宮崎玉樹，菅野仁美，阿曽幸男，合田幸広：国内で流通している経皮吸収型製剤の粘着特性の比較 ―剥離力とタックについて―.
*薬学雑誌* 2018;138(11):1425-33.

Forty-four brands of transdermal patches for twelve kinds of active pharmaceutical ingredients (APIs) are available in Japan as of April 30, 2018. Although approximately one-third of the corresponding pharmaceutical interview forms lack information on how to evaluate the adhesive properties of the patches, the peel test, probe tack test, or inclined ball tack test have generally been adopted. This means that it might be difficult to simply compare the adhesive properties among the patches because the testing methods are not unified in some cases. In this study, measurements of the adhesive properties of 38 transdermal patches of ten different APIs were performed using several unified testing methods (180° peel test, 90° peel test, self-adhesion test, and probe tack test) under unified experimental conditions. The adhesive properties were found to be quite different among the patches, even for the same API, dose, and size. For example, the ratios of the maximum to minimum measured values of tack and 180° peel strength for tulobuterol patches were 5 and 29, respectively. In the case of generic products for which the bioequivalence to a brand-name product is assured, the variation in adhesive properties can extend the range of choices for patients, which is advantageous. Providing information to medical experts on adhesive properties through, for example, pharmaceutical interview forms and package inserts, is considered to be useful for helping patients to make better choices.
Keywords: transdermal patch, peel strength, tack

Miyazaki T, Aso Y, Goda Y: Identifying the origin plant of starches by numerical description of the coloration of iodine-starch reaction solutions.
*Jpn. J. Food Chem. Safety.* 2018; 25(3): 145-51.

Microscopy is the primary technique for identifying the origin plant of a starch sample but requires operators with highly proficient skills and experience, and is unsuitable for discriminating modified starches such as pregelatinized starches. The plant species from which the starch is isolated is reflected in the characteristic color of the iodine-starch reaction solution. However, visual observation is subjective and vague because color perception is organoleptic and color is expressed by ambiguous names such as "orange-red" and "deep blue". Quality management using the color of samples is gaining wider acceptance in the field of natural products as well as industrial products because simple, easy to use, high-performance spectrophotometers are now widely used. In this study, the color of iodine-starch reaction solution of 31 kinds of starches and pregelatinized starches from maize, wheat, potato and rice were measured spectrophotometrically and the color was numerically described using the $L^*a^*b^*$ color system. The characteristic ($a^*$, $b^*$) values grouped together on the color system according to the origin plant for each starch, suggesting that numerical information on the color reaction is useful for estimating the origins of starches.
Keywords: starch, origin plant, $L^*a^*b^*$ color system

Otaki T[*1], Tanabe Y[*2], Kojima T[*1], Miura M[*1], Ikeda Y[*1], Koide T, Fukami T[*2]: In situ monitoring of cocrystals in formulation development using low-frequency Raman spectroscopy.
*Int. J. Pharm.* 2018;542(1-2):56-65.

In recent years, to guarantee a quality-by-design approach to the development of pharmaceutical products, it is important to identify properties of raw materials and excipients in order to determine critical process parameters and critical quality attributes. Feedback obtained from real-time analyses using various process analytical technology (PAT) tools has been actively investigated. In this study, in situ monitoring using low-frequency (LF) Raman spectroscopy (10–200 $cm^{-1}$), which may have higher discriminative ability among polymorphs than near-infrared spectroscopy and conventional Raman spectroscopy (200–1800 $cm^{-1}$), was investigated as a possible application to PAT. This is because LF-Raman spectroscopy obtains information about intermolecular and/or lattice vibrations in the solid state. The monitoring results obtained from Furosemide/Nicotinamide cocrystal indicate that LF-Raman spectroscopy is applicable to in situ monitoring of suspension and fluidized bed granulation processes, and is an effective technique as a PAT tool to detect the

conversion risk of cocrystals. LF-Raman spectroscopy is also used as a PAT tool to monitor reactions, crystallizations, and manufacturing processes of drug substances and products. In addition, a sequence of conversion behaviors of Furosemide/Nicotinamide cocrystals was determined by performing in situ monitoring for the first time.

Keywords: low-frequency Raman spectroscopy, process analytical technology, monitoring

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Azuma M*1, Fujii M*1, Inoue M*1, Hisada H*1, Koide T, Kemper M*2, Yamamoto Y*3, Suzuki N*4, Suzuki T*4, Fukami T*1: Molecular state of active pharmaceutical ingredients in ketoprofen dermal patches characterized by pharmaceutical evaluation.
*Biol. Pharm. Bull.* 2018;41(9):1348–54.

The molecular states of ketoprofen and the interaction between ketoprofen and other pharmaceutical excipients in the matrix layer were examined to determine their effect on the pharmaceutical properties of original and generic ketoprofen dermal patches (generic patches A and B). Molecular states of ketoprofen were evaluated using polarized light microscopy, Raman spectroscopy and powder X-ray diffraction. For the original ketoprofen patch, crystalline components were not observed in the matrix layer. However, crystalline ketoprofen was observed in the two generic ketoprofen patches. Moreover, the ketoprofen exhibited hydrogen bonding with the pharmaceutical excipients or patch materials in the generic products. Skin permeation of ketoprofen from the patches was evaluated using hairless mouse skin. Twelve hours after application, the original patch demonstrated the highest level of cumulative skin permeation of ketoprofen. This was followed by generic patch B while generic patch A showed the lowest level of permeation. Fluxes were calculated from the skin permeation profiles. The original patch was approx. 2.4-times faster compared with generic patch A and approximately 1.9-times faster compared with generic patch B. This investigation suggested that pharmaceutical properties such as skin permeability for these types of products are affected by the precipitation of crystalline ketoprofen in the matrix layer and the interaction of ketoprofen with the pharmaceutical excipients or patch materials.

Keywords: ketoprofen dermal patch, Raman spectroscopy, skin permeability

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Yamamoto Y*1, Hanai A*1, Onuki Y*2, Fujii M*3, Onishi Y*3, Fukami T*3, Metori K*4, Suzuki N*4, Suzuki T*4, Koide T: Mixtures of betamethasone butyrate propionate ointments and heparinoid oil-based cream: Physical stability evaluation.
*Euro. J. Pharm. Sci.* 2018;124:199–207.

Betamethasone butyrate propionate ointment (BBPO) is mainly used for adult patients in dermatology and is often prescribed as a mixture containing a base or moisturizing cream for various reasons. However, in the case of a moisturizing cream, since this formulation is composed of various ingredients, a physical change is expected to occur by mixing it with an ointment. Therefore, in the present study, the physical stability of a mixture of four BBPO formulations and heparinoid oily cream (HPOC) was examined. Layer separation was observed in all mixtures following centrifugation. The near-infrared (NIR) measurement showed a peak at 5200 $cm^{-1}$ on the lower layer side, which strongly suggests the presence of water. The peak at 5200 $cm^{-1}$ in the middle layer was hardly observed in the mixtures of two BBPO generic formulations and HPOC, thus suggesting that the separation was more advanced in those mixtures than in the others. These two mixtures separated into a semisolid layer (upper side) and a liquid layer (lower side) after 3 h of storage at 37℃. The NIR measurement of each layer revealed that most of the semisolid layer was oil while the liquid layer was water. Furthermore, backscattered light measurements were conducted to monitor the behavior of the mixture's layer separation. An evaluation using model formulations revealed that the layer separation of the mixtures was due to the propylene glycol (PG) and surfactant content of the two generic BBPO formulations. Thus, these findings suggest that excipients need to be considered in selecting formulations for mixtures of skin preparations.

Keywords: steroidal ointment, heparinoid oily cream, near-infrared spectroscopy

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Inoue M*1, Hisada H*1, Koide T, Fukami T*1, Roy A*2, Carriere J*2, Heyler R*2: Transmission Low-Frequency Raman Spectroscopy for Quantification of Crystalline Polymorphs in Pharmaceutical Tablets.
*Anal. Chem.* 2019;91(3):1997–2003.

The purpose of this study was to quantify polymorphs of active pharmaceutical ingredients in pharmaceutical tablets using a novel transmission low-frequency Raman spectroscopy method. We developed a novel transmission geometry for low-frequency Raman spectroscopy and compared quantitative ability in transmission mode versus backscattering mode using chemometrics. We prepared two series of tablets, (1) containing different weight-based contents of carbamazepine form III and (2) including different ratios of carbamazepine polymorphs (forms I/III). From the relationship between the contents of carbamazepine form III and partial least-squares (PLS) predictions in the tablets, correlation coefficients in transmission mode (R2 = 0.98) were found to be higher than in backscattering mode (R2 = 0.97). The root-mean-square error of cross-validation (RMSECV) of the transmission mode was 3.9 compared to 4.9 for the backscattering mode. The tablets containing a mixture of carbamazepine (I/III) polymorphs were measured by transmission low-frequency Raman spectroscopy, and it was found that the spectral shape changed according to the ratio of polymorphs: the relationship between the actual content and the prediction showed high correlation. These findings indicate that transmission low-frequency Raman spectroscopy possesses the potential to complement existing analytical methods for the quantification of polymorphs.

Keywords: low-frequency Raman spectroscopy, transmission, polymorph

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Sakai-Kato K, Nanjo K, Takechi-Haraya Y, Goda Y, Okuda H, Izutsu K: Detailed morphological characterization of nanocrystalline active ingredients in solid oral dosage forms using atomic force microscopy.
*AAPS PharmSciTech.* 2019;20:70.

The characterization of nanocrystalline active ingredients in multicomponent formulations for the design and manufacture of products with increased bioavailability is often challenging. The purpose of this study is to develop an atomic force microscopy (AFM) imaging method for the detailed morphological characterization of nanocrystalline active ingredients in multicomponent oral formulations. The AFM images of aprepitant and sirolimus nanoparticles in aqueous suspension show that their sizes are comparable with those measured using dynamic light scattering (DLS) analysis. The method also provides information on a wide-sized range of particles, including small particles that can often only be detected by DLS when larger particles are removed by additional filtration steps. An expected advantage of the AFM method is the ability to obtain a detailed information on particle morphology and stiffness, which allows the active pharmaceutical ingredient and excipient (titanium dioxide) particles to be distinguished. Selective imaging of particles can also be achieved by varying the surface properties of the AFM solid substrate, which allows to control the interactions between the substrate and the active pharmaceutical ingredient and excipient particles. AFM analysis in combination with other methods (e.g., DLS), should facilitate the rational development of formulations based on nanoparticles.

Keywords: nanocrystalline active ingredient, excipient, atomic force microscopy

Ohgita T*1, Takechi-Haraya Y, Nadai R*1, Kotani M*1, Tamura Y*1, Nishikiori K*1, Nishitsuji K*2, Uchimura K*3, Hasegawa K*1, Sakai-Kato K, Akaji K*1, Saito H*1: A novel amphipathic cell-penetrating peptide based on the N-terminal glycosaminoglycan binding region of human apolipoprotein E.
*Biochim. Biophys. Acta–Biomembranes.* 2019;1861:541-9.

In the direct cell membrane penetration, arginine-rich cell-penetrating peptides are thought to penetrate into cells across the hydrophobic lipid membranes. To

investigate the effect of the amphipathic property of argininerich peptide on the cell-penetrating ability, we designed a novel amphipathic cell-penetrating peptide, A2-17, and its derivative, A2-17KR, in which all lysine residues are substituted with arginine residues, based on the glycosaminoglycan binding region in the N-terminal α-helix bundle of human apolipoprotein E. Isothermal titration calorimetry showed that A2-17 variants have a strong ability to bind to heparin with high affinity. Circular dichroism and tryptophan fluorescence measurements demonstrated that A2-17 variants bind to lipid vesicles with a structural change from random coil to amphipathic α-helix, being inserted into the hydrophobic membrane interiors. Flow cytometric analysis and confocal laser scanning microscopy demonstrated the great cell penetration efficiency of A2-17 variants into CHO-K1 cells when incubated at low peptide concentrations (2 μM or less), suggesting that the increased amphipathicity with α-helix formation enhances the cell membrane penetration ability of arginine-rich peptides. Interestingly, A2-17KR exhibited lower efficiency of cell membrane penetration compared to A2-17 despite of their similar binding affinity to lipid membranes. Since high peptide concentrations (typically>10 μM) are usually prerequisite for efficient cell penetration of arginine-rich peptides, A2-17 is a unique amphipathic cell-penetrating peptide that exhibits an efficient cell penetration ability even at low peptide concentrations.

Keywords: arginine-rich peptide, amphipathicity, cell membrane penetration

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Sakai-Kato K, Najo K, Goda Y: Rapid analysis of cyclic peptide cyclosporine A by HPLC using a column packed with nonporous particles.
*Chem. Pharm. Bull.* 2018;66:805-9.

We developed a rapid and efficient analytical technique for cyclosporine A using HPLC on a column packed with 2-μm nonporous octadecylsilyl silica particles. Under optimized conditions, cyclosporine A was separated with high resolution from other cyclic peptides within 3 min, because the mass transfer resistance in the stationary phase was reduced by the use of the small, nonporous particles. Although the plate number increased greatly with the increase in the column temperature, the retention times were not affected. This behavior is different from other cyclic peptides or linear peptides. Based on its physicochemical characteristics, cyclosporine A is a poor hydrogen bond donor, and has a small topological polar surface area, low rotatable bond count, and high log *P* value. These results show that cyclosporine A is structurally rigid and undergoes poor water solvation even at high temperature. In the context of the rapid development of cyclic peptides with similar physicochemical characteristics to cyclosporine A, our developed method is useful for the development of cyclic peptide therapeutics.

Keywords: cyclic peptide, cyclosporine A, nonporous column

Takechi-Haraya Y, Goda Y, Sakai-Kato K: Atomic force microscopy study on the stiffness of nanosized liposomes containing charged lipids.
*Langmuir*. 2018;34:7805-12.

It has recently been recognized that the mechanical properties of lipid nanoparticles play an important role during in vitro and in vivo behaviors such as cellular uptake, blood circulation, and biodistribution. However, there have been no quantitative investigations of the effect of commonly used charged lipids on the stiffness of nanosized liposomes. In this study, by means of atomic force microscopy (AFM), we quantified the stiffness of nanosized liposomes composed of neutrally charged lipids combined with positively or negatively charged lipids while simultaneously imaging the liposomes in aqueous medium. Our results showed that charged lipids, whether negatively or positively charged, have the effect of reducing the stiffness of nanosized liposomes, independently of the saturation degree of the lipid acyl chains; the measured stiffness values of liposomes containing charged lipids are 30−60% lower than those of their neutral counterpart liposomes. In addition, we demonstrated that the Laurdan generalized polarization values, which are related to the hydration degree of the liposomal membrane interface and often used as a qualitative indicator of liposomal membrane stiffness, do not directly correlate with the physical stiffness values of the liposomes prepared in this study. However,

our results indicate that direct quantitative AFM measurement is a valuable method to gain molecular-scale information about how the hydration degree of liposomal interfaces reflects (or does not reflect) liposome stiffness as a macroscopic property. Our AFM method will contribute to the quantitative characterization of the nano－bio interaction of nanoparticles and to the optimization of the lipid composition of liposomes for clinical use.

Keywords: liposome stiffness, charged lipid, atomic force microscopy

Miura Y[*1], Hashii N, Ohta Y[*2], Itakura Y[*3], Tsumoto H[*1], Suzuki J, Takakura D[*4], Abe Y[*5], Arai Y[*5], Toyoda M[*3], Kawasaki N[*2], Hirose N[*5], Endo T[*1]: Characteristic glycopeptides associated with extreme human longevity identified through plasma glycoproteomics.

*Biochim Biophys Acta.* 2018;1862(6): 1462-1471.

BACKGROUND: Glycosylation is highly susceptible to changes of the physiological conditions, and accordingly, is a potential biomarker associated with several diseases and/or longevity. Semi-supercentenarians (SSCs; older than 105 years) are thought to be a model of human longevity. Thus, we performed glycoproteomics using plasma samples of SSCs, and identified proteins and conjugated N-glycans that are characteristic of extreme human longevity.

METHODS: Plasma proteins from Japanese semi-supercentenarians (SSCs, 106-109 years), aged controls (70-88 years), and young controls (20-38 years) were analysed by using lectin microarrays and liquid chromatography/mass spectrometry (LC/MS). Peak area ratios of glycopeptides to corresponding normalising peptides were subjected to orthogonal projections to latent structures discriminant analysis (OPLS-DA). Furthermore, plasma levels of clinical biomarkers were measured.

RESULTS: We found two lectins such as Phaseolus vulgaris, and Erythrina cristagalli (ECA), of which protein binding were characteristically increased in SSCs. Peak area ratios of ECA-enriched glycopeptides were successfully discriminated between SSCs and controls using OPLS-DA, and indicated that tri-antennary and sialylated N-glycans of haptoglobin at Asn207 and Asn211 sites were characterized in SSCs. Sialylated glycans of haptoglobin are a potential biomarker of several diseases, such as hepatocellular carcinoma, liver cirrhosis, and IgA-nephritis. However, the SSCs analysed here did not suffer from these diseases.

CONCLUSIONS: Tri-antennary and sialylated N-glycans on haptoglobin at the Asn207 and Asn211 sites were abundant in SSCs and characteristic of extreme human longevity.

GENERAL SIGNIFICANCE: We found abundant glycans in SSCs, which may be associated with human longevity.

Keywords: liquid chromatography/mass spectrometry, N-glycosylation, semi-supercentenarian

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Tada M, Suzuki T, Ishii-Watabe A: Development and characterization of an anti-rituximab monoclonal antibody panel.

*mAbs.* 2018;10(3):370-379.

During the development of monoclonal antibodies (mAbs) and other therapeutic proteins, immunogenicity, in particular the induction of anti-drug antibodies (ADAs), is an important concern, and thus immunogenicity assessment is a requirement for their approval. Establishment of appropriate methods for detecting and characterizing ADAs is necessary for immunogenicity assessment, but the lack of commonly available reference standards makes it difficult to compare and evaluate the methods. It is also difficult to compare the data with those obtained by other methods or facilities without reference standards. Here, we developed a panel of ADAs against anti-CD20 rituximab (Rituxan®, MabThera®); the panel consisted of eight clones of recombinant human-rat chimeric mAbs that target rituximab. The anti-rituximab mAbs showed different binding properties (specificity, epitope and affinity), and different neutralization potencies for CD20 binding, complement-dependent cytotoxicity and

antibody-dependent cell-mediated cytotoxicity. The molecular size of the immune complex consisting of rituximab and the anti-rituximab mAb differed among the clones, and was well correlated with their level of Fcγ-receptor activation. These results suggest that the ADAs chosen for the newly developed panel are suitable surrogates for human ADAs, which exhibit different potential to affect the efficacy and safety of rituximab. Next, we used this panel to compare several ADA-detecting assays and revealed that the assays had different abilities to detect the ADAs with different binding characteristics. We conclude that our panel of ADAs against rituximab will be useful for the future development and characterization of assays for immunogenicity assessment.

Keywords: anti-drug antibody, rituximab, immunogenicity

Hashimoto Y[*1], Hata T[*1], Tada M, Iida M[*1], Watari A[*1], Okada Y[*1], Doi T[*1], Kuniyasu H[*2], Yagi K[*1], Kondoh M[*1]: Safety evaluation of a human chimeric monoclonal antibody that recognizes the extracellular loop domain of claudin-2.

*Eur J Pharm Sci*. 2018;117:161-167.

Claudin-2 (CLDN-2), a pore-forming tight junction protein with a tetra-transmembrane domain, is involved in carcinogenesis and the metastasis of some cancers. Although CLDN-2 is highly expressed in the tight junctions of the liver and kidney, whether CLDN-2 is a safe target for cancer therapy remains unknown. We recently generated a rat monoclonal antibody (mAb, clone 1A2) that recognizes the extracellular domains of human and mouse CLDN-2. Here, we investigated the safety of CLDN-2-targeted cancer therapy by using 1A2 as a model therapeutic antibody. Because most human therapeutic mAbs are IgG1 subtype that can induce antibody-dependent cellular cytotoxicity, we generated a human-rat chimeric IgG1 form of 1A2 (xi-1A2). xi-1A2 activated Fcγ receptor IIIa in the presence of CLDN-2-expressing cells, indicating that xi-1A2 likely exerts antibody-dependent cellular cytotoxicity. At 24 h after its intravenous injection, xi-1A2 was distributed into the liver, kidney, and tumor tissues of mice bearing CLDN-2-expressing fibrosarcoma cells. Treatment of the xenografted mice with xi-1A2 attenuated tumor growth without apparent adverse effects, such as changes in body weight and biochemical markers of liver and kidney injury. These results support xi-1A2 as the lead candidate mAb for safe CLDN-2-targeted cancer therapy.

Keywords: claudin-2, monoclonal antibody, safety evaluation

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鈴木琢雄，森岡知子[*]，林真由美[*]，東阪嘉子，蛭田葉子，橋井則貴，中川ゆかり[*]，石井明子：日局新規収載候補日局グルカゴン各条試験法に関する研究―液体クロマトグラフィーを用いた合成グルカゴン定量法の検討―.

*医薬品医療機器レギュラトリーサイエンス* 2018;49(7):488-497.

Animal testing has been adopted as an assay method for the synthetic glucagons approved in Japan. On the other hand, high performance liquid chromatography (HPLC) has been used as an assay method for glucagon (Genetical recombination) approved in Japan. HPLC assay has also been adopted in the Glucagon (Genetical recombination) monographs in the European Pharmacopoeia (EP) and United States Pharmacopeia (USP). Therefore, an HPLC assay for synthetic glucagon is required as an alternative to animal testing from the viewpoint of animal welfare and harmonization of the assay method in the synthetic glucagon and Glucagon (Genetical recombination), which have been listed as the new candidate monographs in the Japanese Pharmacopoeia (JP). In this study, we demonstrated the suitability of the proposed HPLC method for synthetic glucagon.

