%, sensitivity of 81.5% and specificity of 98.1%. These results satisfied the targets set by the validation management team for demonstrating transferability, within- and between-laboratory reproducibility, and predictive capacity as well as gave a clear indication that ADRA is easily transferable and sufficiently robust to be used in place of DPRA.

Keywords: ADRA (amino acid derivative reactivity assay), skin sensitization, validation study

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Kojima H, Yamaguchi H*1, Sozu T*2, Kleinstreuer N*3, Chae-Hyung L*4, Chen W*5, Watanabe M*6, Fukuda T*7, Yamashita K*8, Takezawa T*9: Multi-laboratory Validation Study of the Vitrigel-Eye Irritancy Test Method as an Alternative to *In Vivo* Eye Irritation Testing.

Altern Lab Anim. 2019;47:140-157

Collagen vitrigel membranes (CVMs) comprising high-density collagen fibrils equivalent to *in vivo* connective tissues have been widely used in cell culture applications. A human corneal epithelium (hCE) model was previously developed by the Takezawa group, by culturing HCE-T cells (derived from hCE cells) on a CVM scaffold in a chamber that provided an air-liquid interface culture system. This hCE model was used to establish a new test method, known as the Vitrigel-Eye Irritancy Test (Vitrigel-EIT) method, which can be used to estimate the ocular irritation potential of test chemicals by analysing relative changes in transepithelial electrical resistance (TEER) over time. The current study was conducted in order to assess the reliability and relevance of the Vitrigel-EIT method at three participating laboratories by determining the method's within-laboratory reproducibility and between-laboratory reproducibility, as well as its capacity for distinguishing non-irritants from irritants in a bottom-up approach. The initial test sample size was found to be too low to evaluate the predictive capacity of the test method, and so it was evaluated with additional in-house data for a total of 93 test chemicals. The results showed 80-100% within-laboratory reproducibility and an excellent between-laboratory reproducibility that met the acceptance criteria of 80%. However, the method's predictive capacity for distinguishing non-irritants (test chemicals not requiring classification and labelling for eye irritation or serious eye damage, i.e. United Nations Globally Harmonised System of Classification and Labelling of Chemicals (GHS) No Category) from irritants (GHS Categories 1 and 2) in a bottom-up approach was unacceptable because of false negative rates as high as 16.7%. After considerable review of the data with a view to using the method for regulatory purposes, it was determined that a more defined applicability domain, excluding test chemical solutions with a pH of 5 or less and solid test chemicals, improved the false negative rate to 4.2%. These results suggested that, within this carefully defined applicability domain, the Vitrigel-EIT method could be a useful alternative for distinguishing test chemicals that are ocular non-irritants from those that are irritants as part of a bottom-up approach.

Keywords: TEER, eye irritation, validation study

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Toxicol In Vitro. 2020;66:104832, doi: 10.1016/

j.tiv.2020.104832

To evaluate the immunotoxic effects of xenobiotics, we have established the Multi-ImmunoTox assay, in which three stable reporter cell lines are used to evaluate the effects of chemicals on the IL-2, IFN-γ, IL-1β and IL-8 promoters. Here, we report the official validation study of the IL-2 luciferase assay (IL-2 Luc assay). In the Phase I study that evaluated five coded chemicals in three sets of experiments, the average within-laboratory reproducibility was 86.7%. In the Phase II study, 20 coded chemicals were evaluated at multiple laboratories. In the combined results of the Phase I and II studies, the between-laboratory reproducibility was 80.0%. These results suggested that the IL-2 Luc assay was reproducible both between and within laboratories. To determine the predictivity, we collected immunotoxicological information and constructed the reference data by classifying the chemical into immunotoxic compounds targeting T cells or others according to previously reported criteria. When compared with the reference data, the average predictivity of the Phase I and II studies was 75.0%, while that of additional 60 chemicals examined by the lead laboratory was 82.5%. Although the IL-2 Luc assay alone is not sufficient to predict immunotoxicity, it will be a useful tool when combined with other immune tests.

Keywords: immunotoxic assay, luciferase assay, validation study

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J Appl Toxicol. 2020;40:843-854

The amino acid derivative reactivity assay (ADRA), which is an *in chemico* alternative to the use of animals in testing for skin sensitization potential, offers significant advantages over the direct peptide reactivity assay (DPRA) in that it utilizes nucleophilic reagents that are sensitive enough to be used with test chemical solutions prepared to concentrations of 1 mm, which is one-hundredth that of DPRA. ADRA testing of hydrophobic or other poorly soluble compounds requires that they be dissolved in a solvent consisting of dimethyl sulfoxide (DMSO) and acetonitrile. DMSO is known to promote dimerization by oxidizing thiols, which then form disulfide bonds. We investigated the extent to which DMSO oxidizes the cysteine-derived nucleophilic reagents used in both DPRA and ADRA and found that oxidation of both N-(2-(1-naphthyl) acetyl)-l-cysteine (NAC) and cysteine peptide increases as the concentration of DMSO increases, thereby lowering the concentration of the nucleophilic reagent. We also found that use of a solvent consisting of 5% DMSO in acetonitrile consistently lowered NAC concentrations by about 0.4 μm relative to the use of solvents containing no DMSO. We also tested nine sensitizers and four nonsensitizers having different sensitization potencies to compare NAC depletion with and without 5% DMSO and found that reactivity was about the same with either solvent. Based on the above, we conclude that the use of a solvent containing 5% DMSO has no effect on the accuracy of ADRA test results. We plan to review and propose revisions to OECD Test Guideline 442C based on the above investigation.

Keywords: amino acid derivative reactivity assay (ADRA), dimethyl sulfoxide, skin sensitization

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