Keywords: synthetic glucagon, glucagon assay, liquid chromatography

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Kiyoshi M, Shibata H, Harazono A, Torisu T[*1], Maruno T[*2], Akimaru M[*3], Asano Y[*4], Hirokawa M[*5], Ikemoto K[*5], Itakura Y[*1], Iwura T[*6], Kikitsu A[*7], Kumagai T[*8], Mori N[*5], Murase H[*4], Nishimura H[*9], Oda A[*10], Ogawa T[*11], Ojima T[*3], Okabe S[*4], Saito S[*3], Saitoh S[*12], Suetomo H[*6], Takegami K[*11], Takeuchi M[*7], Yasukawa H[*4], Uchiyama S[*13], Ishii-Watabe A: Collaborative Study For Analysis

Of Subvisible Particles Using Flow Imaging And Light Obscuration: Experiences In Japanese Biopharmaceutical Consortium.

*J Pharm Sci.* 2019;108(2):832-841.

The evaluation of subvisible particles, including protein aggregates, in therapeutic protein products has been of great interest for both pharmaceutical manufacturers and regulatory agencies. To date, the flow imaging (FI) method has emerged as a powerful tool instead of light obscuration (LO) due to the fact that (1) protein aggregates contain highly transparent particles and thereby escape detection by LO and (2) FI provides detailed morphological characteristics of subvisible particles. However, the FI method has not yet been standardized nor listed in any compendium. In an attempt to assess the applicability of the standardization of the FI method, we conducted a collaborative study using FI and LO instruments in a Japanese biopharmaceutical consortium. Three types of subvisible particle preparations were shared across 12 laboratories and analyzed for their sizes and counts. The results were compared between the methods (FI and LO), inter-laboratories, and inter-instruments (Micro Flow Imaging and FlowCam). We clarified the marked difference between the detectability of FI and LO when counting highly transparent protein aggregates in the preparations. Although FlowCam provided a relatively higher number of particles compared with MFI, consistent results were obtained using the instrument from the same manufacturer in all 3 samples.

Keywords: protein aggregation, image analysis, particle size

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Aoyama M, Tada M, Tatematsu KI*, Hashii N, Sezutsu H*, Ishii-Watabe A: Effects of amino acid substitutions on the biological activity of anti-CD20 monoclonal antibody produced by transgenic silkworms (Bombyx mori).

*Biochem Biophys Res Commun.* 2018;503(4):2633-2638.

Recombinant monoclonal antibodies (mAbs) have been used in various therapeutic applications including cancer therapy. Fc-mediated effector functions play a pivotal role in the tumor-killing activities of some tumor-targeting mAbs, and Fc-engineering technologies with glyco-engineering or amino acid substitutions at the antibody Fc region have been used to enhance cytotoxic activities including antibody-dependent cellular cytotoxicity (ADCC). We previously reported that the mAbs produced using transgenic silkworms showed stronger ADCC activity and lower complement-dependent cytotoxicity (CDC) activity than mAbs derived from Chinese hamster ovary (CHO) cells due to their unique N-glycan structure (lack of core-fucose and non-reducing terminal galactose). In this study, we generated anti-CD20 mAbs with amino acid substitutions using transgenic silkworms and analyzed their biological activities to assess the effect of the combination of glyco-engineering and amino acid substitutions on the Fc-mediated function of mAbs. Three types of amino acid substitutions at the Fc region (G236A/S239D/I332E, L234A/L235A, and K326W/E333S) modified the Fc-mediated biological activities of silkworm-derived mAbs as in the case of CHO-derived mAbs, resulting in the generation of Fc-engineered mAbs with characteristic Fc-mediated functions. The combination of amino acid substitutions at the Fc region and glyco-engineering using transgenic silkworm made it possible to generate Fc-engineered mAbs with suitable Fc-mediated biological functions depending on the pharmacological mechanism of their actions. Transgenic silkworms were shown to be a promising system for the production of Fc-engineered mAbs.

Keywords: amino acid substitution, transgenic silkworm, Fc-mediated effector function

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Niimi S, Nishimiya K*1, Nishidate M*1, Saito T*2,

Minoura K[*2], Kadotsuji K[*3], Shimakura J[*3], Shigemizu H[*4], Hosogi J[*5], Adachi M[*5], Hashimoto T[*6], Mori T[*6], Harada H[*6], Yamamoto KI[*6], Nakamura T[*7], Nomura T[*7], Yamaguchi I[*8], Sonehara K[*8], Ishii-Watabe A, Kawasaki N[*9]: Collaborative study using common samples to evaluate the performance of anti-drug antibody assays constructed by different companies.
*Drug Metab Pharmacokinet*. 2018;33(2):125-132.

This study was undertaken to evaluate the performance of anti-drug antibody (ADA) assays constructed by each participating company using common samples including ADA, drug and human serum. The ADA assays constructed by each company showed good sensitivity and precision for evaluation of ADA. Cut points for screening and confirmatory assays and assay selectivity were determined by various calculation methods. In evaluations of blind ADA samples, nearly similar results were obtained by the study companies in determinations of whether samples were positive or negative except at the lowest sample concentration (5 ng/mL). In measurement of drug tolerance, for almost samples containing ADA and drugs, more positive results were obtained in assays using acid dissociation compared to those without acid dissociation. Overall, the performance of ADA assays constructed by the 10 companies participating in this study was acceptable in terms of sensitivity and reproducibility for detection and evaluation of immunogenicity in both patients and healthy subjects. On the other hand, based on results for samples containing ADA and drugs, validity of results for ADA assays conducted without acid dissociation was less meaningful and more difficult to evaluate. Thus, acid dissociation was confirmed to be useful for improving drug tolerance.
Keywords: immunogenicity, ADA assay, cut point

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Hashii N, Suzuki J, Hanamatsu H[*], Furukawa JI[*], Ishii-Watabe A: In-depth site-specific O-Glycosylation analysis of therapeutic Fc-fusion protein by electron-transfer/higher-energy collisional dissociation mass spectrometry.
*Biologicals*. 2019; 58; 35-43.

Unexpected O-glycosylations, including O-xylosylations and mucin-type O-glycosylations, have been reported in recent glycosylation analyses of Fc-fusion proteins produced in mammalian cell expression systems. This observation suggests that therapeutic proteins with novel structures can undergo unintended O-glycosylations, having implications regarding their efficacy and safety. Therefore, the implementation of O-glycosylation analysis during product developmental is essential. However, detail site-specific O-glycosylation analysis is difficult because no consensus sequence for mucin-type O-glycosylations is known, and O-glycopeptides often contain multiple or continuous glycosylation sites. Recently, a new mass spectrometric fragmentation method called electron-transfer/higher-energy collisional dissociation (EThcD) has been used for site-specific glycosylation analysis. In this study, we conducted site-specific O-glycosylation analysis of commercially available GLP1-Fc fusion protein with (G4S)3 linker peptide using liquid chromatography/mass spectrometry (LC/MS) with EThcD and a glycoproteomic database search. We successfully identified unexpected O-xylosylations at Ser residues in the (G4S)3 linker peptide, mucin-type O-glycosylations at Thr and Ser residues in the GLP-1 peptide, and Ser residues in the (G4S)3 linker peptide. This study is the first to report these unexpected O-xylosylations and mucin-type O-glycosylations in this therapeutic fusion protein. Mammalian-cell production of therapeutic fusion proteins that contain novel structures may require exhaustive O-glycosylation analysis to ensure their quality, efficacy, and safety.
Keywords: glucagon-like peptide-1, O-glycosylation, therapeutic Fc-fusion protein

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Harazono A, Shibata H, Kiyoshi M, Muto T, Fukuda J[*1], Torisu T[*2], Saitoh S[*3], Nishimura H[*4], Uchiyama S[*5], Ishii-Watabe A: Interlaboratory comparison about feasibility of insoluble particulate matter test

for injections with reduced test volume in light obscuration method. Biologicals.
*Biologicals*. 2019;57:46-49.

Insoluble particulate matter test for injections in pharmacopoeia is mandatory for parenteral drug products. In this test using light obscuration, four measurements of at least 5-mL are required. Since therapeutic protein injections of low dosage volumes are getting more popular, reduction of test volumes is desired. In this collaborative study, the impact of lower measurement volume on the accuracy and precision of particle count was evaluated using 2, 5, 10, and 25-μm polystyrene count standards for the validity of test with reduced sample volumes. Good accuracy (3000 particles/mL ± 10%) was obtained at all measurement volumes, and the inter-run variability (RSD) was the same levels between 5 and 1 mL. Although the inter-run variability increased at 0.2 mL, it was below 5%. These results indicated that light obscuration method can be used with 5 mL-0.2 mL, and that it is feasible for monitoring particles ≥2 μm.
Keywords: light obscuration, reduced-volume method, insoluble particulate matter test

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Yoshida K*1, Kuroda D*1,2, Kiyoshi M, Nakakido M*1, Nagatoishi S*1,3, Soga S*4, Shirai H*4, Tsumoto K*5,6,7: Exploring designability of electrostatic complementarity at an antigen-antibody interface directed by mutagenesis, biophysical analysis, and molecular dynamics simulations.
*Sci Rep*. 2019 14;9(1):4482.

Antibodies protect organisms from a huge variety of foreign antigens. Antibody diversity originates from both genetic and structural levels. Antigen recognition relies on complementarity between antigen-antibody interfaces. Recent methodological advances in structural biology and the accompanying rapid increase of the number of crystal structures of proteins have enabled atomic-level manipulation of protein structures to effect alterations in function. In this study, we explored the designability of electrostatic complementarity at an antigen-antibody interface on the basis of a crystal structure of the complex. We designed several variants with altered charged residues at the interface and characterized the designed variants by surface plasmon resonance, circular dichroism, differential scanning calorimetry, and molecular dynamics simulations. Both successes and failures of the structure-based design are discussed. The variants that compensate electrostatic interactions can restore the interface complementarity, enabling the cognate antigen-antibody binding. Retrospectively, we also show that these mutational effects could be predicted by the simulations. Our study demonstrates the importance of charged residues on the physical properties of this antigen-antibody interaction and suggests that computational approaches can facilitate design of antibodies that recognize a weakly immunogenic antigen.
Keywords: antibody, affinity, molecular dynamics

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Nishimura K, Shibata H, Aoyama M*1, Hosogi J*2, Kadotsuji K*3, Minoura K*4, Mori T*5, Nakamura T*6, Nishimiya K*7, Nomura T*6, Saito T*4, Soma M*8, Wakabayashi H*5, Sakamoto N*9, Niimi S, Katori N, Saito Y, Ishii-Watabe A: Elucidation of the statistical factors that influence anti-drug antibody cut point setting through a multi-laboratory study.
*Bioanalysis*. 2019;11(6):509-524.

Appropriateness of anti-drug antibody (ADA) assay is critical for immunogenicity assessment of biopharmaceuticals. Although cut point setting in ADA assay has a large impact on the results, a standard statistical approach for its setting has not been well established. In this multi-laboratory study, to elucidate factors influencing the cut point setting, we compared the statistical approaches and calculated cut points for multiple datasets of ADA assays using the individual

procedure employed at each laboratory. Conclusion: We showed that outlier exclusion, false-positive rate and investigating data distribution have the greatest impact on both screening and confirmatory cut points. Our results would be useful for industry researchers and regulators engaged in immunogenicity assessment of biopharmaceuticals.
Keyword: immunogenicity, anti-drug antibody assay, cut point

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Takemoto H*1, Takahashi J*1, Hyuga S*2, Odaguchi H*2, Uchiyama N, Maruyama T, Yamashita T*3, Hyuga M, Oshima N*4, Amakura Y*5, Hakamatsuka T, Goda Y, Hanawa T*2, Kobayashi Y*1: Ephedrine Alkaloids-Free Ephedra Herb extract, EFE, has no adverse effects such as excitation, insomnia and arrhythmias.

*Biol. Pharm. Bull.* 2018; 41: 247-53

Ephedrine alkaloids-free Ephedra Herb extract (EFE) has been developed to eliminate the adverse effects caused by ephedrine alkaloid-induced sympathetic hyperactivation. Previously, we reported that EFE possesses analgesic, anti-influenza, and cancer metastatic inhibitory effects at comparable levels to that of Ephedra Herb extract (EHE). However, it has not yet been demonstrated that EFE is free from the known side effects of EHE, such as excitation, insomnia, and arrhythmias. In this study, the incidence of these adverse effects was compared between mice administered EHE and those administered EFE. Increased locomotor activity in an open-field test, reduced immobility times in a forced swim test, and reduced sleep times in a pentobarbital-induced sleep test were observed in EHE-treated mice, when compared to the corresponding values in vehicle-treated mice. In contrast, EFE had no obvious effects in these tests. In electrocardiograms, atrial fibrillation (*i.e.*, irregular heart rhythm, absence of P waves, and appearance of f waves) was observed in the EHE-treated mice. It was suggested that this atrial fibrillation was induced by stimulation of adrenaline $\beta_1$ receptors, but not by hypokalemia. However, EFE did not affect cardiac electrophysiology. These results suggest that the abovementioned side effects are caused by ephedrine alkaloids in EHE, and that EFE is free from these adverse effects, such as excitation, insomnia, and arrhythmias. Thus, EFE is a promising new botanical drug with few adverse effects.
Keywords: Ephedra Herb, adverse effect, ephedrine alkaloid-free Ephedra Herb extract (EFE)

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Tokumoto H, Shimomura H, Hakamatsuka T, Ozeki Y*, Goda Y: Fluorescence coupled with macro and microscopic examinations of morphological phenotype give key characteristics for identification of crude drugs derived from scorpions.

*Biol. Pharm. Bull.* 2018;41:510-23

Microscopic examination of crude drug components has been the traditional method to identify the origin of biological materials. For the identification of components in a given mixture *via* microscopy, standard reference photographs of fragments derived from different organs and tissues of individual species are required. In addition to these reference photographs, a highly observant eye is needed to compare the morphological characteristics observed under the microscope with those of the references and to then identify the origin of the materials. Therefore, if other indexes are available to be coupled with microscope examination, the accuracy of identification would be significantly improved. Here, we prepared standard reference photographs for microscopic examination to identify powdered and fragmented materials in the crude drug "Quanxie" derived from individual organs of dried scorpion (*Buthus martensii* KARSCH). Since a remarkable characteristic of scorpion bodies is that they fluoresce under UV light, two methods to identify "Quanxie" were established,

including fluorescence fingerprint analysis and microscopic fluorescent luminance imaging analysis. In the former, at least 0.1 g of powered materials was used, which could be recovered after the measurement, and in the latter, only small amounts of powders were used for microscopic examinations. Both methods could distinguish powders of "Quanxie" from those of other micro-morphologically similar crude drugs, namely, "Chantui," "Sangpiaoxiao," and "Jiangcan." The combination of these methods should improve the swiftness and accuracy of "Quanxie" identification.

Keywords: *Buthus martensii*, fluorescence fingerprint, microscopic morphology

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Oshima N*[1], Yamashita T*[2], Uchiyama N, Hyuga S*[3], Hyuga M, Yang J*[2], Hakamatsuka T, Hanawa T*[3], Goda Y: Non-alkaloidal composition of Ephedra Herb is influenced by differences in habitats.

*J. Nat. Med.* 2019;73:303-11

Ephedra Herb is a crude drug defined as the terrestrial stem of *Ephedra sinica*, *E. intermedia*, or *E. equisetina*. It is often used to treat headaches, bronchial asthma, nasal inflammation, and the common cold. In this study, we isolated characteristic non-alkaloidal constituents from the extracts and identified them in relation to the habitat of Ephedra Herb. Extracts were prepared from Ephedra Herb collected from Inner Mongolia and Gansu. High-performance liquid chromatography was performed to quantitatively analyse the amount of ephedrine alkaloids in each extract. We compared the chemical compositions of the extracts by thin layer chromatography (TLC) to find spot characteristics depending on the habitat. $^{1}$H-NMR, $^{13}$C-NMR, and 2D-NMR spectra of the samples were also examined. The ephedrine content of all extracts satisfied the quality standard stated in the Japanese Pharmacopoeia. Nonetheless, we found each notable constituent characteristic to the Ephedra Herbs from both habitats. In order to identify them, Ephedra Herb extracts were separated by column chromatography, resulting in the isolation of (±)-α-terpineol-β-D-*O*-glucopyranoside (**1**) and (*E*)-7-hydroxy-3,7-dimethyloct-2-en-1-yl-β-D-*O*-glucopyranoside (**2**) as the characteristic constituents in Ephedra Herb from Inner Mongolia. Epheganoside (**3**), a new eudesmane-type sesquiterpene glycoside, and scopoletin (**4**) were found to be the characteristic constituents in Ephedra Herb from Gansu. The results obtained from this study can be used to distinguish between the habitats of Ephedra Herb.

Keywords: Ephedra Herb, habitat, terpenoid

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Tsujimoto T, Yoshitomi T, Maruyama T, Yamamoto Y*, Hakamatsuka T, Uchiyama N：$^{13}$C NMR-based metabolic fingerprinting of *Citrus*-type crude drug towards their quality control.

*J. Pharm. Biomed. Anal.*, 2018;161:305-12

Five Citrus-type crude drugs (40 samples) were classified using13C-NMR spectra-based metabolomics. The following eight metabolites were identified from the loading plots of multivariate analysis of the13C-NMR spectra; naringin, neohesperidin, narirutin, synephrine, sucrose, α-glucose, β-glucose, andlimonene.$^{13}$C-NMR spectra-based metabolic fingerprinting is a promising strategy for classifying crudedrugs.

Keywords: metabolomics, NMR, multivariate analysis

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Odaguchi H*[1], Sekine M*[1], Hyuga S*[1], Hanawa T*[1], Hoshi K*[2], Sasaki Y*[1], Aso M*[3], Yang J*[4], Hyuga M, Kobayashi Y*[5], Hakamatsuka T, Goda Y, Kumagai Y*[3]: A Double-Blind, Randomized, Crossover Comparative Study for Evaluating the Clinical Safety of Ephedrine Alkaloids-Free Ephedra Herb Extract (EFE).

*Evid Based Complement Alternat Med.* 2018;2018:4625358

Ephedra Herb is an important crude drug; it is used in various Traditional Japanese Medicine (Kampo) formulations. Its significant pharmacological effects have been believed to be attributed to ephedrine and pseudoephedrine, which sometimes induce adverse effects. On the other hand, it has been reported

that some of these pharmacological effects are not dependent on ephedrine or pseudoephedrine. Ephedrine alkaloids-free Ephedra Herb extract has been newly developed. It has been reported to have analgesic, anti-influenza, and antimetastatic effects. This clinical trial was aimed at verifying the noninferiority of EFE's safety compared to that of Ephedra Herb extract (EHE) in humans. This was a single-institution, double-blinded, randomized, two-drug, two-stage, crossover comparative study. Twelve healthy male subjects were equally and randomly allocated into two groups: prior administration of EFE (EFE-P) and prior administration of EHE (EHE-P). In Stage 1, EFE and EHE were orally administered to the EFE-P and EHE-P groups, respectively, for six days. After a 4-week washout period, Stage 2 was initiated wherein the subjects were given a study drug different from Stage 1 study drug for six days. Eleven adverse events with a causal relationship to the study drugs (EHE: 8; EFE: 3) were noted; all events were mild in severity. With regard to the incidence of adverse events, EHE and EFE administration, respectively, accounted for 4 cases (out of 12 subjects, similarly below) and 1 case of increased pulse rate (p=0.32) and 3 cases and 1 case of insomnia (p=0.59). Further, there was one case of hot flashes (p=1.00) due to EFE administration and one case of dysuria (p=1.00) due to EHE administration. There were no significant differences in the incidences of adverse events between EHE administration and EFE administration. Therefore, we concluded that EFE is not inferior to EHE in terms of safety.

Keywords: Ephedra Herb, clinical safety, ephedrine alkaloids-free Ephedra Herb extract

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Maeda H*, Nagashima E*, Hayashi Y. K*, Kikura-Hanajiri R, Yoshida K*. MDMB-CHMICA induces thrashing behavior, bradycardia, and slow pressor response in a $CB_1$- and $CB_2$-receptor-dependent manner in conscious rats.

*Forensic Toxicology*, 2018;36:313-9

MDMB-CHMICA, a new synthetic cannabinoid (SC), has become prevalent since 2014 as an ingredient of recreational drugs. Reports on intoxication due to the drug have been increasing, which show diverse cardiovascular, psychiatric, and neuronal symptoms. Reports on sudden death and accidental death related to psychiatric disorders in MDMB-CHMICA intoxication have also increased, but the underlying mechanisms are largely unknown. As there has been no experimental study on the drug, we investigated the effects of peripheral injection of MDMB-CHMICA in conscious rats. MDMB-CHMICA induced rapid bradycardia and a slow pressor response. Cardiovascular responses to other SCs have been shown to be inhibited only by cannabinoid receptor-1 ($CB_1$)-antagonists. However, the MDMB-CHMICA-induced bradycardia was inhibited not only by a $CB_1$-antagonist, AM281, but also by a $CB_2$-antagonist, AM630. Unlike other SCs, MDMB-CHMICA induced a gradual increase in mean blood pressure, which was marginally enhanced by the $CB_1$- and $CB_2$- antagonists. For the first time, we demonstrated that MDMB-CHMICA induces a thrashing hypermobile behavior in a $CB_1$- and $CB_2$-receptor-dependent manner, following catalepsy-like hypomobile behavior. This unexpected response to MDMB-CHMICA may help explain the mechanisms underlying the sudden deaths and accidents associated with its use.

Keywords: synthetic cannabinoid, bradycardia, thrashing hypermobile behavior

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Lysaght T*1, Munsie M*2, Castricum A*3, Hui JHP*4, Okada K*5, Sato Y, Sawa Y*5, Stewart C*6, Tan LK*4, Tan LHY*7, Sugii S*8: A roundtable on responsible innovation with autologous stem cells in Australia, Japan and Singapore.

*Cytotherapy* 2018 20(9):1103-09.

We report on a roundtable event hosted in Singapore that sought to identify some of the ethical and regulatory challenges in translating autologous cell-based interventions, particularly those claiming to involve stem cells, into safe and effective therapies and to propose some solutions to encourage responsible innovation with these products. Challenges are identified in the three areas of cell manufacturing

and processing, innovative uses of autologous cells in clinical practice and standards of evidence. Proposed solutions are discussed within a co-operative model of statutory laws and regulations that can enable product development with autologous cells and professional codes and standards that can encourage ethical conduct in clinical practice. Future research should be directed toward establishing regional networks for the development of internationally consistent standards in manufacturing and ethical codes of conduct for innovating with stem cells, and other autologous cells, and fostering ongoing exchange between jurisdictions.
Keywords: autologous cells, cell manufacturing and processing, safe and effective therapies

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Yasuda S, Kusakawa S, Kuroda T, Miura T, Tano K, Takada N, Matsuyama S, Matsuyama A*1, Nasu M*2, Umezawa A*2, Hayakawa T*3, Tsutsumi H*4, Sato Y: Tumorigenicity-associated characteristics of human iPS cell lines.
*PLOS ONE* 2018;13:e0205022.

Human induced pluripotent stem cells (hiPSCs) represent promising raw materials of human cell-based therapeutic products (hCTPs). As undifferentiated hiPSCs exhibit intrinsic tumorigenicity properties that enable them to form teratomas, hCTPs containing residual undifferentiated hiPSCs may cause tumor formation following transplantation. We first established quantitative and sensitive tumorigenicity testing of hiPSCs dissociated into single cells using NOD/Shi-scid IL2Rγnull (NOG) mice by inhibiting apoptosis of hiPSCs with a Rho kinase inhibitor. To examine different features in tumorigenicity of various hiPSCs, 10 commonly available hiPSC lines were subjected to in vivo tumorigenicity testing. Transplanted hiPSC lines showed remarkable variation in tumor incidence, formation latency, and volumes. Most of the tumors formed were classified as immature teratomas. However, no signs of malignancies, such as carcinoma and sarcoma, were recognized in the tumors. Characteristics associated tumorigenicity of hiPSCs were investigated with microarray analysis, karyotype analysis, and whole exome sequencing. Gene expression profiling and pathway analysis supported different features of hiPSC lines in tumorigenicity. hiPSC lines showed chromosomal abnormalities in some lines and 61-77 variants of cancer-related genes carrying effective nonsynonymous mutations, which were confirmed in the COSMIC databases. In this study, the chromosomal abnormalities and cancer-related gene mutations observed in hiPSC lines did not lead to the malignancy of tumors derived from hiPSCs. Our results suggest that the potential tumorigenicity risk of hCTPs containing residual undifferentiated hiPSCs is dependent on not only amounts of undifferentiated hiPSCs but also features of the cell lines used as raw materials, a finding that should be considered from the perspective of quality of hCTPs used.
Keywords: hCTP, hiPSC, tumorigenicity

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Nishimura A*1, Shimauchi T*1, Tanaka T*1, Shimoda K*1, Toyama T*1, Kitajima N*1, Ishikawa T*2, Shindo N*2, Numaga-Tomita T*1, Yasuda S, Sato Y, Kuwahara K*3, Kumagai Y*4, Akaike T*5, Ide T*6, Ojida A*2, Mori Y*7, Nishida M*1: Hypoxic stress-induced interaction of filamin/actin cytoskeleton with Drp1 causes mitochondrial hyperfission-associated myocardial senescence.
*Sci. Signal.* 2018;11:eaat5158.

Defective mitochondrial dynamics through aberrant interactions between mitochondria and actin cytoskeleton is increasingly recognized as a key determinant of cardiac fragility after myocardial infarction (MI). Dynamin-related protein 1 (Drp1), a mitochondrial

fission–accelerating factor, is activated locally at the fission site through interactions with actin. Here, we report that the actin-binding protein filamin A acted as a guanine nucleotide exchange factor for Drp1 and mediated mitochondrial fission–associated myocardial senescence in mice after MI. In peri-infarct regions characterized by mitochondrial hyperfission and associated with myocardial senescence, filamin A colocalized with Drp1 around mitochondria. Hypoxic stress induced the interaction of filamin A with the GTPase domain of Drp1 and increased Drp1 activity in an actin-binding–dependent manner in rat cardiomyocytes. Expression of the A1545T filamin mutant, which potentiates actin aggregation, promoted mitochondrial hyperfission under normoxia. Furthermore, pharmacological perturbation of the Drp1–filamin A interaction by cilnidipine suppressed mitochondrial hyperfission–associated myocardial senescence and heart failure after MI. Together, these data demonstrate that Drp1 association with filamin and the actin cytoskeleton contributes to cardiac fragility after MI and suggests a potential repurposing of cilnidipine, as well as provides a starting point for innovative Drp1 inhibitor development.

Keywords: Drp1, mitochondrial fission, myocardial infarction

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Ito E*, Miyagawa S*, Takeda M*, Kawamura A*, Harada A*, Iseoka H*, Yajima S*, Sougawa N*, Mochizuki-Oda N*, Yasuda S, Sato Y, Sawa Y*: Tumorigenicity assay essential for facilitating safety studies of hiPSC-derived cardiomyocytes for clinical application.
*Sci. Rep.* 2019;9:1881.

Transplantation of cardiomyocytes (CMs) derived from human induced pluripotent stem cells (hiPSC-CMs) is a promising treatment for heart failure, but residual undifferentiated hiPSCs and malignant transformed cells may lead to tumor formation. Here we describe a highly sensitive tumorigenicity assay for the detection of these cells in hiPSC-CMs. The soft agar colony formation assay and cell growth analysis were unable to detect malignantly transformed cells in hiPSC-CMs. There were no karyotypic abnormalities during hiPSCs subculture and differentiation. The hiPSC markers TRA1-60 and LIN28 showed the highest sensitivity for detecting undifferentiated hiPSCs among primary cardiomyocytes. Transplantation of hiPSC-CMs with a LIN28-positive fraction > 0.33% resulted in tumor formation in nude rats, whereas no tumors were formed when the fraction was < 0.1%. These findings suggested that combination of these *in vitro* and *in vivo* tumorigenecity assays can verify the safety of hiPSC-CMs for cell transplantation therapy.

Keywords: cardiomyocytes, hiPSC, tumorigenicity

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Kono K, Sawada R, Kuroda T, Yasuda S, Matsuyama S, Matsuyama A*1, Mizuguchi H*2, Sato Y: Development of selective cytotoxic viral vectors for concentration of undifferentiated cells in cardiomyocytes derived from human induced pluripotent stem cells.
*Sci. Rep.* 2019;9(1):3630.

Cell-processed therapeutic products (CTPs) derived from human pluripotent stem cells (hPSCs) have innovative applications in regenerative medicine. However, undifferentiated hPSCs possess tumorigenic potential; thus, sensitive methods for the detection of residual undifferentiated hPSCs are essential for the clinical use of hPSC-derived CTPs. The detection limit of the methods currently available is $1/10^5$ (0.001%, undifferentiated hPSCs/differentiated cells) or more, which could be insufficient for the detection of residual hPSCs when CTPs contain more than $1 \times 10^5$ cells. In this study, we developed a novel approach to overcome this challenge, using adenovirus and adeno-associated virus (AdV and AAV)-based selective cytotoxic vectors. We constructed AdV and AAV vectors that possess a suicide gene, iCaspase 9 (iCasp9), regulated by the CMV promoter, which is dormant in hPSCs, for the selective expression of

iCasp9 in differentiated cells. As expected, AdV/CMV-iCasp9 and AAV/CMV-iCasp9 exhibited cytotoxicity in cardiomyocytes but not in human induced pluripotent stem cells (hiPSCs). The vectors also induced apoptosis in hiPSC-derived cardiomyocytes, and the surviving cells exhibited higher levels of hPSC marker expression. These results indicate that the AdV- and AAV-based cytotoxic vectors concentrate cells expressing the undifferentiated cell markers in hiPSC-derived products and are promising biological tools for verifying the quality of CTPs.

Keywords: viral vectors, hiPSC-derived products, tumorigenicity

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Ohashi F*1, 2, 3, 4, Miyagawa S*1, Yasuda S, Miura T, Kuroda T, Itoh M*4, Kawaji H*4, 5, Ito E*1, Yoshida S*1, Saito A*1, Sameshima T*3, Kawai J*4, Sawa Y1, Sato Y: CXCL4/PF4 is a predictive biomarker of cardiac differentiation potential of human induced pluripotent stem cells.

*Scientific Reports*. 2019:9:4638

Selection of human induced pluripotent stem cell (hiPSC) lines with high cardiac differentiation potential is important for regenerative therapy and drug screening. We aimed to identify biomarkers for predicting cardiac differentiation potential of hiPSC lines by comparing the gene expression profiles of six undifferentiated hiPSC lines with different cardiac differentiation capabilities. We used three platforms of gene expression analysis, namely, cap analysis of gene expression (CAGE), mRNA array, and microRNA array to efficiently screen biomarkers related to cardiac differentiation of hiPSCs. Statistical analysis revealed candidate biomarker genes with significant correlation between the gene expression levels in the undifferentiated hiPSCs and their cardiac differentiation potential. Of the candidate genes, PF4 was validated as a biomarker expressed in undifferentiated hiPSCs with high potential for cardiac differentiation in 13 additional hiPSC lines. Our observations suggest that PF4 may be a useful biomarker for selecting hiPSC lines appropriate for the generation of cardiomyocytes.

Keywords: hiPSCs, differentiation propensity, CXCL4/PF4

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Yoshida T, Naito Y*1, Sasaki K, Uchida E, Sato Y, Naito M, Kawanishi T, Obika S*2, Inoue T: Estimated number of off-target candidate sites for antisense oligonucleotides in human mRNA sequences.

*Genes Cells* 2018:23,448-455

Antisense oligonucleotide (ASO) therapeutics are single stranded oligonucleotides which bind to RNA through sequence-specific Watson-Crick base pairings. A unique mechanism of toxicity for ASOs is hybridization-dependent off-target effects that can potentially occur due to the binding of ASOs to complementary regions of unintended RNAs. To reduce the off-target effects of ASOs, it would be useful to know the approximate number of complementary regions of ASOs, or off-target candidate sites of ASOs, of a given oligonucleotide length and complementarity with their target RNAs. However, the theoretical number of complementary regions with mismatches has not been reported to date. In this study, we estimated the general number of complementary regions of ASOs with mismatches in human mRNA sequences by mathematical calculation and *in silico* analysis using several thousand hypothetical ASOs. By comparing the theoretical number of complementary regions estimated by mathematical calculation to the actual number obtained by *in silico* analysis, we found that the number of complementary regions of ASOs could be broadly estimated by the theoretical number calculated mathematically. Our analysis showed that the number of complementary regions increases dramatically as the number of tolerated mismatches increases, highlighting the need for expression analysis of such genes to assess the safety of ASOs

Keywords: antisense oligonucleotides, hybridization,

off-target

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Nagasaka M*, Hashimoto R*, Inoue Y*, Ishiuchi K*, Matsuno M*, Itoh Y*, Tokunaga M*, Ohoka N, Morishita D*, Mizukami H*, Makino T*, Hayashi H*: Anti-Tumorigenic Activity of Chrysin from *Oroxylum indicum* via Non-Genotoxic p53 Activation through the ATM-Chk2 Pathway.
*Molecules*. 2018:23:pii: E1394.

The p53 tumor suppressor plays critical roles in cell cycle regulation and apoptotic cell death in response to various cellular stresses, thereby preventing cancer development. Therefore, the activation of p53 through small molecules is an attractive therapeutic strategy for the treatment of cancers retaining wild-type p53. We used a library of 700 Myanmar wild plant extracts to identify small molecules that induce p53 transcriptional activity. A cell-based screening method with a p53-responsive luciferase-reporter assay system revealed that an ethanol extract of *Oroxylum indicum* bark increased p53 transcriptional activity. Chrysin was isolated and identified as the active ingredient in the *O. indicum* bark extract. A treatment with chrysin increased p53 protein expression and the p53-mediated expression of downstream target genes, and decreased cell viability in MCF7 cells, but not in p53-knockdown MCF7 cells. We also found that chrysin activated the ATM-Chk2 pathway in the absence of DNA damage. Hence, the inactivation of the ATM-Chk2 pathway suppressed p53 activation induced by chrysin. These results suggest the potential of chrysin as an anti-cancer drug through the activation of p53 without DNA damage.
Keywords: ATM, Chk2, p53

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Kumar S*1, Muthuselvam P*1, Pugalenthi V*1, Subramanian N*1, Ramkumar KM*2, Suresh T, Suzuki T, Rajaguru P*1: Toxicoproteomic analysis of human lung epithelial cells exposed to steel industry ambient particulate matter (PM) reveals possible mechanism of PM related carcinogenesis.
*Environ Pollut*., 2018: 239, 483-492.

Toxicoproteomic analysis of steel industry ambient particulate matter (PM) that contain high concentrations of PAHs and metals was done by treating human lung cancer cell-line, A549 and the cell lysates were analysed using quantitative label-free nano LC-MS/MS. Enrichment analyses revealed that proteins associated to redox homeostsis, metabolism, and cellular energy generation were inhibited while, proteins related to DNA damage and repair and other stresses were over expressed. Altered activities of several tumor associated proteins were observed. Together it could be inferred that PM exposure induced oxidative stress which could have lead into DNA damage and tumor related changes.
Keywords: toxicoproteomics, oxidative stress, ambient particulate matter

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Shibata N, Shimokawa K*, Nagai K*, Ohoka N, Hattori T, Miyamoto N*, Ujikawa O*, Sameshima T*, Nara H*, Cho N*, Naito M: Pharmacological difference between degrader and inhibitor against oncogenic BCR-ABL kinase.
*Scientific Reports* 2018:8,13549.

Chronic myelogenous leukemia (CML) is characterized by the oncogenic fusion protein, BCR-ABL protein kinase, against which clinically useful inhibitors have been developed. An alternative approach to treat CML is to degrade the BCR-ABL protein. Recently, potent degraders against BCR-ABL have been developed by conjugating dasatinib to ligands for E3 ubiquitin ligases. Since the degraders contain the dasatinib moiety, they also inhibit BCR-ABL kinase activity, which complicates our understanding of the impact of BCR-ABL degradation by degraders in CML growth inhibition. To address this issue, we chose DAS-IAP, as a potent BCR-ABL degrader, and developed a structurally related inactive degrader, DAS-meIAP, which inhibits kinase activity but does not degrade the BCR-ABL protein. DAS-IAP showed slightly weaker activity than DAS-meIAP in inhibiting cell growth when CML cells were treated for 48h. However, DAS-IAP showed sustained growth inhibition even when the drug was removed after short-term treatment, whereas CML cell growth rapidly resumed following

removal of DAS-meIAP and dasatinib. Consistently, suppression of BCR-ABL levels and downstream kinase signaling were maintained after DAS-IAP removal, whereas kinase signaling rapidly recovered following removal of DAS-meIAP and dasatinib. These results indicate that BCR-ABL degrader shows more sustained inhibition of CML cell growth than ABL kinase inhibitor.

Keywords: BCR-ABL, dasatinib, ubiquitin-proteasome system

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Hattori T, Watanabe-Takahashi M*, Nishikawa K*, and Naito M: Acquired Resistance to Shiga Toxin-Induced Apoptosis by Loss of CD77 Expression in Human Myelogenous Leukemia Cell Line, THP-1.

*Biological & pharmaceutical bulletin*. 2018:41:1475-1479.

Shiga toxin (Stx) is a main virulence factor of Enterohemorrhagic Escherichia coli (EHEC) that causes diarrhea and hemorrhagic colitis and occasionally fatal systemic complications. Stx induces rapid apoptotic cell death in some cells, such as human myelogenous leukemia THP-1 cells expressing CD77, a receptor for Stx internalization, and the induction of apoptotic cell death is thought to be crucial for the fatal systemic complications. Therefore, in order to suppress the fatal toxicity, it is important to understand the mechanism how cells can escape from apoptotic cell death in the presence of Stx. In this study, we isolated resistant clones to Stx-induced apoptosis from highly sensitive THP-1 cells by continuous exposure with lethal dose of Stx. All of the ten resistant clones lost the expression of CD77 as a consequence of the reduction in CD77 synthase mRNA expression. These results suggest that downregulation of CD77 or CD77 synthase expression could be a novel approach to suppress the fatal toxicity of Stx in EHEC infected patient.

Keywords: CD77 synthase, Shiga toxin, Shiga toxin receptor CD77

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*日本輸血細胞治療学会誌*, 2018, 64(3):502-509.

厚生労働省の血漿分画製剤の安全性確保対策の小委員会では，国内で使用されている輸血用血液製剤と血漿分画製剤の原料となる血漿に対するウイルス核酸増幅検査（NAT）の精度管理等に使用する国内標準品を1999年より作製し，国立感染症研究所が交付している．HCV，HBV及びHIVの第１次NAT国内標準品は，当時のWHO国際共同研究に準じエンドポイント法によって国際標準品に対する相対力価が定められた．2014年にNATガイドラインの改正と輸血用血液スクリーニングへの個別NAT導入に伴うNAT感度の改正が行われ，より厳格な精度管理に合わせ，NAT国内標準品の再評価の必要性が高まった．そこで，NAT国内標準品の力価を多施設共同研究にて再評価した．最新の高精度のリアルタイムPCR定量法で測定した結果，第１次HBV-DNA 国内標準品1,060,000 IU/mL，第１次HIV-RNA国内標準品75,000 IU/mL，第１次HCV-RNA国内標準品260,000 IU/mLに力価が改正された．信頼性の高い国際単位に校正された国内標準品を活用することで，NATの精度管理や試験法の改良の進展が期待される．

Keywords: human blood product，NAT，Japanese National Standard

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Furihata C, Toyoda T, Ogawa K, Suzuki T: Using RNA-Seq with 11 marker genes to evaluate 1,4-dioxane compared with typical genotoxic and non-genotoxic rat hepatocarcinogens.

*Mutat Res*., 2018: 834, 51-55.

The present study aimed to evaluate rat genotoxic hepatocarcinogen (GTHCs) and non-genotoxic hepatocarcinogens (NGTHCs) via selected gene expression patterns in the liver, as determined by next generation sequencing-targeted mRNA sequencing (RNA-Seq) and principal component analysis

(PCA). Previously, we selected 11 marker genes to discriminate GTHCs and NGTHCs. In the present study, we quantified changes in the expression of these genes following 1,4-dioxane (DO) treatment, and compared them with treatment with two typical rat GTHCs, N-nitrosodiethylamine (DEN) and 3,3'-dimethylbenzidine·2HCl (DMB), and a typical rat NGTHC, di(2-ethylhexyl)phthalate (DEHP). RNA-Seq was conducted on liver samples from groups of five male F344 rats after 4 weeks' feeding of chemicals. Significant changes in gene expression in experimental groups compared with the control group were observed in eight genes (Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Phlda3 and Plk2). Gene expression profiles of the 11 genes under DO treatment differed significantly from those with DEN and DMB, as well as DEHP. The present results suggest that RNA-Seq and PCA are useful to evaluate rat typical GTHCs and typical NGTHCs. DO was suggested to result in a different intermediate gene expression profile from typical GTHCs and NGTHC.

Keywords: toxicogeonomics, 1,4-dioxane, next generation sequencer

Nagasaka M*, Tsuzuki K*, Tokunaga M*, Ohoka N, Inoue Y*, Hayashi H*: Lysine-Specific Demethylase 1 (LSD/KDM1A) Is a Novel Target Gene of c-Myc.
*Bio Pharm Bull.* 2019:42:481-8.

Lysine-specific demethylase 1 (LSD1/KDM1A) is a histone demethylase and specifically catalyzes the demethylation of mono- and di-methylated histone H3 lysine 4 (H3K4). The LSD1-mediated demethylation of H3K4 promotes the assembly of the c-Myc-induced transcription initiation complex. Although LSD1 and c-Myc are both strongly expressed in human cancers, the mechanisms by which their activities are coordinated remain unclear. We herein demonstrated that LSD1 is a direct target gene of c-Myc. The knockdown of c-Myc decreased the expression of LSD1 in several cancer cell lines. We identified two non-canonical E-boxes in the proximal promoter region of the LSD1 gene. A chromatin immunoprecipitation assay showed that c-Myc bound to these E-boxes in the LSD1 promoter. Importantly, LSD1 mRNA expression correlated with c-Myc expression in human acute myeloid leukemia (AML), glioblastoma, stomach adenocarcinoma, and prostate adenocarcinoma. The present results suggest that LSD1 is induced by c-Myc and forms a positive feedback mechanism in transcription reactions by c-Myc.

Keywords: The Cancer Genome Atlas (TCGA) database, c-Myc, lysine-specific demethylase 1

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Furihata C, Suzuki T: Evaluation of 12 mouse marker genes in rat toxicogenomics public data, Open TG-GATEs: Discrimination of genotoxic from non-genotoxic hepatocarcinogens.
*Mutat Res.*, 2019: 838, 9-15.

Previously, we proposed 12 marker genes (Aen, Bax, Btg2, Ccnf, Ccng1, Cdkn1a, Gdf15, Lrp1, Mbd1, Phlda3, Plk2 and Tubb4b) to discriminate mouse genotoxic hepatocarcinogens (GTHC) from non-genotoxic hepatocarcinogens (NGTHC). For this paper, we conducted an application study of the 12 mouse marker genes to rat data, Open TG-GATEs. We analyzed five typical rat GTHC, and not only seven typical rat NGTHC but also 11 non-genotoxic non-hepatocarcinogens (NGTNHC) from Open TG-GATEs. GTHC-specific dose-dependent gene expression changes were observed and significance assessed with the Williams test. Similar significant changes were observed during 3-24h and 4-29 days, assessed with Welch's t-test, except not for NGTHC or NGTNHC. Significant differential changes in gene expression were observed between GTHC and NGTHC in 11 genes (except not Tubb4b) and between GTHC and NGTNHC in all 12 genes at 24h and 10 genes (except Ccnf and Mbd1) at 29 days, per Tukey's test. PCA successfully discriminated GTHC from NGTHC and NGTNHC at 24h and 29 days. The results demonstrate that 12 previously proposed mouse marker genes are useful for discriminating rat GTHC from NGTHC and NGTNHC from Open TG-GATEs.

Keywords: toxicogeonomics, Open TG-GATEs, PCA

Ohoka N, Ujikawa O*, Shimokawa K*, Sameshima T*, Shibata N, Hattori T, Nara H*, Cho N*, Naito M: Different Degradation Mechanisms of Inhibitor of Apoptosis Proteins (IAPs) by the Specific and Nongenetic IAP-Dependent Protein Eraser (SNIPER).
*Chem Pharm Bull.* 2019:67:203-9.

Targeted protein degradation by small molecules is an emerging modality with significant potential for drug discovery. We previously developed chimeric molecules, termed specific and non-genetic inhibitor of apoptosis protein (IAP)-dependent protein erasers (SNIPERs), which induce the ubiquitylation and proteasomal degradation of target proteins. This degradation is mediated by the IAPs; the target proteins include bromodomain-containing protein 4 (BRD4), an epigenetic regulator protein. The SNIPER that degrades this particular protein, SNIPER(BRD)-1, consists of an IAP antagonist LCL-161 derivative and a bromodomain and extra-terminal (BET) inhibitor, (+)-JQ-1. SNIPER(BRD)-1 also degrades a cellular inhibitor of apoptosis protein 1 (cIAP1) and an X-linked inhibitor of apoptosis protein (XIAP), the mechanisms of which are not well understood. Here, we show that the degradation of cIAP1 and XIAP by SNIPER(BRD)-1 is induced via different mechanisms. Using a chemical biology-based approach, we developed two inactive SNIPERs, SNIPER(BRD)-3 and SNIPER(BRD)-4, incapable of degrading BRD4. SNIPER(BRD)-3 contained an N-methylated LCL-161 derivative as the IAP ligand, which prevented it from binding IAPs, and resulted in the abrogated degradation of cIAP1, XIAP, and BRD4. SNIPER(BRD)-4, however, incorporated the enantiomer (-)-JQ-1 which was incapable of binding BRD4; this SNIPER degraded cIAP1 but lost the ability to degrade XIAP and BRD4. Furthermore, a mixture of the ligands, (+)-JQ-1 and LCL-161, induced the degradation of cIAP1, but not XIAP and BRD4. These results indicate that cIAP1 degradation is triggered by the binding of the IAP antagonist module to induce autoubiquitylation of cIAP1, whereas a ternary complex formation is required for the SNIPER-induced degradation of XIAP and BRD4.

Keywords: E3 ligase, chimeric molecule, proteasome

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Morishita Y, Nomura Y, Fukui C, Fujisawa A[*1], Watanabe K[*2], Fujimaki H[*2], Kumada H[*3], Inoue K, Morikawa T, Takahashi M, Kawakami T, Sakoda H, Mukai T[*4], Yuba T[*4], Inamura K[*4], Tanoue A[*5], Miyazaki K[*6], Chung UI[*1], Ogawa K, Yoshida M, Haishima Y: Alternative plasticizer, 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester, for blood containers with protective effects on red blood cells and improved cold resistance.

*J Biomed Mater Res Part B*. 2018;106:1052-63.

Di (2-ethylhexyl) phthalate (DEHP), a typical plasticizer used for polyvinyl chloride (PVC), is eluted from PVC-made blood containers and protects against red blood cell (RBC) hemolysis. However, concerns have arisen regarding the reproductive and developmental risks of DEHP in humans, and the use of alternative plasticizers for medical devices has been recommended worldwide. In this study, we propose that the use of a novel plasticizer, 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester (DL9TH), could help produce more useful and safe blood containers. PVC sheet containing DL9TH and di (2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTH) provides comparable or superior protective effects to RBCs relative to PVC sheet containing DEHP or di-isononyl-cyclohexane-1,2-dicarboxylate (DINCH®, an alternative plasticizer that has been used in PVC sheets for blood containers). The total amount of plasticizer eluted from DOTH/DL9TH-PVC sheets is nearly the same as that eluted from DEHP-PVC sheets. In addition, DOTH/DL9TH-PVC has better cold resistance than DEHP- and DINCH® -PVC sheets. In vitro and in vivo tests for biological safety based on International Organization for Standardization guidelines (10993 series) suggest that the DOTH/DL9TH-PVC sheet can be used safely. Subchronic toxicity testing of DL9TH in male rats in accordance with the principles of Organisation for Economic Co-operation and Development Test Guideline 408 showed that DL9TH did not induce adverse effects up to the highest dose level tested (717 mg/kg body weight/day). There were no effects on testicular histopathology and sperm counts, and no indications of endocrine effects: testosterone, thyroid-stimulating hormone, follicle-stimulating hormone, and 17$\beta$-estradiol were unchanged by the treatment, compared with the control group.

Keywords: 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester, alternative plasticizer, blood container

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De Jong WH*1, Hoffmann S*2, Lee M*3, Kandárová H*3, Pellevoisin C*5, Haishima Y, Rollins B*6, Zdawczyk A*7, Willoughby J*8, Bachelor M*9, Schatz T*10, Skoog S*11, Parker S*12, Sawyer A*13, Pescio P*14, Fant K*15, Kim KM*16, Kwon JS*16, Gehrke H*17, Hofman-Hüther H*18, Meloni M*18, Julius C*19, Briotet D*20, Letasiova S*4, Kato R, Miyajima A, De La Fonteyne LJJ*21, Videau C*5, Tornier C*5, Turley AP*3, Christiano N*22, Rollins TS*3, Coleman KP*23: Round Robin study to evaluate the Reconstructed Human Epidermis (RhE) model as an in vitro skin irritation test for detection of irritant activity in medical device extracts.

*Toxicol In Vitro*, 2018;50:439-49.

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

Keywords: medical device, irritation, alternative testing

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Nomura Y, Toida H, Fukui C, Kai S*1, Nakaoka R, Kato R, Uematsu M, Ono K*2, Kanai A*2, Haishima Y: Evaluation of pigment distribution and depth analysis methods for decorative soft contact lenses.

*Eye Contact Lens*. 2018;44:S105-12.

Objectives: This study evaluates pigment component distribution and depth in decorative soft contact lenses (DSCLs) using a variety of analytical methods.

Methods: We sampled 18 DSCLs using optical microscopy, optical coherence tomography (OCT) analysis, Z-stack analysis, X-ray photoelectron spectroscopy (XPS), scanning electron microscopy/energy-dispersive X-ray spectroscopy (SEM/EDX), and time-of-flight secondary ion mass spectrometry

（TOF-SIMS）to evaluate the distribution and depth of pigment components.

Results: Pigment distribution in DSCLs was easily observed with optical methods including Z-stack analysis. XPS, SEM/EDX, and TOF-SIMS were used to evaluate the level of pigment exposure on the lens surface and the results showed significant differences between the methods. Pigment components were detected in 16 samples by SEM/EDX, but not by XPS. Pigment components were only detected in 8 samples using TOF-SIMS.

Conclusions: It may be necessary to show that a nanometer-thick monomolecular film does not exist on the surface of DSCLs, to demonstrate the exposure of a pigment particle. Taking into account the principle behind each of the measurement methods and the resolution and sensitivity of each of the analytical methods compared, TOF-SIMS may be the most appropriate method to accurately judge pigment exposure on DSCLs. The Z-stack method may be useful for estimating the depth of pigment components in DSCLs.

Keywords: decorative soft contact lens, pigment component, monomolecular film

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Nomura Y, Lee M*1, Fukui C, Watanabe K*2, Olsen D*1, Turley A*1, Morishita Y, Kawakami T, Yuba T*3, Fujimaki H*2, Inoue K, Yoshida M, Ogawa K, Haishima Y: Proof of concept testing of a positive reference material for in vivo and in vitro skin irritation testing. *J Biomed Mater Res Part B*. 2018;106:2807-14.

In vivo and in vitro irritation testing is important for evaluating the biological safety of medical devices. Here, the performance of positive reference materials for skin irritation testing was evaluated. Four reference standards, referred to as Y-series materials, were analyzed: a polyvinyl chloride (PVC) sheet spiked with 0 (Y-1), 1.0 (Y-2), 1.5 (Y-3), or 10 (Y-4) parts of Genapol X-080 per 100 parts of PVC by weight. Y-1, Y-2, and Y-3 did not induce skin irritation responses in an in vitro reconstructed human epidermis (RhE) tissue model, as measured by tissue viability or interleukin-1 $\alpha$ release, or in an in vivo intracutaneous response test using rabbits. In contrast, Y-4 extracts prepared with saline or sesame oil at 37℃ and 50℃ clearly elicited positive irritation responses, including reduced viability (< 50%) and significantly higher interleukin-1 $\alpha$ release compared with the solvent alone group, in the RhE tissue model and an intracutaneous response test, where substantial necrosis was observed by histopathology. The positive skin irritation responses induced in vitro under various extraction conditions, as well as those elicited in vivo, indicate that Y-4 is an effective extractable positive control material for in vivo and in vitro skin irritation tests of medical devices.

Keywords: irritation test, positive control, biological safety evaluation

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Miyajima A, Kuroda Y, Sakemi-Hoshikawa K, Usami M, Mitsunaga K*1, Irie T, Y Ohno Y*2, Sunouchi M: In vitro metabolism of 4-methyl- and 5-methyl-2-mercaptobenzimidazole, thyrotoxic and hepatotoxic rubber antioxidants, in rat liver microsomes. *Fundam. Toxicol. Sci.* 2018;5:113–6.

The metabolism of 4-methyl-2-mercaptobenzimidazole (4-MeMBI), 5-methyl-2-mercaptobenzimidazole (5-MeMBI), and 2-mercaptobenzimidazole (MBI) was examined in vitro in rat liver microsomes. The test chemicals were incubated in the presence of liver microsomes from male Sprague-Dawley rats and their metabolism was analyzed by HPLC. The increase in metabolism was dependent on the incubation duration and was similar among the test chemicals. SKF-525A, a non-selective inhibitor of cytochrome P450 (CYP) enzymes, decreased the metabolism rate of all the test chemicals, which indicated the involvement of liver microsomal CYP enzymes. When liver microsomes from rats treated with CYP-inducers (ß-naphthoflavone, phenobarbital, and isoniazid) were used, 4-MeMBI resulted in a greater decrease than 5-MeMBI, particularly in the phenobarbital-treated group. These results, together with the reported inducibility of the drug-metabolizing activity of the test chemicals, partly explained the counteraction of the toxic effects of 4-MeMBI and 5-MeMBI in the in vivo study.

Keywords: methyl-2-mercaptobenzimidazole, in vitro metabolism, cytochrome P450

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追田秀行，岡本吉弘，菅野伸彦*：抜去された親水性表面処理ライナーの表面解析．
*臨床バイオメカニクス* 2018;39:49-53.

摺動面に親水性表面処理を施した人工股関節ライナーは，*in vitro*摩耗試験や，5年の臨床成績で，極めて良好な耐摩耗性が報告されている．しかし，数年以内の表面処理層の消失を示唆する臨床報告も少数ながら存在する．従って，抜去インプラント解析による*in vivo*での処理層の耐久性評価と摩耗特性への影響を調査する必要があるが，その方法は充分に確立されていない．

本研究では，抜去されたライナー1例を対象に，表面観察，表面元素分析，接触角測定，表面粗さ測定，フーリエ変換赤外分光分析を行い，表面処理層の残存状態と摩耗量について分析すると共に，その評価法の有用性について検討した．

その結果，いずれの測定法でも，親水性表面処理層の非荷重部での残存と荷重部での消失が示唆され，処理層の残存状態の評価に有用であることが示された．また，表面粗さ測定では，残存する機械加工痕の山高さから摩耗量の推定が可能と考えられた．

Keywords: total hip replacement, contact angle, surface roughness, element analysis, wear

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Sakoda H, Osaka Y*, Uetsuki K*, Okamoto Y, Haishima Y: Evaluating the durability of UHMWPE biomaterials used for articulating surfaces of joint arthroplasty using delamination tests.
*J Biomed Mater Res PartB*. 2019;107:65-72.

Ultra-high molecular weight polyethylene (UHMWPE) is the most popular material used for the articulating surface of joint replacements. Delamination is a common fatigue-related failure mode in UHMWPE components; however, the relationship between delamination resistance and fatigue crack growth has not been reported. Here, the delamination resistance of contemporary UHMWPE materials, including highly cross-linked UHMWPE (HXLPE), vitamin E blended UHMWPE (VEPE), and vitamin E blended HXLPE (VEXLPE), was measured to verify a previously proposed accelerated test method using a U-shaped sliding motion; the results were compared with those of fatigue crack growth tests. The oxidative stability of each material was estimated using Fourier transform infrared analysis. UHMWPE sterilized by gamma irradiation in an inert atmosphere and annealed HXLPE had lower delamination resistance than virgin UHMWPE after artificial aging. This was consistent with previous findings from retrieval studies, and *in vitro* knee simulator and ball-on-flat unidirectional reciprocation wear studies. In contrast, remelted HXLPE, VEPE, and VEXLPE showed excellent delamination resistance after artificial aging. The results of the delamination tests were not consistent with those of fatigue crack growth tests, indicating the complex delamination mechanism and importance of evaluating these factors separately.

Keywords: delamination, fatigue property, ball-on-flat wear test

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Kawakami T, Isama K*, Ikarashi Y: Determination of benzotriazole UV absorbers in textile products made of polyurethane fibers by high-performance liquid chromatography with a photo diode array detector
*J Liq Chromatogr Relat Technol*, 2018;41:831-8.

In this study, we aimed to develop a simple method for the simultaneous analysis of seven benzotriazole (BZT) UV absorbers that are commonly used in urethane fiber products. Acetone/chloroform shaking extraction, tetrahydrofuran dissolution, and acetone/hexane soaking extraction methods were examined, and acetone/hexane soaking extraction was used for analysis because its operation was simply and the low-toxicity and small consumption volume of extraction solvents. All the BZT UV absorbers investigated in this study were subjected to qualitative/quantitative analysis by high-performance liquid-chromatography with a photodiode array detector. To avoid interference by co-elution substances, a wavelength of 340 nm was used for quantification. Good linearity of standard curve of every chemical was observed in the concentration range of 0.02–10 μg/mL. Sufficient reproducibility was achieved because their recoveries and its variance of

coefficients were good values. Furthermore, limits of detection and limits of quantification obtained from recovery tests were 0.095 to 0.56 μg/g and 0.29 to 1.7 μg/g, respectively. Among the 39 polyurethane textile products analyzed, only 2-(2'-hydroxy-5'-methylphenyl)benzotriazole (UV-P), 2-(2'-hydroxy-3'-tert-butyl-5'-methylphenyl)-5-chlorobenzotriazole (UV-326), and 2-(2'-hydroxy-3',5'-di-α-cumylphenyl)-2H-benzotriazole (UV-234) were detected in 2 samples (1.2 to 1.7 μg/g), 8 samples (0.62 to 384 μg/g), and 16 samples (trace amounts to 935 μg/g), respectively.

Keywords: high-performance liquid-chromatograph photodiode array detector, polyurethane fiber, benzotriazole UV absorber

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田原麻衣子，杉本直樹，香川（田中）聡子[*1]，酒井信夫，五十嵐良明，神野透人[*2]：ホルムアルデヒド及びアセトアルデヒドの定量分析におけるqNMRを用いたトレーサビリティの確保.

*YAKUGAKU ZASSHI* 2018;138:551–7.

Currently, indoor air quality guidelines for formaldehyde and acetaldehyde are set by the Ministry of Health, Labour and Welfare of Japan. Aldehydes are widely used in adhesives and preservatives, and exposure to these compounds via indoor air is a matter of concern. Considering that contact with indoor air is part of daily life, evaluation of indoor air quality is extremely important. 2,4-Dinitrophenylhydrazine (DNPH) derivatization is widely used for quantitative analysis of aldehydes. A certified reference material with traceability to the International System of Units (SI) is required for this method. However, currently, there are no certified reference materials available for aldehyde-DNPH derivatives, which means that the quantified values obtained by this method are not sufficiently reliable. In this study, we determined the actual content and purity of commercially available aldehyde-DNPH derivatives using $^{1}$H-quantitative NMR (qNMR), which can be measured with SI-traceability. Although the commercial DNPH derivatives of formaldehyde and acetaldehyde were low concentration solutions, we were able to determine their purities using $^{1}$H-qNMR. Furthermore, we were able to separate and quantify the acetaldehyde isomers generated by the derivatization reaction. In conclusion, it is possible to obtain highly accurate results using $^{1}$H-qNMR with commercially available reagents that are not certified metrologically.

Keywords: aldehyde, quantitative NMR, International System of Units traceability

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Fujita M[*1], Yamamoto Y[*1], Watanabe S[*2], Sugawara T[*2], Wakabayashi K[*3], Tahara Yu[*3], Horie N[*4], Fujimoto K[*4], Kusakari K[*5], Kurokawa Y[*5], Kawakami T, Kojima K[*6], Kojima H, Ono A[*7], Katsuoka Y[*1], Tanabe H[*1], Yokoyama H[*1], Kasahara T[*1]: Cause of and countermeasures for oxidation of the cysteine-derived reagent used in the amino acid derivative reactivity assay

*J Appl Toxicol*, 2019;39:191-208.

The amino acid derivative reactivity assay (ADRA) is an in chemico alternative to animal testing for skin sensitization that solves certain problems found in the use of the direct peptide reactivity assay (DPRA). During a recent validation study conducted at multiple laboratories as part of the process to include ADRA in an existing OECD test guideline, one of the nucleophilic reagents used in ADRA —*N*-(2-(1-naphthyl)acetyl)-L-cysteine(NAC)— was found to be susceptible to oxidation in much the same manner that the cysteine peptide used in DPRA was. Owing to this, we undertook a study to clarify the cause of the promotion of NAC oxidation. In general, cysteine and other chemicals that have thiol groups are known to oxidize in the presence of even minute quantities of metal ions. When metal ions were added to the ADRA reaction solution, $Cu^{2+}$ promoted NAC oxidation significantly. When 0.25 μM of EDTA was added in the presence of $Cu^{2+}$, NAC oxidation was suppressed. Based on this, we predicted that the addition of EDTA to the NAC stock solution would suppress NAC oxidation. Next, we tested 82 chemicals used in developing ADRA to determine whether EDTA affects ADRA's ability to predict sensitization. The results showed that the addition of EDTA has virtually no effect on the reactivity of NAC with a test chemical, yielding an accuracy of 87% for predictions of skin sensitization, which was roughly the same as ADRA.

Keywords: ADRA（amino acid derivative reactivity assay), in chemico alternative to animal testing, skin sensitization

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小林憲弘，土屋裕子，堀池秀樹*，増田潤一*，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141農薬の一斉分析法の開発.
*水環境学会誌* 2019;42:13–25.

水道水中の農薬は，検査対象項目数が多く検査方法が多岐にわたっており，検査の労力が非常に大きいことから，水道水をLC/MS/MSに直接注入して一斉分析する方法を検討し，141農薬の一斉分析条件を確立できた．さらに，一斉分析条件が確立できた農薬について水道水への添加回収試験を行い，その分析精度について評価を行った．その結果，アスコルビン酸ナトリウムおよびチオ硫酸ナトリウムのどちらの脱塩素処理剤を用いて残留塩素を除去した場合も，目標値の１/100の添加濃度において126農薬が定量可能であり，そのうち120農薬について良好な検査精度が得られたことから，本分析法はこれらの農薬の水道水質検査に適用可能と考えられる．ただし，一部の農薬については，添加した脱塩素処理剤により試験結果に違いが見られため，残留塩素を除去して検査する場合には，検査対象農薬によって脱塩素処理剤を適切に選択する必要がある．

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

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久保田晶子*，岡部亮*，柿本洋一郎*，根本了，青柳光敏*：LC-MS/MSを用いた畜産物中のヘキサジノンおよび主要代謝物の分析法.
*食品衛生学雑誌* 2018;59(4):167-173

A method for the determination of hexazinone and three metabolites (hexazinone metabolite B, hexazinone metabolite C, hexazinone metabolite F) in livestock products by LC-MS/MS was developed. Hexazinone and the three metabolites were extracted from a sample with acetonitrile in the presence of *n*-hexane, and lipid was removed by acetonitrile/*n*-hexane partition. The acetonitrile extract was cleaned up using a SAX/PSA cartridge column. Average recoveries (n = 5) of hexazinone and the three metabolites from cattle meat, fat, liver and milk spiked at the maximum residue limits (MRLs) or at 0.0025 mg/kg ranged from 85.6 to 96.0 %, and the relative standard deviations ranged from 0.8 to 4.9% .

Keywords : hexazinone, livestock, LC-MS/MS

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Saito-Shida S, Shiono K, Narushima J, Nemoto S, Akiyama H: Determination of total florfenicol residues as florfenicol amine in bovine tissues and eel by liquid chromatography-tandem mass spectrometry using external calibration.
*J Chromatogr B.* 2019;1109:37-44

A reliable liquid chromatography-tandem mass spectrometry method was developed to determine total florfenicol residues in bovine tissues and eel. Florfenicol and its metabolites (florfenicol amine, monochloroflorfenicol, florfenicol oxamic acid, and florfenicol alcohol) were analyzed as the marker residue, florfenicol amine, as defined by several regulatory agencies. After hydrolysis with hydrochloric acid, samples were defatted and subjected to solid-supported liquid extraction and Oasis MCX-cartridge cleanup before analysis. The method was validated for florfenicol and its metabolites at two levels in eel and bovine muscle, fat, and liver. Excellent recoveries were obtained (93-104%), with relative standard deviations of <6% for all compounds. Negligible matrix effects and minimal analyte loss during sample preparation enabled accurate quantification by external calibration using solvent standards. No interfering peaks were observed around the retention time of florfenicol amine, indicating the high selectivity of the method. Retention times in the spiked samples corresponding to that of the calibration standard in solvent did not exceed ±0.1 min. Ion ratios from the spiked sample were within ±10% (relative) of the calibration standards. Calibration curves were linear in the range of 0.5 to 100 ng/mL, with coefficients of determination higher than 0.998. The limits of quantification and limits of

detection of the proposed method were estimated to be 0.01 mg/kg and 0.0005 mg/kg, respectively, in all food samples. Thus, the developed method is considered reliable and suitable for regulatory use.

Keywords：florfenicol, florfenicol amine, LC-MS/MS

Saito-Shida S, Kashiwabara N, Nemoto S, Akiyama H: Determination of the total tulathromycin residues in bovine muscle, fat, and liver by liquid chromatography-tandem mass spectrometry.

*J Chromatogr B*. 2019;1110-1111:51-58

A reliable and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method was developed to quantify total tulathromycin residues in bovine tissues. Specifically, the above method relied on the quantification of CP-60,300, a marker produced by tulathromycin hydrolysis, for which maximum residue limits (MRLs) were established by the European Union and several other countries. Sample preparation and LC-MS/MS conditions were thoroughly optimized to allow for accurate quantification. The optimized procedure involved sample homogenization with 2 mol/L hydrochloric acid and ethyl acetate, heating of the resulting aqueous layer to convert tulathromycin and its metabolites into the marker residue, cleanup by a polymer-based cation-exchange cartridge, and subsequent analysis by LC-MS/MS. The developed method was validated for tulathromycin A and the marker residue in bovine muscle, fat, and liver at two levels, namely at the MRL set in Japan and at 0.01 mg/kg. Excellent analytical performance was observed, with the average recoveries of tulathromycin A and the marker residue ranging from 98 to 107%, and relative standard deviations ranging from 1 to 3%. Matrix effects were negligible, and analyte loss during sample preparation was minimal for all matrices tested, which allowed for accurate determination by external standard calibration using a solvent standard. No interfering peaks were observed close to the retention time of the marker residue for all matrices, which was indicative of high specificity. Overall, the developed method was proven suitable for regulatory purpose analysis of total tulathromycin residues.

Keywords：tulathromycin, LC-MS/MS, bovine tissue

Kikuchi H, Sakai T, Nemoto S, Akiyama H: Total determination of residual flutolanil and its metabolites in livestock products and seafood using liquid chromatography-tandem mass spectrometry.

*Food Addit. Contam*. Part A. 2018;35:2366-2374

We have developed a simple and sensitive LC-MS/MS analytical method for the determination of residual flutolanil and its principal metabolites, including α, α, α-trifluoro-3'-hydroxy-o-toluanilide (M-4) and its conjugates, in livestock and seafood products. Both flutolanil and its metabolites contain the 2-(trifluoromethyl)benzoic acid (2-TFMBA) moiety. In this method, flutolanil and its metabolites are converted to 2-TFMBA by hydrolysis. The method involves direct hydrolysis with sodium hydroxide at 200℃, acidification, partitioning into a mixture of ethyl acetate-*n*-hexane (1:9, v/v), clean-up using a strong anion exchange cartridge (InertSep SAX), and then quantification using LC-MS/MS. The optimal conditions for the complete hydrolysis of flutolanil to 2-TFMBA are an incubation time of 6 h and a temperature of 200℃. The developed method was evaluated using seven types of food: bovine samples of muscle, fat, liver and milk, as well as egg, eel, and freshwater clam. Samples were spiked both at 0.01 mg/kg and at the Japanese maximum residue limit (MRL) established for each food type. The validation results show excellent recoveries (88–107%) and precision (< 10%) for flutolanil and M-4. The limit of quantification (S/N ≥ 10) of the developed method is 0.01 mg/kg. The developed method is applicable to the definition of residual flutolanil for animal-based food commodities and MRLs established by the Codex Alimentarius, and will be useful for the regulatory monitoring of residual flutolanil and its metabolites in food products.

Keywords：flutolanil, metabolites, LC-MS/MS

Kikuchi H, Sakai T, Okura T, Nemoto S, Akiyama H: Total determination of triclabendazole and its metabolites in bovine tissues using liquid chromatography-tandem mass spectrometry.

*J. Chromatogr B*. 2019;1109:54-59

A reliable LC-MS/MS analytical method for the determination of residual triclabendazole and its principal metabolites (triclabendazole sulfoxide, triclabendazole sulfone and keto-triclabendazole) in bovine tissues was developed, in which triclabendazole and its metabolites are oxidized to keto-triclabendazole

as a marker residue. The method involves sample digestion with hot sodium hydroxide, thus releasing the bound residues of various triclabendazole metabolites in bovine tissues. The target compounds are extracted from the digest mixture with ethyl acetate, defatted by liquid-liquid partitioning using *n*-hexane and acetonitrile, then oxidized with hydrogen peroxide in a mixture of ethanol and acetic acid. The reaction mixture is cleaned up using a strong cation exchange cartridge (Oasis MCX) and the analytes are quantified using LC-MS/MS. The optimal conditions for the complete oxidation of triclabendazole and its metabolites to keto-triclabendazole are an incubation time of 16 h and a temperature of 90℃. The developed method was evaluated using three bovine samples: muscle, fat, and liver. Samples were spiked with triclabendazole and its principal metabolites at 0.01 mg/kg and at the Japanese Maximum Residue Limits (MRLs) established for each sample. The validation results show excellent recoveries (81–102%) and precision (<10%) for all target compounds. The limit of quantification (S/N ≥ 10) of the developed method is 0.01 mg/kg. These results suggest the developed method is applicable to quantifying residual triclabendazole in bovine tissues in compliance with the MRLs established by the Codex Alimentarius and EU and Japanese regulations, and thus the proposed method will be a useful tool for the regulatory monitoring of residual triclabendazole and its metabolites.

Keywords：triclabendazole, metabolites, LC-MS/MS

Tsutsumi T, Matsuda R, Yanagi T, Iizuka S, Isagawa S, Takatsuki S, Watanabe T, Teshima R, Akiyama H: Dietary intake of dioxins in Japan in 2016 with time trends since 1998.

*Food Addit. Contam. Part A* 2018;35:1553-1564

Total diet samples collected from seven regions throughout Japan in 2016 were analysed for polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and dioxin-like polychlorinated biphenyls (DL-PCBs), known collectively as dioxins. This led to estimates of the latest dietary intake of these contaminants for the general Japanese population (≥ 1 year old). The average daily intake of dioxins for a person weighing 50 kg, calculated at non-detected congener concentrations assumed to be equal to zero, was estimated to be 0.54 pg TEQ (toxic equivalents) $kg^{-1}$ body weight (bw) $day^{-1}$. This value was well below the tolerable daily intake of 4 pg TEQ $kg^{-1}$ bw $day^{-1}$ for dioxins in Japan. The average intake was highest from fish and shellfish, followed by meat and eggs. The TEQ contribution of the fish and shellfish group to the total dietary TEQs was significant (89%). The DL-PCBs accounted for about 67% of the dioxin intake. The latest dioxin intake level was compared with previous estimates from total diet study results obtained annually since 1998 to determine the time trends in the dietary intake of dioxins in Japan. Overall, the average dioxin intake appeared to be decreasing gradually during the period of study. The previous average intakes of dioxins ranged from 0.58 to 1.9 pg TEQ $kg^{-1}$ bw $day^{-1}$. The latest average intake was the lowest since 1998 and was about one-third of the average intake in 1998. This decreasing trend in the dietary intake of dioxins was mainly influenced by the decreased dioxin intakes from two food groups, fish and shellfish, and meat and eggs.

Keywords：dioxin, total diet study, dietary intake

今村正隆，鍋師裕美，堤智昭，植草義徳，松田りえ子，前田朋美，曽我慶介，手島玲子，蜂須賀暁子，穐山浩：市場流通食品中の放射性セシウム調査（2014–2016年度）．

*食品衛生学雑誌* 2018;59(5):239-247

2011年３月の東京電力福島第一原子力発電所事故により，放射性物質による食品汚染が発生した．地方自治体による出荷前放射性物質検査の有効性を検証するため，放射性セシウムが検出される蓋然性が高い食品・地域を重点的調査対象とした買い上げ調査を行った．2014年度は1,516試料，2015年度は900試料，2016年度は654試料を調査した結果，一般食品における放射性セシウムの基準値を超過した試料数は2014年度では９試料（0.6%），2015年度は12試料（1.3%），2016年度は10試料（1.5%）であった．放射性セシウムが検出される蓋然性が高い食品・地域を重点的に選択したが，基準値超過率は１%程度であったことから，各地方自治体における出荷前の検査体制は適切に整備され，かつ有効に機能していることが確認された．原木栽培や天然のきのこ，天然の山菜，野生獣肉などは放射性セシウム濃度が高い試料が存在したことから，継続的な監視が必要であると考えられた．

Keywords：放射性セシウム，スクリーニング法，流通食品

Kataoka, Y, Watanabe, T, Hayashi, K, Akiyama, H:

Surveillance of Cadmium Concentration in Chocolate and Cocoa Powder Products Distributed in Japan.
*J. Food Hyg. Soc. Japan* 2018;59:269-274.

Chocolate and cocoa are manufactured from cacao beans produced by the cacao tree (Theobroma cacao). These products may contain cadmium (Cd), which originates from contaminated soil. Here, we surveyed the Cd concentrations in dark chocolate, milk chocolate, white chocolate and cocoa powder products purchased at retail stores in Japan, using inductively coupled plasma mass spectrometry. The Cd concentrations in these chocolate and cocoa powder products ranged from 0.00021 to 2.3 mg/kg and from 0.015 to 1.8 mg/kg, respectively. A weak positive correlation was found between the Cd concentration and the content of cocoa solids stated on the product labels. A comparison between these results and the maximum levels (MLs) set by the European Union revealed that the Cd concentrations in chocolate and cocoa powder products on the Japanese market exceeded the MLs for eight of the 180 chocolate products and 26 of the 140 cocoa powder products.
Keywords：cadmium, chocolate, surveillance

Osumi M[*1], Yamaguchi M[*1], Sugimoto N[*1], Suzukawa M[*2], Arai H[*1], Akiyama H[*3], Nagase H[*1], Ohta K[*1,2]: Allergy to carminic acid: *in vitro* evidence of involvement of protein-binding hapten.
*Asia Pac Allergy.* 2019;9:e2

We previously described a rare case of anaphylaxis presumably induced by carminic acid in cochineal dye used as a food additive. In this study, highly pure carminic acid was added to an albumin-containing buffer at various concentrations, followed by serial dilution. Varying the mixing ratio of carminic acid and albumin affected the extent of histamine release from passively sensitized basophils. Similar basophil histamine release occurred with carminic acid-globulin solutions. These results provide experimental evidence indicating that basophil activation is dependent on hapten (carminic acid) and carrier (protein) interaction.
Keywords：Anaphylaxis, Basophils, Cochineal dye

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Takeo N[*1], Nakamura M[*2], Nakayama S[*3], Okamoto O[*4], Sugimoto N, Sugiura S[*5], Sato N[*2], Harada S[*7], Yamaguchi M[*7], Mitsui N[*8], Kubota Y[*9], Suzuki K[*10], Terada M[*11], Nagai A[*12], Sowa-Osako J[*13], Hatano Y[*1], Akiyama H, Yagami A[*10], Fujiwara S[*1], Matsunaga K[*2]: Cochineal dye-induced immediate allergy: Review of Japanese cases and proposed new diagnostic chart.
*Allergol Int.* 2018;67:496-505

BACKGROUND: Cochineal dye is used worldwide as a red coloring in foods, drinks, cosmetics, quasi-drugs, and drugs. The main component of the red color is carminic acid (CA). Carmine is an aluminum- or calcium-chelated product of CA. CA and carmine usually contain contaminating proteins, including a 38-kDa protein thought to be the primary allergen. Severe allergic reactions manifest as anaphylaxis. The aim of this study was to review all Japanese reported cases and propose useful diagnostic chart. METHODS: All reported Japanese cases of cochineal dye-induced immediate allergy were reviewed, and newly registered cases were examined by skin prick test (SPT) with cochineal extract (CE) and measurement of CE and carmine-specific serum IgE test. Two-dimensional (2D) western blotting using patient serum was conducted to identify the antigen. RESULTS: Twenty-two Japanese cases have been reported. SPT and the level of specific IgE test indicated that six cases should be newly registered as cochineal dye allergy. All cases were adult females, and all cases except three involved anaphylaxis; 13 cases involved past history of local symptoms associated with cosmetics use. Japanese strawberry juice and fish-meat sausage, and European processed foods (especially macarons made in France) and drinks were recent major sources of allergen. 2D western blotting showed that patient IgE reacted to the 38-kDa protein and other proteins. Serum from healthy controls also weakly reacted with these proteins. CONCLUSIONS:SPT with CE and determination of the level of CE and carmine-specific IgE test are useful methods for the diagnosis of cochineal dye allergy.
Keywords: Carminic acid, Cochineal dye, Immediate allergy

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松原優里*1，阿江竜介*1，大矢幸弘*2，穐山浩，今井孝成*3，松本健治*2，福家辰樹*2，青山泰子*1，牧野伸子*1，中村好一*1，斎藤博久*2: 日本における食物アレルギー患者数の推計：疫学調査の現状と課題
*アレルギー*, 2018;67:767-773.

背景・目的：アレルゲンを含む食物の食品表示は重要だが，現状では明確な表示基準はない．これらの制定に，内閣府は食物アレルギー患者の「重症度と有病率」が重要としている．本研究では，本邦の食物アレルギー患者の頻度分布（有病率）を明らかにし，新たな調査方法を検討する．方法：政府統計等利用可能な資料を用いて，食物アレルギー患者数を推計する．結果：乳幼児期では，「自己申告」で約80万人「医師の診断」で約30万～50万人，学齢期では，「自己申告」で約60万人，「医師の診断」で約35万人と推計された．成人では，消費者庁が即時型症状の受診者数を調査しているが，対象が限定されており，患者数の推計は困難であった．結語：乳幼児はエコチル調査に症状や診断の有無・血液検査を追加することで，年次変化を把握でき，学齢期では文部科学省の調査が有効である．成人期では大規模調査は少なく，国民健康・栄養調査や国民生活基礎調査などに付随した調査が有効である．一方で個々の情報源の抱える問題点も明らかにした．

Keywords: epidemiology, food allergy, prevalence

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Nishizaki Y, Sato-Masumoto N, Yokota A*1, Mikawa T*1, Nakashima K*1, Yamazaki T*2, Kuroe M*2, Numata M*2, Ihara T*2, Ito Y*3, Sugimoto N, Sato K: HPLC/PDA determination of carminic acid and 4-aminocarminic acid using relative molar sensitivities with respect to caffeine.
*Food Addit. Contam. Part A* 2018;35:838-847.

To accurately determine carminic acid (CA) and its derivative 4-aminocarminic acid (4-ACA), a novel, high-performance liquid chromatography with photodiode array detector (HPLC/PDA) method using relative molar sensitivity (RMS) was developed. The method requires no analytical standards of CA and 4-ACA; instead it uses the RMS values with respect to caffeine (CAF), which is used as an internal standard. An off-line combination of $^{1}$H-quantitative nuclear magnetic resonance spectroscopy ($^{1}$H-qNMR) and HPLC/PDA was able to precisely determine the RMSs of $CA^{274nm}$/$CAF^{274nm}$ and 4-$ACA^{274nm}$/$CAF^{274nm}$. To confirm the performance of the HPLC/PDA method using RMSs, the CA and 4-ACA contents in test samples were tested using four different HPLC-PDA instruments and one HPLC-UV. The relative standard deviations of the results obtained from five chromatographs and two columns were less than 2.7% for $CA^{274nm}$/$CAF^{274nm}$ and 1.1% for 4-$ACA^{274nm}$/$CAF^{274nm}$. The $^{1}$H-qNMR method was directly employed to analyse the CA and 4-ACA contents in test samples. The differences between the quantitative values obtained from both methods were less than 5% for CA and 3% for 4-ACA. These results demonstrate that the HPLC/PDA method using RMSs to CAF is a simple and reliable quantification method that does not require CA and 4-ACA certified reference materials.

Keywords: relative molar sensitivity, cochineal extract, carminic acid

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Nishizaki Y, Masumoto N, Nakajima K, Ishizuki K, Yamazaki T*, Kuroe M*, Numata M*, Ihara T*, Tada A, Sugimoto N, Sato K: Relative molar sensitivities of carnosol and carnosic acid with respect to diphenylamine allow accurate quantification of antioxidants in rosemary extract.
*Food Addit. Contam. Part A* 2019;36:203–211.

We have been developing a high-performance liquid chromatography/photodiode array (HPLC/PDA) employing relative molar sensitivities (RMSs) and adopted it to the accurate quantification of carnosol (CL) and carnosic acid (CA) which are the antioxidants in rosemary extract. The method requires no references of CL or CA and instead uses RMSs with respect to diphenylamine (DPA) whose certified reference material is available from a reagent manufacturer. The molar and response ratios of the analytes to the reference in an artificial mixture of them were determined using $^{1}$H-quantitative nuclear magnetic resonance spectroscopy ($^{1}$H-qNMR) and HPLC/PDA at a wavelength of 284 nm under isocratic condition, respectively, and then RMSs were calculated to be 0.111 for CL/DPA and 0.0809 for CA/DPA as averaged values in three HPLC-PDA instruments. The RMS values varied by up to 1.1% as relative standard deviation. To evaluate the performance of HPLC/PDA with the RMSs, the CL and CA contents in rosemary extracts were determined using DPA as a reference. The CL and CA contents were compared with those determined using calibration curves of CL and CA obtained by HPLC measurement of standard solutions prepared from their reagents whose absolute purities were determined using $^{1}$H-qNMR. The differences between the two methods for CL and CA were ≤3% as relative error. This chromatographic method with RMSs allows a simple and reliable quantification when reference of the analyte is unavailable.
Keywords: relative molar sensitivity, rosemary extract, antioxidant

* National Institute of Advanced Industrial Science and Technology

Masumoto N, Nishizaki Y, Sugimoto N, Sato, K: Phytochemical profiling of rosemary extract products distributed as food additives in the Japanese market.
*Jpn. J. Food Chem. Safety* 2018;25:105–113.

Rosemary extract is one of the natural food additives on the List of Existing Food Additives used in Japan. There are two types of rosemary extract products on the Japanese market: water-soluble type and water-insoluble type. Since the two types are thought to have different chemical compositions, investigation of their compositions is essential in order to ensure the safety and efficacy of the products. In this study, LC/MS and GC/MS analyses were performed on products distributed as rosemary extract on the Japanese market. The results showed that carnosol and carnosic acid, which are thought to be main components of rosemary extract, were only present in the water-insoluble-type products. Many kinds of volatile compounds were also detected in the waterinsoluble-type products, and the ratios of these compounds varied even among the products having similar amounts of carnosol and its relatives. In the water-soluble-type products, rosmarinic acid and flavonoids were observed instead of carnosol, carnosic acid and volatile compounds.
Keywords: rosemary extract, existing food additive, *Rosmarinus officinalis* L.

Saito N*1,2, Kitamaki Y*1, Otsuka S*1, Yamanaka N*1, Nishizaki Y, Sugimoto N, Imura H*2, Ihara T*1: Extended internal standard method for quantitative $^{1}$H NMR assisted by chromatography (EIC) for analyte overlapping impurity on $^{1}$H NMR spectra.
*Talanta* 2018;184:484–490.

We devised a novel extended internal standard method of quantitative $^{1}$H NMR (qNMR) assisted by chromatography (EIC) that accurately quantifies $^{1}$H signal areas of analytes, even when the chemical shifts of the impurity and analyte signals overlap completely. When impurity and analyte signals overlap in the $^{1}$H NMR spectrum but can be separated in a chromatogram, the response ratio of the impurity and an internal standard (IS) can be obtained from the chromatogram. If the response ratio can be converted into the $^{1}$H signal area ratio of the impurity and the IS, the $^{1}$H signal area of the analyte can be evaluated accurately by mathematically correcting the contributions of the $^{1}$H signal area of the impurity overlapping the analyte in the $^{1}$H NMR spectrum. In this study, gas chromatography and liquid chromatography were used. We used 2-chlorophenol

and 4-chlorophenol containing phenol as an impurity as examples in which impurity and analyte signals overlap to validate and demonstrate the EIC, respectively. Because the $^{1}$H signals of 2-chlorophenol and phenol can be separated in specific alkaline solutions, 2-chlorophenol is suitable to validate the EIC by comparing analytical value obtained by the EIC with that by only qNMR under the alkaline condition. By the EIC, the purity of 2-chlorophenol was obtained with a relative expanded uncertainty ($k$=2) of 0.24%. The purity matched that obtained under the alkaline condition. Furthermore, the EIC was also validated by evaluating the phenol content with the absolute calibration curve method by gas chromatography. Finally, we demonstrated that the EIC was possible to evaluate the purity of 4-chlorophenol, with a relative expanded uncertainty ($k$=2) of 0.22%, which was not able to be separated from the $^{1}$H signal of phenol under any condition.

Keywords: EIC, quantitative $^{1}$H NMR, overlapping impurity

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黒江美穂*，斎藤直樹*，山﨑太一*，西﨑雄三，杉本直樹，沼田雅彦*，井原俊英*：デュアル検出の高速液体クロマトグラフィーと定量核磁気共鳴分光法から求めた相対モル感度を利用したヘプタオキシエチレンドデシルエーテル標準液の値付け．

*分析化学* 2018;67:541–549.

多成分の有機化合物の測定方法として，我々は定量核磁気共鳴分光法（qNMR）とクロマトグラフィーを併用した分析法の研究を進めてきた．本法はqNMRとクロマトグラフィーを併用することで，分析対象成分ごとの標準物質が不要であるというqNMRの利点に加え，qNMRの測定ではシグナルどうしが重なるような多成分溶液の濃度測定が可能となる．本研究では，鎖長の異なる成分を不純物として含むために$^{1}$H qNMR のみでは分離定量が難しい非イオン界面活性剤標準液の値付けに本法の適用を試みた．検討対象とした非イオン界面活性剤であるヘプタオキシエチレンドデシルエーテルは，紫外吸光光度検出器で汎用される波長域に特徴的な吸収がないことから，真空紫外域の波長で検出を行った．このとき示差屈折率検出器を併用したデュアル検出とすることで，測定値の検証が行えるように工夫した．本法を用いて1000 mg/Lのヘプタオキシエチレンドデシルエーテル標準液の認証標準物質を測定したところ，相対拡張不確かさ1.1%（$k$=2）での定量が可能であり，得られた濃度は認証値と満足できる一致を得た．

Keywords：非イオン界面活性剤，相対モル感度，不確かさ

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Takahashi M*, Nishizaki Y, Sugimoto N, Sato K, Inoue K*: Single reference quantitative analysis of xanthomonasin A and B in Monascus yellow colorant using high-performance liquid chromatography with relative molar sensitivity based on high-speed countercurrent chromatography.

*J. Chromatogr. A* 2018;1555:45–52.

Monascus yellow (MY) is a natural yellow food coloring. The main components from MY are xanthomonasin A (XA) and xanthomonasin B (XB) for natural yellow colorant of food additives. However, few chromatographic assays of XA and XB exist in food additive products because of unavailable standards for calibration curves. In this study, the single reference (SR) quantitative analysis of XA and XB in MY product is proposed by high-performance liquid chromatography with photodiode array detection (HPLC/PDA) using relative molar sensitivity (RMS). Moreover, high-speed countercurrent chromatography (HSCCC) purification with $^{1}$H quantitative NMR (qNMR) evaluation is necessary to separate the two analytes for the RMS to be demonstrated. For HSCCC separation, the biphasic solvent system (hexane/ethyl acetate/methanol/0.1% formic acid in water, 1/5/1/5) was used to obtain XA and XB fractions that were subjected to qNMR for the determination of their contents in each test solution. Using these solutions and SR solution of carbazochrome acid (CBZ), the RMS of XA and XB are calculated from slopes ratios of calibration curves (three ranges from 0 to 177 μM for XA and 0-126 μM for XB, $r^2$>0.998). The averaged RMS of XA/CBZ and XB/CBZ were 8.75 ± 0.07 and 14.8 ± 0.26, respectively. The concentrations of XA and XB in MY can be determined from RMS, peak area and content of CBZ added in the samples; the concentrations were found to be 7.26 μmol/g and 2.53 μmol/g, respectively. The performance of HPLC/PDA using RMS was

compared with an absolute calibration curve method. This developed HPLC/PDA using RMS is simple and reliable quantification that does not require native XA and XB standards based on HSCCC purification and qNMR evaluation.

Keywords: relative molar sensitivity, xanthomonasin A and B, single reference standard

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Takahashi M*, Nishizaki Y, Morimoto K*, Sugimoto N, Sato K, Inoue K*: Design of synthetic single reference standards for the simultaneous determination of sesamin, sesamolin, episesamin, and sesamol by HPLC using relative molar sensitivity.

*Separation Science Plus* 2018;1:498–405.

A single reference standard can be used as an internal standard for both quantitative proton NMR spectroscopy and high-performance liquid chromatography to estimate the relative molar sensitivity for a simultaneous determination of multiple analytes. However, we find it difficult to choose a candidate single reference standard from currently existing compounds. The present work aims to design and synthesize idealized single reference standards for the simultaneous determination of sesamin, sesamolin, episesamin and sesamol by high-performance liquid chromatography using relative molar sensitivity. These analytes all contain a 1,3-benzodioxole derivative that has an absorption wavelength near 290 nm. Using this core structure, piperoanol and synthetic methyl, butyl and hexyl sesamol derivatives were evaluated by quantitative proton NMR spectroscopy. The purities of these candidate single reference standards were higher than 97.0%. The relative molar sensitivity of the analyte was calculated from slope ratios of the calibration curves (three ranges from 0 to 100mM, $r^2$ = 0.999). The averaged relative molar sensitivity values of the analytes and other single reference standards ranged from 0.73 ± 0.01 to 2.26 ± 0.01. Using these relativemolar sensitivity values, the concentrations of sesamin, sesamolin, episesamin and sesamol in sesame oil, health foods, and food additives could be evaluated by high-performance liquid chromatography within the expanded uncertainty. This approach for the design of single reference standards based on structural information can be applied for the simultaneous determination of similar chemical compositions where native standards do not yet exist.

Keywords: relative molar sensitivity, sesamin, single reference standard

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中西徹*，河村葉子，城市香*，渡邊雄一*，杉本敏明*，阿部裕，六鹿元雄：油脂および脂肪性食品用器具・容器包装のための植物油への総溶出物試験法の確立.

*日本食品衛生学雑誌* 2018;59:193–199.

食品衛生法では，器具・容器包装からの総溶出物試験として蒸発残留物試験が規定されている．油脂および脂肪性食品の最適な食品擬似溶媒は植物油であるが，蒸発乾固が困難であることから，合成樹脂ではヘプタン，ゴムでは20％エタノールが浸出用液として用いられている．一方，欧州連合では，油脂および脂肪性食品に使用される合成樹脂に対してオリブ油への総溶出物試験が規定されており，その試験法は欧州標準規格EN1186-2に収載されている．しかし，試験操作上の問題が多いことから，試料の恒量化を43％硫酸デシケーターで行い，溶出後試料に残存する植物油を内標準浸漬抽出法で抽出し，植物油のメチルエステル化にナトリウムメトキシドを用い，GC測定条件を変更するなどの改良を行った．その結果，操作が簡便で試験時間が大幅に短縮され，試薬の有害性が低減され，合成樹脂だけでなくゴムにも適用可能な試験法を確立することができた．さらに，本法とEN1186-2に示された試験法を６種類の試料を用いて比較したところ，同等の試験性能をもつ優れた試験法であることが確認された．

Keywords：植物油，総溶出物試験，食品用器具・容器包装

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Yamazaki A*1, Honda M*2, Kobayashi N*3, Ishizaki N*3, Asakura H, Sugita-Konishi Y*3: The sensitivity of commercial kits in detecting the genes of pathogenic bacteria in venison.

*J Vet Med Sci.* 2018;80:706-709.

The expansion of the wild deer population is a major problem for the Japanese farm and forestry industries because their damage to farm products and vegetation results in huge economic loss. To promote game meat consumption, hygiene inspections should be performed

to detect main bacterial pathogens before products are shipped. In this study, we aimed to evaluate the ability of commercial test kits to genetically detect EHEC, Salmonella and Listeria monocytogenes in venison. Our results demonstrated that the kits for three pathogens could be useful for venison as well as other domestic meat products. Our comparative study showed that the LAMP kits were more sensitive than the RT-qPCR kits in the detection of all of these pathogens.

Keywords: EHEC O157, LAMP, *Listeria monocytgenes*, venison

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Honda M*1, Sawaya M*2, Taira K*2, Yamazaki A*3, Kamata Y*4, Shimizu H*5, Kobayashi N*2, Sakata R*2, Asakura H, Sugita-Konishi Y*2: Effects of temperature, pH and curing on the viability of *Sarcocystis*, a Japanese sika deer (*Cervus Nippon centralis*) parasite, and the inactivation of their diarrheal toxin.

*J Vet Med Sci.* 2018;80:1337-1344.

Recently, *Sarcocystis* parasite in horse and deer meat has been reported to be a causative agent of acute food poisoning, inducing nausea, vomiting and diarrhea. However, sability of the parasite in deer meat under various conditions, remains underestimated. Here, we assessed the viability of *Sarcocystis* spp. and the activity of their diarrhea toxin in deer meat under conditions of freezing, cold storage, pH change and curing. The heat tolerance was simultaneously assayed. The results showed that the species lost viability by freezing at below −20℃ for <1h, heating at 70℃ for 1min, alkylation for 4days, or salt soaking with 2.0% for <1day. Immunoblot assays showed that the diarrhea toxin disappeared with the loss of viability. However, the parasite survived cooling and acidification for more than 7 days with the diarrhea toxin intact. These imply to develop practical applications for the prevention of food poisoning by *Sarcocystis* spp. in deer meat during cooking and preservation.

Keywords: *Sarcocystis*, deer meat, cooking conditions

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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.

*J Vet Med Sci.* 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: *Sarcocystis*, STEC, LAMP-DNA strip

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山本詩織，森篤志*，朝倉宏：国内市販鶏挽肉におけるカルバペネム耐性菌の汚染実態調査.

*日本防菌防黴学会誌* 2019;47:47-52.

本研究では，国内の鶏挽肉におけるカルバペネム耐性菌（CRB）の汚染実態及び分離株の遺伝特性を検討した．CRBは226検体中4検体（1.8％）より検出され，*Stenotrophomonas maltophilia* 1株，*Pseudomonas otitidis* 2株，*P. protegens* 1株，*P. putida* 1株の計5株が分離された．*S. maltophilia*株及び*P. otitidis*株はメロペネムに対するMIC値が＞64 μg/mlと高く，それぞれ$bla_{L2}$又は$bla_{POM}$遺伝子を保有していた．他の分離株では，代表的なカルバペネマーゼ遺伝子を認めなかった．本研究は，国内の鶏挽肉製品におけるCRB汚染実態に関する初めての報告である．当該菌の食品汚染を通じた，

ヒトのカルバペネム耐性腸内細菌科細菌の蔓延との関連性把握には，疫学知見の更なる集積が必要であろう．
Keywords：ESBL耐性菌，鶏肉汚染，耐性遺伝子

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窪亜紀[*1]，川端舞香[*1]，川村研二[*1]，古木孝二[*1]，谷内正人[*1]，二川眞子[*1]，井上慎也[*2]，中澤佑介[*2]，山本詩織：男性外来患者における基質特異性拡張型β-ラクタマーゼ（ESBL）産生菌の直腸内長期保菌について．
*恵寿総合病院医学雑誌* 2019;In press.

前立腺特異抗原（PSA）高値の男性外来患者において，ESBL産生菌の直腸内保菌期間について検討した．ESBL産生菌陰性化例60%，ESBL産生菌陽性継続例は40%であり，陽性継続期間は12～29ヵ月間であった．重篤な基礎疾患のない外来通院患者でも，長期間ESBL産生菌を保有している群が存在していることが明らかとなった．今後，生体内におけるESBL産生菌の遺伝動態等に関するさらなる研究が必要である．
Keywords：ESBL産生菌，直腸内長期間保菌，外来患者

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佐々木貴正，岡田由美子，上間匡，朝倉宏，野田衛：鶏肝臓のカンピロバクターおよび腸内細菌科菌群に対する高圧処理効果．
*日本食品微生物学会雑誌* 2018;35:187-192.

鶏肝臓のカンピロバクターおよび腸内細菌科菌群に対する高圧処理効果を調査した．市販の鶏肝臓40検体のうち，カンピロバクターは23（58%）検体，腸内細菌科菌群は36（90%）検体の内部から分離された．陽性検体における平均汚染菌数については，カンピロバクターが1.70 log CFU/g，腸内細菌科菌群が1.97 log CFU/gであった．鶏肝臓のカンピロバクターに対する高圧処理効果を定量的に調査するため，*C. jejuni*または*C. coli* を肝臓乳剤に接種し，300 MPaで５分または10分の高圧処理をした．*C. jejuni*では５分または10分の処理でそれぞれ1.90 または3.20 log CFU/gの殺菌効果が得られた．*C. coli*では５分または10分の高圧処理でそれぞれ2.34 または3.00 log CFU/gの殺菌効果が得られた．

以上の結果から，300 MPaで10分の高圧処理は,鶏肝臓のカンピロバクター殺菌技術として有用であることが示唆された．
Keywords：カンピロバクター，高圧処理

Shiraishi R[*1], Yamazaki Y[*1], Sasaki Y, Haruna M[*2], Nakamura M[*1]：Imperfection of commercial inactivated *Salmonella* vaccine against *Salmonella* Infantis during induced molting in chickens and proposed evaluation method.
*Avian Dis.* 2018;62:340-34.

We evaluated the continuance and efficacy of inactivated vaccine against *Salmonella* Infantis（SI）in chickens raised on a commercial farm. Chickens（88-days-old）were inoculated with 1 or 0.5 doses of commercially available trivalent inactivated *Salmonella* vaccine; anti-SI antibody titer was examined continuously for 11 months thereafter. Molting was induced 11 months after vaccination, and SI was administered orally. SI colony-forming units（CFUs）were measured in cecal feces, cecal contents, liver, and spleen samples. Anti-SI antibodies in the 1 dose vaccination group could be detected in at least 90% of cases until the end of testing. SI discharge was significantly reduced in birds treated with either dose of vaccine. However, SI CFUs were elevated in the induced molting group, regardless of vaccination dose, particularly in the cecal feces, cecal contents, and spleen. To achieve sufficient SI protective efficacy, we recommend inoculation with 1 dose of vaccine. Moreover, the efficacy of inactivated *Salmonella* vaccine is recommended to be evaluated by challenging chickens with live *Salmonella* in addition to *Salmonella* antibody titration.
Keywords: chicken, comercial farm, efficacy, induced molting, *Salmonella infantis*, vaccine

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Teramura H[*1], Fukuda N[*2], Okada Y, Ogihara H[*2]: Comparison of Chromogenic Selective Media for the Detection of *Cronobacter* spp.（*Enterobacter sakazakii*）.
*Biocontrol Science* 2018; 23:27-33.

The four types of chromogenic selective media that are commercially available in Japan were compared for establishing a Japanese standard method for detecting *Cronobacter* spp. based on ISO/TS 22964:2006.
Keywords: *Cronobacter* spp. *Enterobacter sakazakii*, chromogenic medium, detection

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Suzuki H*, Okada Y: Comparative toxicity of dinophysistoxin-1 and okadaic acid in mice.
*Journal of Veterinary Medical Science* 2018; 80: 616-619.

The mouse bioassay for diarrhetic shellfish poisoning toxins has been used worldwide. In this study, dinophysistoxin-1 (DTX-1) and okadaic acid (OA) were compared for toxicity. The lethality rate increased and the median survival time decreased in a dose-dependent manner in both DTX-1 and OA.

Keywords: diarrhetic shellfish poisoning (DSP) toxin, dinophysistoxin-1 (DTX-1), mouse bioassay, okadaic acid (OA), survival curve

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Takayuki Kobayashi*, Hideaki Yoshitomi*, Asako Nakamura*, Yuki Ashizuka*, Jumboku Kajiwara* and Mamoru Noda: Genetic characterization of rarely reported GI.Pc_GI.5 norovirus strain detected from a foodborne suspected outbreak in Japan.
*Jpn J Infect Dis,* 71(5):390-392(2018)

A foodborne outbreak associated with the recombinant human norovirus GI.Pc-GI.5 was occurred in Fukuoka city, Japan, in January 2017, where 28 (68.3%) of 41 individuals who consumed a common meal at a barbecue restaurant were affected. In order to understand the genetic characteristics of the detected GI norovirus, the entire RdRp and VP1 coding regions were determined. The nucleotide sequences of noroviruses isolated from all 11 samples were identical and identified as FAnT99 GI.Pc-GI.15 strain (accession number LC331067) closest relative SzUG1 strains.

Keywords: norovirus, foodborne outbreak, genetic characteristics

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Imamura S*1, Kanezashi H*1, Goshima T*1, Suto A*2, Ueki Y*3, Sugawara N*3, Ito H*4, Zou B*5, Kawasaki C*5, Okada T*6, Uema M, Noda M, Akimoto K*: Effect of High Pressure Processing on a Wide Variety of Human Noroviruses Naturally Present in Aqua-Cultured Japanese Oysters.
*Foodborne Pathog Dis.* 2018

Wide variety of human noroviruses naturally contaminated in Japanese oysters could not be detected after high pressure processing using a polymerase chain reaction-based methods with enzyme pretreatment, to distinguish between infectious viruses.

Keywords: norovirus, oyster, high pressure processing

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上間匡，永田文宏，朝倉宏，野田衛：カキの糞便汚染指標としてのPepper mild mottle virusの評価
*獣医疫学雑誌*. 2018:22(2):102-107.

NoV等の糞便由来病原ウイルスに汚染されるリスクの高い食品であるカキの糞便汚染指標としてヒト糞便や環境中に存在するPepper mild mottle virus (PMMoV) が利用出来るかを市販カキ138バッチについてNoVとPMMoVの検出率を比較し，PMMoVがカキ生産海域およびカキに広く浸潤していることを示した.

Keywords: fecal indicator, norovirus, oyster, pepper mild mottle virus

Konishi N*1, Obata H*1, Kai A*1, Ohtsuka K*2, Nishikawa Y*3, Terajima J*4, Hara-Kudo Y: Major Vehicles and O-Serogroups in Foodborne Enterotoxigenic: Escherichia coli Outbreaks in Japan, and Effective Detection Methods of the Pathogen in Food Associated with An Outbreak.
*Food Hygiene and Safety Science* (*shokuhin eiseigaku Zasshi*). 2018;59:161-166

Enterotoxigenic Escherichia coli (ETEC) is a common pathogen in developing countries, and causes foodborne infections through contaminated vegetables and water. ETEC also caused some foodborne infections in developed countries, though the vehicles are often unclear. We analyzed ETEC foodborne outbreaks in Japan based on the National Food Poisoning Statistics. Vegetables and private well water accounted for 50% and 22.2% of vehicles, respectively. The main vehicles were similar

to those in developing countries. Serogroups of ETEC were also analyzed, and O6, O25, O27, O148, O153, O159, and O169 were the seven major O-serogroups. We investigated suitable detection methods for the pathogen (O148) in food samples associated with an outbreak of ETEC in Japan in 2011. We show that ETEC O148 could be effectively detected in cut leeks by means of a two-step enrichment and real-time PCR assay targeting heat-stable enterotoxin gene. Our survey of the vehicles and the major O-serogroups of ETEC outbreaks in Japan indicates that ETEC survives in the environment in Japan.

Keywords: enterotoxigenic *Escherichia coli*, foodborne outbreak, O-serogroup

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Yamazaki A*1, Izumiyama S*2, Yagita K*2, Kishida N*3, Kubosaki A, Hara-Kudo Y, Kamata Y*4, Terajima J*1: The Molecular Detection of *Cryptosporidium* and *Giardia* in Sika Deer (*Cervus Nippon Centralis*) in Japan.

*Food Safety*. 2018;6:88–95

Fecal specimens (271 samples) from wild deer, *Cervus nippon centralis*, were collected from nine different areas in Japan; these samples were subjected to a real-time reverse transcription PCR for *Cryptosporidium*-and *Giardia*-specific 18S ribosomal RNA to investigate the prevalence of *Cryptosporidium* and *Giardia* infection. The incidence of *Cryptosporidium* and *Giardia* in the nine areas ranged from 0% to 20.0% and 0% to 3.4%, respectively. The prevalence of *Cryptosporidium* among male and female deer was 8.1% and 3.9%, respectively, while that of *Giardia* was 0.7% and 0.8%. Sequence analysis identified the *Cryptosporidium* deer genotype, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium meleagridis* from the sequence of *Cryptosporidium*-specific partial 18S ribosomal RNA and *Giardia intestinalis* assemblage A from the partial sequence of *Giardia*-specific 18S rRNA. The variation in regional prevalence indicates that *Cryptosporidium* infection depends on environmental factors, and that bovine *Cryptosporidium* was detected more frequently than cervine *Cryptosporidium*. These data suggest that wild deer might be a healthy carrier of bovine *Cryptosporidium*.

Keywords: *Cervus nippon centralis*, *Cryptosporidium*, *Giardia*, wild deer

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Parvej MS*1, Nakamura H*2, Alam MA*a, Wang L*1,3, Zhang S*1, Emura K*1, Kage-Nakadai E*1, Wada T*4, Hara-Kudo Y, Nishikawa Y*1: Host Range-Associated Clustering Based on Multilocus Variable-Number Tandem-Repeat Analysis, Phylotypes, and Virulence Genes of Atypical Enteropathogenic *Escherichia coli* Strains.

*Applied and Environmental Microbiology*. 2019;85: pii: e02796-18

Atypical enteropathogenic *Escherichia coli* (aEPEC) strains (36 Japanese and 50 Bangladeshi) obtained from 649 poultry fecal samples were analyzed by molecular epidemiological methods. Clermont's phylogenetic typing showed that group A was more prevalent (58%, 50/86) than B1 (31%, 27/86). Intimin type $\beta$1, which is prevalent among human diarrheal patients, was predominant in both phylogroups B1 (81%, 22/27) and A (70%, 35/50). However, about 95% of B1-$\beta$1 strains belonged to virulence group I, and 77% of them were Japanese strains, while 17% (6/35) of A-$\beta$1 strains did. Multilocus variable-number tandem-repeat analysis (MLVA) distributed the strains into 52 distinct profiles, with Simpson's index of diversity (D) at 73%. When the data were combined with those of 142 previous strains from different sources, the minimum spanning tree formed five zones for porcine strains, poultry strains (excluding B1-$\beta$1), strains from healthy humans, bovine and human patient strains, and the B1-$\beta$1 poultry strains. Antimicrobial resistance to nalidixic acid was most common (74%) among the isolates. Sixty-eight percent of them demonstrated resistance to ≧3 antimicrobial agents, and most of them (91%) were from Bangladesh. The strains were assigned into two groups by hierarchical clustering. Correlation matrix analysis revealed that the virulence genes were

negatively associated with antimicrobial resistance. The present study suggested that poultry, particularly Japanese poultry, could be another reservoir of aEPEC (phylogroup B1, virulence group I, and intimin type $\beta$1); however, poultry strains seem to be apart from patient strains that were closer to bovine strains. Bangladeshi aEPEC may be less virulent for humans but more resistant to antibiotics.

Keywords: *Escherichia coli*, MLVA, molecular epidemiology

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Tran THT.[*], Yanagawa H[*], Nguyen KT [*], Hara-Kudo Y, Taniguchi T[*], Hayashidani H[*]: Prevalence of *Vibrio parahaemolyticus* in seafood and water environment in the Mekong Delta, Vietnam.
*Journal of Veterinary Medical Science*. 2018;80:1737-1742

A total of 449 samples including 385 seafood and 64 water samples in the Mekong Delta of Vietnam collected in 2015 and 2016 were examined. Of 385 seafood samples, 332 (86.2%) samples were contaminated with *Vibrio parahaemolyticus* and 25 (6.5%) samples were pathogenic *V. parahaemolyticus* carrying tdh and/or trh genes. The tdh gene positive *V. parahaemolyticus* strains were detected in 22 (5.7%) samples and trh gene positive *V. parahaemolyticus* strains were found in 5 (1.3%) samples. Of 25 pathogenic *V. parahaemolyticus* strains, two strains harbored both tdh and trh genes and the other 23 strains carried either tdh or trh gene. Of 64 water samples at aquaculture farms, 50 (78.1%) samples were contaminated with *V. parahaemolyticus*. No tdh gene positive *V. parahaemolyticus* strains were detected; meanwhile, trh gene positive *V. parahaemolyticus* strain was detected in 1 (1.6%) sample. Twenty-six pathogenic *V. parahaemolyticus* strains isolated were classified into 6 types of O antigen, in which the serotype O3:K6 was detected in 4 strains. All pathogenic strains were group-specific PCR negative except for 4 O3:K6 strains. The result of antimicrobial susceptibility test indicated that pathogenic strains showed high resistance rates to streptomycin (84.6%), ampicillin (57.7%) and sulfisoxazole (57.7%). These findings can be used for understanding microbiological risk of seafood in the Mekong Delta, Vietnam.

Keywords: tdh, trh, *Vibrio parahaemolyticus*

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*医薬品医療機器レギュラトリーサイエンス* 2018；49：708-718

エンドトキシン試験法は，カブトガニの血球抽出成分から調製したライセート試薬を用いてグラム陰性菌由来のエンドトキシンを検出する．ライセート試薬に含まれるFactor C の組換えタンパク質からなる３種類のエンドトキシン測定試薬（組換え試薬）を使用する妥当性を評価するため，各種の菌由来LPSからなるエンドトキシンパネルで反応性を比較すると共に，施設間差とロット間差を調べた．一部の精製LPSについては，試薬間で大きな差が認められ，測定したエンドトキシン活性が非常に低値となった試薬もあった．エンドトキシンを検出できない，すなわち偽陰性という結果を与える可能性が高い試薬をエンドトキシン試験法に適用することはできないが，本研究においては，そのような試薬はなかった．しかし，試薬の組成がエンドトキシンの活性に影響を与える可能性が示唆されたことから，今後の検討が必要と考えられた．

Keywords：エンドトキシン試験法，組換え試薬，ライセート試薬

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Onami J[*1], Watanabe M, Yoshinari T, Hashimoto R[*2], Kitayama M[*3], Kobayashi N[*4], Sugita-Konishi Y[*4], Kamata Y[*5,6], Takahashi H, Kawakami H[*3],

Terajima J[*5]: Fumonisin-production by *Aspergillus* section *Nigri* isolates from Japanese Foods and Environments.

*Food Safety*. 2018; 6:74-82

Recently *Aspergillus niger* has been reported to be a fumonisin B2 (FB2) producer. *Aspergillus niger* is a member of *Aspergillus* section *Nigri*. Members of this section are common food contaminants and are also distributed widely in the environment. This study aimed to determine 1) optimum culture conditions of A. niger for fumonisin production including growth medium, temperature and incubation period and 2) fumonisin production among isolates of *Aspergillus* section *Nigri* and closely related species isolated from Japanese food and environmental samples. Growth on Czapek yeast extract broth +5% NaCl (CYBS) at 28℃ for 7 days resulted in the highest levels of FB2 production. Phylogenic analysis of *Aspergillus* section *Nigri* isolates from food and environmental samples in this study indicated that fumonisin producing strains could be grouped into the *A. niger* clade. Nineteen of 35 (54%) isolates classified as *A. niger* were FB2 producers. The current study suggests that FB2-producing *A. niger* are distributed throughout several regions of Japan.

Keywords : *Aspergillus niger*, fumonisin B2, tip culture method

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Kobayashi N[*1], Kubosaki A, Takahashi Y[*2], Yanai M[*3], Konuma R[*4], Uehara S[*2], Chiba T[*2], Watanabe M, Terajima J, Sugita-Konishi Y[*1]: Distribution of Sterigmatocystin-producing *Aspergilli* in Japan.

*Food Safety*. 2018; 6:67-73

Sterigmatocystin is produced as a precursor to aflatoxin B1 or as an end product by certain Aspergilli. *Aspergillus* section *Versicolores* is one of the major sections including sterigmatocystin-producing species and is thus a potential health and environmental hazard. Recently, the taxonomy of this section was revised and classified into 14 species on the basis of molecular phylogenetic analysis. However, investigation of the distribution and sterigmatocystin production of each species has been limited; in particular, its distribution in foods has been scarcely reported. In this study, we collected isolates of *Aspergillus* section *Versicolores* from various foods and environments in Japan and investigated their distribution and sterigmatocystin production. The isolates were classified into nine species or species groups, which revealed that *A. creber, A. puulaauensis/tennesseensis* and *A. sydowii* are the main species/species groups in Japan. In addition, *A. versicolor* sensu stricto was detected with some frequency, specifically in foods. Furthermore, the two species *A. creber* and *A. versicolor* sensu stricto frequently produced sterigmatocystin.

Keywords: sterigmatocystin, *Aspergillus* section *Versicolores*, distribution

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Tsurikisawa N[*1,2,3], Oshikata C[*1,2,3], Watanabe M, Tsuburai T[*2,4], Kaneko T[*3], Saito H[*5]: Innate immune response reflects disease activity in eosinophilic granulomatosis with polyangiitis.

*Clinical and Experimental Allergy*. 2018; 48:1305-1316

Eosinophilic granulomatosis with polyangiitis (EGPA) is a disease characterized by allergic granulomatosis, necrotizing vasculitis, and peripheral blood eosinophilia. Interleukin (IL)-33, thymic stromal lymphopoietin (TSLP), and type 2 innate lymphoid cells (ILC2) are involved in the innate and type 2 immune responses in EGPA. However, the relationships among these molecules and the mechanisms underlying the development of EGPA remain unknown. We investigated the relationships among peripheral blood eosinophil count, serum IL-33 and TSLP concentration, and peripheral blood ILC2 count in patients with EGPA, chronic eosinophilic pneumonia (CEP), or bronchial asthma (BA). Peripheral blood eosinophil count or ILC2 count, and serum sST2 or TSLP concentration were higher in patients with EGPA at onset than in those with EGPA

at relapse or remission, or in those with BA or CEP. Serum IL-33 concentration was higher in patients with EGPA at relapse than in those with EGPA at onset or remission, or in those with BA or CEP. In a logistic regression model, EGPA disease activity was correlated with serum IL-33 concentration and peripheral blood ILC2 count, but not daily systemic and inhaled corticosteroid dose or immunosuppressant use. Eosinophil count was correlated with peripheral blood ILC2 count and serum TSLP concentration, but not serum IL-33 concentration. Increased peripheral blood ILC2 count and serum IL-33 concentration were associated with disease activity in EGPA. Increases in serum IL-33 concentration may indicate the presence of active vasculitis rather than peripheral or tissue eosinophilia.

Keywords: Churg-Strauss syndrome, IL-10, IL-33

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Yoshinari T, Takeda N*, Watanabe M, Sugita-Konishi Y*: Development of an analytical method for simultaneous determination of the modified forms of 4,15-diacetoxyscirpenol and their occurrence in Japanese retail food.

*Toxins* (*Basel*). 2018;10:E178

4,15-Diacetoxyscirpenol (4,15-DAS) is a type A trichothecene mycotoxin produced by *Fusarium* species. Four modified forms of 4,15-DAS were purified from cultures of *F. equiseti*. An analytical method using a multifunctional column has been developed for the simultaneous determination of 4,15-DAS, its four modified forms, T-2 toxin, HT-2 toxin and neosolaniol in cereals. The four modified forms of 4,15-DAS were detected in samples of Job's tears products.

Keywords: 4,15-diacetoxyscirpenol, modified mycotoxin, cereal

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Bryła M*1, Ksieniewicz-Woźniak E*1, Yoshinari T, Waśkiewicz A*2, Szymczyk K*1: Contamination of wheat cultivated in various regions of Poland during 2017 and 2018 agricultural sasons with selected trichothecenes and their modified forms.

*Toxins* (*Basel*). 2019;11:E88

Cross-interaction of antibodies within the immunoaffinity columns used in this study facilitated the simultaneous determination of nivalenol (NIV), deoxynivalenol (DON), their glucoside derivatives (NIV-3G, DON-3G), and 3-acetyl-deoxynivalenol (3-AcDON) in wheat grain harvested in various regions of Poland. DON was strongly correlated with DON-3G (correlation coefficient r = 0.9558), while NIV was strongly correlated with NIV-3G (r = 0.9442). The percentage occurrence of NIV-3G- and DON-3G-positive samples was 14% in 2017 and 49% in 2018. The NIV-3G/NIV ratio was 5.9-35.7%, while the DON-3G/DON ratio range was 3.2-53.6%.

Keywords: deoxynivalenol-3-glucoside, nivalenol-3-glucoside, trichothecenes

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*病原微生物検出情報*　2018;39:18-9

長野県で発生した*K. iwataiが*関与していると考える事例について紹介した.

Keywords：*Kudoa*，粘液胞子虫，食中毒

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Okitsu K*1, Hattori T, Misawa T, Shoda T, Kurihara M*2, Naito M, Demizu Y: Development of small hybrid molecule that mediates degradation of His-Tag fused proteins.

*J. Med. Chem*. 2018, *61*, 576-582.

In recent years, the induction of target–protein degradation via the ubiquitin–proteasome system (UPS) mediated by small molecules has attracted attention, and this approach has applications in pharmaceutical development. However, this technique requires a ligand for the target protein that can be

incorporated into tailor-made molecules, and there are many proteins for which such ligands have not been found. In this study, we developed a protein-knockdown method that recognizes a His-tag fused to a protein of interest. This strategy theoretically allows comprehensive targeting of proteins of interest by a particular molecule recognizing the tag. As expected, our hybrid molecule **10** [SNIPER(CH6)] efficiently degraded His-tagged CRABP-II and Smad2 in cells. This system provides an easy method to determine the susceptibility of proteins of interest to UPS-mediated degradation. Furthermore, we hope that this method will become an efficient tool to analyze the function of the UPS.

Keywords: His-tag fused protein, protein knockdown, ubiquitin-proteasome system, CRABP-II, Smad 2

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Eto R*1, Misawa T, Noguchi-Yachide T*2, Ohoka N, Kurihara M*3, Naito M, Tanaka M*1, Demizu Y: Design and synthesis of estrogen receptor ligands with a 4-heterocycle-4-phenylheptane skeleton.
*Bioorg. Med. Chem.* 2018, *26*, 1638-1642.

The estrogen receptor (ER), a member of the nuclear receptor (NR) family, is involved in the regulation of physiological effects such as reproduction and bone homeostasis. Approximately 70% of human breast cancers are hormone-dependent and ER $\alpha$-positive, and, thus, ER antagonists are broadly used in breast cancer therapy. We herein designed and synthesized a set of ER antagonists with a 4-heterocycle-4-phenylheptane skeleton.

Keywords: antagonist, estrogen receptor, heterocycles

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Koba Y*1, Ueda A*1, Oba M*1, Doi M*2, Kato T*2, Demizu Y, Tanaka M*1: Left-handed helix of three-membered ring amino acid homopeptide interrupted by an N-H···ethereal O type hydrogen bond.
*Org Lett.* 2018 *20*, 7830-7834.

A chiral three-membered ring C$\alpha$,$\alpha$-disubstituted $\alpha$-amino acid (*R*,*R*)-$Ac_3c^{dMOM}$, in which the $\alpha$-carbon is not a chiral center, but two side chain $\beta$-carbons are chiral centers, was synthesized from dimethyl L-(+)-tartrate, and its homopeptides were prepared. X-ray crystallographic analysis of (*R*,*R*)-$Ac_3c^{dMOM}$ pentapeptide showed bent left-handed (*M*) $3_{10}$-helical structures with an unusual intramolecular hydrogen bond of the N–H···O (ethereal) type. The left-handedness of the bent helices was exclusively controlled by the side-chain $\beta$-carbon chiral centers.

Keywords: peptide, helical structure, non-proteinogenic amino acids, X-ray diffraction

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Kobayashi H, Misawa T, Oba M*1, Hirata N, Kanda Y, Tanaka M*1, Matsuno K*2, Demizu Y: Structural development of cell-penetrating peptides containing cationic proline derivatives.
*Chem. Pharm. Bull.* 2018, *66*, 575-580.

We designed and synthesized a series of cell-penetrating peptides containing cationic proline derivatives ($Pro^{Gu}$) that exhibited responsive changes in their secondary structures to the cellular environment. Effects of the peptide length and steric arrangement of the side chain in cationic proline derivatives [$Pro^{4SGu}$ and $Pro^{4RGu}$] on their secondary structures and cell membrane permeability were investigated. Moreover, peptides **3** and **8** exhibited efficient intracellular delivery of plasmid DNA.

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Misawa T, Tsuji G, Takahashi T, Ochiai E*1, Takagi K, Horie K*1, Kakuda S*1, Takimoto-Kamimura M*1, Kurihara M*2, Demizu Y: Structural development of non-secosteroidal vitamin D receptor (VDR) ligands without any asymmetric carbon.
*Bioorg. Med. Chem.* 2018, *26*, 6146-6152.

Non-secosteroidal VDR ligands without any assymmetric carbon were designed and synthesized based on the structure of the previously reported non-secosteroidal VDR agonist LG190178. The VDR-agonistic activity of all synthesized compounds was evaluated, and **7b** emerged as a potent agonist activity with an $EC_{50}$ value of 9.26 nM. Moreover, a docking

simulation analysis was also performed to determine the binding mode of **7b** with VDR-LBD.

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Tsuji G, Misawa T, Doi T*, Demizu Y: Extent of helical induction of 2-aminoisobutyric acid into oligovaline sequence.
*ACS Omega* 2018 *3*, 6395-6399.

The preferred conformations of a dodecapeptide composed of l-valine (l-Val) and α-aminoisobutyric acid (Aib) residues, Boc-(l-Val-l-Val-Aib)$_4$-OMe, were analyzed in solution and in the crystalline state. Peptide **3** predominantly folded into a mixture of α- and $3_{10}$-(*P*) helical structures in solution and a (*P*) α helix in the crystalline state.
Keywords: peptide, helical structure, non-proteinogenic amino acids, X-ray diffraction

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Tsuji G, Hattori T, Kato M, Hakamata W*1, Inoue H*2, Naito M, Kurihara, M*3, Demizu Y, Shoda T: Synthesis of Cell-permeable Fluorescent Nitrilotriacetic Acid Derivatives.
*Bioorg Med. Chem*. 2018 *26*, 5494-5498.

Fluorescence labeling of the target molecules using a small molecule-based probe is superior than a method using genetically expressed green fluorescence protein (GFP) in terms of convenience in its preparation and functionalization. Fluorophore-nitrilotriacetic acid (NTA) conjugates with several ester protecting groups were synthesized and evaluated for their cell membrane permeability by fluorescence microscopy analysis. One of the derivatives, acetoxymethyl (AM) -protected NTA conjugate is hydrolyzed, resulting in intracellular accumulation, thus providing localized fluorescence intensity in cells. This modification is expected as an effective method for converting a non-cell membrane permeable NTA-BODIPY conjugates to a cell membrane permeable derivatives.
Keywords: acetoxymethyl group, BODIPY, cell membrane permeability, NTA

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Takabatake R*1, Kagiya Y*2, Minegishi Y*3, Futo S*2, Soga K, Nakamura K, Kondo K, Mano J*1, Kitta K*1: Rapid Screening Detection of Genetically Modified Crops by Loop-Mediated Isothermal Amplification with a Lateral Flow Dipstick.
*J Agric Food Chem*. 2018; 66: 7839-7845.

We developed a novel loop-mediated isothermal amplification (LAMP)-based detection method using lateral flow dipstick chromatography for genetically modified (GM) soybean and maize events. The single-stranded tag hybridization (STH) for the chromatography printed-array strip (C-PAS) system was used for detections targeting the cauliflower mosaic virus 35S promoter, mannose-6-phosphate isomerase gene, *Pisum sativum* ribulose 1, 5-bisphosphate carboxylase terminator, a common sequence between the Cry1Ab and Cry1Ac genes, and a GA21-specific sequence. The STH C-PAS system was applicable for multiplex analyses to perform simultaneous detections. The limit of detection was 0.5% or less for each target. By using the developed method, the LAMP amplification was visually detected. Moreover, the detection could be carried out without any expensive instruments, even for the DNA amplification steps, by virtue of the isothermal reaction. We demonstrated that the rapid and useful method developed here would be applicable for screening GM crops.
Keywords: dipstick DNA chromatography, genetically modified, LAMP

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Nakanishi K*1, Fujii U*2, Nakamura K, Ohtsuki T*3, Kimata S, Soga K, Kishine M*4, Mano J*4, Takabatake R*4, Kitta K*4, Ohmori K*5, Kawakami H*2, Akiyama H, Ikeda M*1, Kondo K: Effect of sodium carboxymethyl cellulose in processed rice foods on detection of genetically modified rice-derived DNA.
*Jpn J Food Chem*. 2018; 25: 77-85.

The effect of sodium carboxymethyl cellulose (CMC), a food additive used as a thickener and emulsion stabilizer, on detection of genetically modified

(GM) foods was evaluated. The addition of CMC to processed rice foods at 2% (w/w) concentration inhibited the yield of DNA in the DNA purification step by up to 40% and 70% using ion-exchange resin-type DNA purification kit and silica membrane-type DNA purification kit, respectively. The DNA yield from the rice vermicelli commodities containing CMC was significantly lower than that from the CMC-free rice vermicelli commodities. When 2% (w/w) CMC was contained in the rice flour with < 5,000 copies of transgenic genes for GM rice, the false negative rate in the real-time polymerase chain reaction detection targeting the genes was more than 10%. The CMC attenuates the DNA purification efficiency from the rice food samples, and may interfere with the GM rice testing using DNA samples prepared from processed rice foods containing CMC.

Keywords: genetically modified rice, sodium carboxymethylcellulose, real-time PCR

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Nakamura K, Ishigaki T, Kobayashi T, Kimata S, Soga K, Fujii U*1, Kishine M*2, Takabatake R*2, Mano J*2, Kitta K*2, Kawakami H*1, Nishimaki-Mogami T, Kondo K：Identification of chickpea (*Cicer arietinum*) in foods using a novel real-time polymerase chain reaction detection method.

*J Food Compost Anal.* 2018; 71: 8-16.

A novel real-time polymerase chain reaction (PCR) method for detecting chickpea was developed. From homologous sequences of 9-cis-epoxycarotenoid dioxygenase gene (NCED) among leguminous species, chickpea's NCED was cloned, and the Southern-blot analysis showed that NCED is a single copy gene in a haploid genome. Its coding sequences at the 5' terminus were found unique to chickpea and conserved among various chickpea varieties. Developed real-time PCR method targeting the unique sequences was specific to chickpea and had detection limit of approximately 30 copies per a reaction, and applicable for qualitative detection of chickpea in various forms of food products including dried, powdered, retort-packed, canned, fermented and pasted. Our results showed that the developed method enables identification of chickpea in foods.

Keywords: food analysis, *Cicer arietinum*, real-time PCR

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Kishine M*, Noguchi A, Mano J*, Takabatake R*, Nakamura K, Kondo K, Kitta K*: Detection of DNA in highly processed foods.

*Food Hyg Safety Sci.* 2018; 59: 151-156. (邦文)

Highly processed foods, including soy sauce, cornflakes, starch sugar, beet sugar and vegetable oil, are not currently subject to genetically modified (GM) food labeling, because DNA could not be detected in these food products. Here we re-examined the method of DNA extraction from starch syrup, beet sugar and vegetable oil using commercially available DNA extraction kits. We found that DNA was not stably detected by PCR targeting a species-specific endogenous plant gene. The reason for this may have been that the DNA yield was below the detection limit, because PCR inhibition was not observed.

Keywords: DNA extraction, genetically modified food, real-time PCR

* Food Research Institute, NARO

Kawaguchi N*, Tomita C*, Naradate R*, Higami T*, Nakamura K, Date K*, Aikawa K*, Ogawa H：A novel protocol for the preparation of active recombinant human pancreatic lipase from *Escherichia coli*.

*J Biochem.* 2018; 164: 407-414.

An active recombinant human pancreatic lipase (recHPL) was successfully prepared for the first time from the *Escherichia coli* expression system using short Strep-tag II (ST II). The recHPL–ST II was solubilized using 8 M urea from *E.coli* lysate and purified on a Strep-Tactin-Sepharose column. After refolding by stepwise dialyses in the presence of glycerol and $Ca^{2+}$ for 2 days followed by gel filtration, 1.8–6 mg of active recHPL–ST II was obtained from 1 L of culture. The recHPL was non-glycosylated, but showed almost equal specific activity, pH-

dependency and time-dependent stability compared to those of native porcine pancreatic lipase (PPL) at 37℃. However, the recHPL lost its lipolytic activity above 50℃, showing a lower heat-stability than that of native PPL, which retained half its activity at this temperature.

Keywords: *Escherichia coli*, human pancreatic lipase, lipolytic activity

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Koizumi D*, Shirota K*, Oda H*, Adachi R, Sakai, S, Akiyama H, Nishimaki-Mogami T, Teshima R: Development and Evaluation of an Enzyme-Linked Immunosorbent Assay Using a Nonpoisonous Extraction System for the Determination of Crustacean Protein in Processed Foods.

*J AOAC Int.* 2018; 101(3): 798-804.

Crustacean proteins are food allergens that cause severe allergic reactions in patients with food allergies; therefore, the identification of crustaceans such as shrimp, crab, and lobster as ingredients in processed food products is mandatory in Japan. We previously developed and validated an ELISA method coupled with an extraction process using the surfactant sodium dodecyl sulfate and the reductant 2-mercaptoethanol (2-ME) to quantify crustacean protein. However, 2-ME was designated as poisonous in Japan in 2008. Therefore, in this study, we developed and evaluated an ELISA method for detecting and quantifying crustacean protein that uses sodium sulfite (Na2SO3) in place of 2-ME for extraction. The proposed ELISA method showed high sensitivity, with an LOQ of 0.66 μg protein/g food sample. Furthermore, the proposed method showed high specificity for the Decapoda order within the subphylum Crustacea, with recoveries ranging from 83.8 to 100.8% for model processed foods, as well as high reproducibility (intra- and interassay CVs of ≤8.2%) and high correlation with our previously validated ELISA method for processed foods (correlation coefficient of 0.996). The proposed ELISA method does not require the use of poisonous reagents, provides acceptable accuracy, and is useful for the routine monitoring of food products.

Keywords: crustacean protein, ELISA, processed food

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Kamemura N*1, Sugimoto M*2, Tamehiro N, Adachi R, Tomonari S*2, Watanabe T*2, Mito T*2: Cross-allergenicity of crustacean and the edible insect Gryllus bimaculatus in patients with shrimp allergy.

*Mol Immunol.* 2019; 106: 127-134.

Food scarcity is a serious problem for many developing as well as developed countries. Edible insects have attracted attention recently as a novel food source. Crickets are especially high in nutritional value and easy to breed and harvest. In this study, we evaluated the risk of allergic reactions associated with cricket consumption in individuals with crustacean allergy. We evaluated food allergy risk in the consumption of *Gryllus bimaculatus* (cricket) in patients with shrimp allergy, using enzyme-linked immunosorbent assay (ELISA) and IgE crosslinking-induced luciferase expression assay (EXiLE). Sera from individuals with shrimp allergy (positive for shrimp-specific IgE by ImmunoCAP (>0.35 UA/mL; n=9) or without shrimp allergy (negative for shrimp-specific IgE; n=6) were obtained. There was a strong correlation between shrimp- and Gryllus-specific IgE levels obtained by ELISA (rs=0.99; P< 0.001). The binding of shrimp-specific IgE on shrimp allergen was dose-dependently inhibited by Gryllus allergen (0-1.0 mg/mL). There was a strong correlation between shrimp- and Gryllus-specific IgE responses, as assessed by EXiLE assays (rs=0.89; P<0.001). We determined that a protein of approximately 40kDa reacted with the positive, but not negative, sera for shrimp-specific IgE by ImmunoCAP. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis identified the major allergen in shrimp and Gryllus to be tropomyosin. Our data suggest that the cricket allergen has the potential to induce an allergic reaction in individuals with crustacean allergy. Therefore, allergy risk and shrimp-specific IgE levels should be considered before consumption of cricket meal.

Keywords: allergy, cricket meal, edible insect

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Tamehiro N, Adachi R, Kimura Y, Sakai S, Teshima R, Kondo K: Determining Food Allergens by Skin Sensitization in Mice.

*Curr Protoc Toxicol.* 2018; 76: e48.

A food allergy is a chronic inflammatory disease against dietary antigens with high prevalence in industrialized countries. Because there is currently no cure for food allergies, avoiding the allergen is crucial for the prevention of an allergic reaction. Therefore, a further understanding of the pathogenesis and risk factors that augment the sensitization to food allergens is required. We have previously developed a food allergy mouse model using transdermal sensitization, which influences the susceptibility to food allergies. In this model, mice sensitized with partially hydrolyzed wheat protein (HWP) successfully resembled the major features of HWP-sensitized and wheat allergy-induced patients. In this article, we describe transdermal sensitization of food allergens and induction of immediate-type food allergies in mice. The methodology detailed here was mainly adapted from an original work by Adachi and colleagues with some modifications to the dressing methods to reduce stress.
Keywords: allergen, animal model, food allergy

牟田朱美*，宮﨑悦子*，中牟田啓子*，渡邉敬浩：Carrez抽出を用いた加工食品中の保存料・甘味料一斉分析に伴う不確かさの推定
*日本食品化学学会誌* 2018;25:167-173

多様な食品を対象に実施される検査への信頼をより確実なものとするために，Carrez抽出法を用いた保存料（ソルビン酸［SOA］，安息香酸［BA］，デヒドロ酢酸［DHA］）及び甘味料（アセスルファムカリウム［Aces-K］，サッカリンナトリウム［Sac-Na］）一斉分析の不確かさをトップダウンアプローチにより推定した．保存料0.15 g/kg，甘味料0.10 g/kgを23種類の食品に対し添加し分析した結果，82～98%の回収率が得られた．併行精度は0.9～3.1%，室内精度は4.2～6.8%であり，拡張不確かさを考慮した分析値の範囲は，Aces-K：0.090～0.11 g/kg，Sac-Na：0.082～0.11 g/kg，SOA：0.12～0.16 g/kg，BA：0.13～0.16 g/kg，DHA：0.11～0.14 g/kgであった．
Keywords：食品添加物，加工食品，不確かさ

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Okiyama Y, Nakano T, Watanabe C*1, Fukuzawa K*2, Mochizuki Y*3, Tanaka S*4: Fragment molecular orbital calculations with implicit solvent based on the Poisson–Boltzmann equation: Implementation and DNA study.
*J. Phys. Chem. B* 2018;122:4457–4471.

A fragment molecular orbital methodology coupled with the Poisson–Boltzmann implicit solvent model was developed to analyze the electronic properties of large biomolecules in solution. We applied this methodology to a deoxyribonucleic acid duplex: the energy levels of frontier molecular orbitals on each fragment are successfully shifted down to those guaranteeing stable electronic states, and the solvation free energy also shows good agreement with that in the explicit solvent study.
Keywords: fragment molecular orbital (FMO) method, Poisson–Boltzmann implicit solvent model, deoxyribonucleic acid (DNA)

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Sun Y, Woess K*1, Kienzl M*1, Leb-Reichl VM*1, Feinle A*3, Wimmer M*1, Zauner R*1, Wally V*1, Luetz-Meindl U*3, Mellerio JE*4, Fuentes I*5, South AP*6, Bauer JW*1, Reichelt J*1, Furihata T*2, Guttmann-Gruber C*1, Piñón Hofbauer J*1: Extracellular Vesicles as Biomarkers for the Detection of a Tumor Marker Gene in Epidermolysis Bullosa-Associated Squamous Cell Carcinoma.
*J Invest Dermatol.* 2018;138:1197-1200.

Recessive dystrophic epidermolysis bullosa (RDEB) patients develop highly aggressive squamous cell carcinoma (SCC) because of repeated cycles of wounding, infection, and chronic inflammation. Arising tumors resemble non-healing wounds or exuberant granulation tissue, requiring invasive biopsies and histological analyses to confirm diagnosis. Therefore, we investigated the feasibility of utilizing tumor-derived EVs as liquid biopsies for the detection of a recently described tumor marker gene Ct-SLCO1B3 (also known as Ct-OATP1B3 mRNA). We have evaluated Ct-SLCO1B3 RNA products for their potential to discriminate between malignant and non-malignant tissue and cells. It was found that Ct-SLCO1B3 mRNA was specifically detected in EB-SCC cells and tumor tissue but not in non-malignant EB and wild type keratinocytes and tissue. Moreover, we could further detect Ct-SLCO1B3 mRNA in extracellular

vehicles (EVs) harvested from conditioned medium of EB-SCC cells cultured in vitro, as well as from the serum of EB-SCC-bearing xenograft mice. In contrast, Ct-SLCO1B3 mRNA was distinctly absent in EVs derived from all control samples tested. Our data show that tumor-specific Ct-SLCO1B3 transcripts exist in RDEB-SCC derived EVs, highlighting the feasibility of this minimally invasive method in the detection of RDEB-SCC particularly once it has metastasized beyond the skin.

Keywords: extracellular vesicles, Ct-SLCO 1 B3, cancer biomarker

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Goda K*, Saito K, Muta K*, Kobayashi A*, Saito Y, Sugai S*: Ether-phosphatidylcholine characterized by consolidated plasma and liver lipidomics is a predictive biomarker for valproic acid-induced hepatic steatosis.

*J Toxicol Sci.* 2018 43:395-405.

Valproic acid (VPA) is known to induce hepatic steatosis due to mitochondrial toxicity in rodents and humans. In the present study, we administered VPA to SD rats for 3 or 14 days at 250 and 500 mg/kg and then performed lipidomics analysis to reveal VPA-induced alteration of the hepatic lipid profile and its association with the plasma lipid profile. VPA induced hepatic steatosis at the high dose level without any degenerative changes in the liver on day 4 (after 3 days dosing) and at the low dose level on day 15 (after 14 days dosing). We compared the plasma and hepatic lipid profiles obtained on day 4 between the VPA-treated and control rats using a multivariate analysis to determine differences between the two groups. In total, 36 species of plasma lipids and 24 species of hepatic lipids were identified as altered in the VPA-treated group. Of these lipid species, ether-phosphatidylcholines (ePCs), including PC(16:0e/22:4) and PC(16:0e/22:6), were decreased in both the plasma and liver from the low dose level on day 4, however, neither an increase in hepatic TG level nor histopathological hepatic steatosis was observed at either dose level on day 4. Hepatic mRNA levels of glycerone-phosphate O-acyltransferase (Gnpat), which is a key enzyme for biosynthesis of ePC, was also decreased by treatment with VPA along with the decrease in ePCs. In conclusion, the changes in ePCs, (PC[16:0e/22:4] and PC[16:0e/22:6]), have potential utility as predictive biomarkers for VPA-induced hepatic steatosis.

Keywords: biomarker, DILI, hepatic steatosis

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*JAMA Neurol.* 2018;75:842-849.

Carbamazepine, a commonly used antiepileptic drug, is one of the most common causes of cutaneous adverse drug reactions (cADRs) worldwide. The allele HLA-A*31:01 is reportedly associated with carbamazepine-induced cADRs in Japanese and European populations; however, the clinical utility of HLA-A*31:01 has not been evaluated. To assess the use of HLA-A*31:01 genetic screening to identify Japanese individuals at risk of carbamazepine-induced cADRs. This cohort study was conducted across 36 hospitals in Japan from January 2012 to November 2014 among 1202 patients who had been deemed suitable to start treatment with carbamazepine. Preemptive HLA-A*31:01 genetic screening was performed for 1187 participants. Patients who did not start treatment with carbamazepine or alternative drugs were excluded. Participants were interviewed once weekly for 8 weeks to monitor the development of cADRs. Data analysis was performed from June 8, 2015, to December 27, 2016. Neuropsychiatrists were

asked to prescribe carbamazepine for patients who tested negative for HLA-A*31:01 and alternative drugs for those who tested positive for HLA-A*31:01. Of the 1130 included patients who were prescribed carbamazepine or alternative drugs, the mean (range) age was 37.4 (0-95) years, 614 (54.3%) were men, and 198 (17.5%) were positive for HLA-A*31:01. Expert dermatologists identified 23 patients (2.0%) who had carbamazepine-induced cADRs, of which 4 patients required hospitalization. Drug-induced hypersensitivity syndrome was observed for 3 patients, maculopapular eruption for 9 patients, erythema multiforme for 5 patients, and an undetermined type of cADR for 6 patients. No patient developed Stevens-Johnson syndrome or toxic epidermal necrolysis. Compared with historical controls, the incidence of carbamazepine-induced cADRs was significantly decreased (for BioBank Japan data: incidence, 3.4%; odds ratio, 0.60; 95% CI, 0.36-1.00; P＝.048; for the Japan Medical Data Centre claims database: incidence, 5.1%; odds ratio, 0.39; 95% CI, 0.26-0.59; P＜.001). Preemptive HLA-A*31:01 genetic screening significantly decreased the incidence of carbamazepine-induced cADRs among Japanese patients, which suggests that it may be warranted in routine clinical practice.

Keywords: pharmacogenomics, carbamazepine, pre-screening test

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Imatoh T, Nishi T*1, Yasui M*2, Maeda T*3, Sai K, Saito Y, Une H*4, Babazono A*2: Association between dipeptidyl peptidase-4 inhibitors and urinary tract infection in elderly patients: A retrospective cohort study.

*Pharmacoepidemiol Drug Saf.* 2018;27:931-939.

Dipeptidyl peptidase-4 (DPP-4) inhibitors are a new class of antidiabetic drugs. Although they have been reported to increase the risk of infection, the findings are controversial. Given that urinary tract infections (UTIs) are common in the elderly, we conducted a retrospective cohort study by using health care insurance claims data, to elucidate the association between the DPP-4 inhibitors and the incidence of UTI in latter-stage elderly patients. We analyzed 25,111 Japanese patients aged 75 years and older between the fiscal years 2011 and 2016. Patients using DPP-4 inhibitors and sulfonylureas (SUs) were matched at a 1:1 ratio using propensity scoring. The Incidence rate ratio (IRR) of UTI was compared between users of SUs and users of DPP-4 inhibitors by Poisson regression. Moreover, subgroup analyses stratified by sex were conducted to evaluate whether the combination of prostatic hyperplasia and DPP-4 inhibitors is associated with the incidence of UTI in male patients. The use of DPP-4 inhibitors was associated with an increased risk of UTI (adjusted IRR 1.23, 95% CI [1.04-1.45]). After propensity score matching, the association remained significant (adjusted IRR 1.28, 95% CI [1.05-1.56]). Moreover, elderly male patients with prostatic hyperplasia who received DPP-4 inhibitors had a higher risk of UTI than SU users without prostatic hyperplasia (Matched: crude IRR 2.90, 95% CI [1.78-4.71]; adjusted IRR 2.32, 95% CI [1.40-3.84]). The long-term use of DPP-4 inhibitors by elderly patients, particularly male patients with prostatic hyperplasia, may increase the risk of UTI.

Keywords: dipeptidyl peptidase-4, urinary tract infections, pharmacoepidemiological study

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Kitamura K*, Ito R*, Umehara K*, Morio H*, Saito K, Suzuki S*, Hashimoto M*, Saito Y, Anzai N*, Akita H*, Chiba K*, Furihata T*: Differentiated HASTR/ci35 cells: A promising in vitro human astrocyte model for facilitating CNS drug development studies. *J Pharmacol Sci*. 2018 137:350-358.

Astrocytes have shown longstanding promise as therapeutic targets for various central nervous system diseases. To facilitate drug development targeting astrocytes, we have recently developed a new conditionally immortalized human astrocyte cell line, termed HASTR/ci35 cells. In this study, in order to further increase their chances to contribute to various astrocyte studies, we report on the development of a culture method that improves HASTR/ci35 cell differentiation status and provide several proofs related to their astrocyte characteristics. The culture method is based on the simultaneous elimination of serum effects and immortalization signals. The results of qPCR showed that the culture method significantly enhanced several astrocyte marker gene expression levels. Using the differentiated HASTR/ci35, we examined their response profiles to nucleotide treatment and inflammatory stimuli, along with their membrane fatty acid composition. Consequently, we found that they responded to ADP or UTP treatment with a transient increase of intracellular Ca2+ concentration, and that they could show reactive response to interleukin-1$\beta$ treatments. Furthermore, the membrane phospholipids of the cells were enriched with polyunsaturated fatty acids. To summarize, as a unique human astrocyte model carrying the capability of a differentiation induction properties, HASTR/ci35 cells are expected to contribute substantially to astrocyte-oriented drug development studies.

Keywords: astrocyte, central nervous system, drug development

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Saito K, Ikeda M*1, Kojima Y*2, Hosoi H*1, Saito Y, Kondo S*1: Lipid profiling of pre-treatment plasma reveals biomarker candidates associated with response rates and hand-foot skin reactions in sorafenib-treated patients.
*Cancer Chemother Pharmacol*. 2018;82:677-684.

Sorafenib is a multi-kinase inhibitor for treatment of advanced hepatocellular carcinoma (HCC). Beyond its clinical benefit against advanced HCC, the efficacy and safety of sorafenib chemotherapy are critical concerns. In this study, we addressed the lipid profiles associated with the efficacy and safety of sorafenib chemotherapy. Plasma samples from HCC patients before sorafenib chemotherapy (N=44) were collected and subjected to lipidomic analysis. We measured the levels of 176 lipids belonging to 8 classes of phosphoglycerolipids, 2 classes of sphingolipids, 3 classes of neutral lipids, and 4 other classes of lipids. To characterize lipids associated with efficacy, we compared the responder group (N=21; partial response and stable disease) with non-responder group (N=22; progressive disease). To characterize lipids associated with hand-foot skin reaction (HFSR), we compared the susceptible group (N=12; grade 2 and 3) with non-susceptible group (N=32; grade 0 and 1). The levels of 8 lipids, including phosphatidylcholine (PC)[34:2], PC[34:3]a, PC[35:2], PC[36:4]a, PC[34:3e], acylcarnitine (Car)[18:0], cholesterol ester[20:2], and diacylglycerol (DG)[34:2], were significantly lower in the responder group, and 6 out of 8 these lipids contained FA(18:2). In addition, the levels of 7 lipids (Car[12:0], Car[18:0], Car[18:1], Car[20:1] and fatty acid amides (FAA[16:0], FAA[18:0], and FAA[18:1]b)) were significantly lower in the group susceptible to HFSR. Our comprehensive lipidomics study using samples from sorafenib-treated patients with HCC revealed that significant differences in the lipid profiles of pre-treatment plasma were associated with sorafenib efficacy and sorafenib-induced HFSR. Validation using another set of patient plasma samples and elucidating the molecular basis of these changes will lead to better treatment with sorafenib chemotherapy.

Keywords: efficacy, hand–foot skin reaction, hepatocellular carcinoma

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Yokoyama U*1, Arakawa N, Ishiwata R*1, Yasuda S*1, Minami T*2, Goda M*1, Uchida K*2, Suzuki S*1, Matsumoto M*3, Koizumi N*1, Taguri M*1, Hirano H*1, Yoshimura K*4,5, Ogino H*1, Masuda M*1,3, Ishikawa Y*1: Proteomic analysis of aortic smooth muscle cell secretions reveals an association of myosin heavy chain 11 with abdominal aortic aneurysm.

*Am J Physiol Heart Circ Physiol.* 2018;315:H1012-H1018.

Abdominal aortic aneurysm (AAA) is a life-threatening disease, and no disease-specific circulating biomarkers for AAA screening are currently available. We have identified a smooth muscle cell (SMC)-specific biomarker for AAA. We cultured aneurysmal tunica media that were collected from eight patients undergoing elective open-repair surgeries. Secreted proteins in culture medium were subjected to liquid chromatography/tandem mass spectrometry. Myosin heavy chain 11 (myosin-11) was identified as a SMC-specific protein in the tunica media-derived secretions of all patients. We then examined myosin-11 protein concentrations by ELISA in plasma samples from patients with AAA (n=35) and age-matched healthy control subjects (n=34). Circulating myosin-11 levels were significantly higher in patients with AAA than control subjects. The area under the receiver-operating characteristic curve (AUC) of myosin-11 was 0.77, with a specificity of 65% at a sensitivity of 91%. Multivariate logistic regression analysis showed a significant association between the myosin-11 level and presence of AAA. When the myosin-11 level was combined with hypertension, it improved the prediction of AAA (AUC 0.88) more than hypertension per se. We then investigated the correlation between aortic diameter and circulating myosin-11 levels using AAA serum samples from patients undergoing endovascular aneurysm repair (n=20). Circulating myosin-11 levels were significantly correlated with maximum aortic diameter. Furthermore, changes in myosin-11 concentrations from the baseline 12 mo after endovascular aneurysm repair were associated with those in aortic diameter. These data suggest that circulating levels of myosin-11, which is a SMC-specific myosin isoform, may be useful as a biomarker for AAA. NEW & NOTEWORTHY Extensive studies have revealed that inflammation- or proteolysis-related proteins are proposed as biomarkers for abdominal aortic aneurysm (AAA). Changes in these protein concentrations are not specific for smooth muscle, which is a major part of AAA pathologies. Hence, no disease-specific circulating markers for AAA are currently available. We found, using secretome-based proteomic analysis on human AAA tunica media, that myosin heavy chain 11 was associated with AAA. Circulating myosin heavy chain 11 may be a new tissue-specific AAA marker.

Keywords: abdominal aortic aneurysm, biomarkers, myosin heavy chain

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Sun Y, Piñón Hofbauer J*1, Harada M*2, Wöss K*1, Koller U*1, Morio H*2, Stierschneider A*1, Kitamura K*2, Hashimoto M*2, Chiba K*2, Akita H*2, Anzai N*2, Reichelt J*1, Bauer JW*1, Guttmann-Gruber C*1, Furihata T*2: Cancer-type organic anion transporting polypeptide 1B3 is a target for cancer suicide gene therapy using RNA *trans*-splicing technology.

*Cancer Lett.* 2018;433:107-116.

Cancer-type organic anion transporting polypeptide 1B3 (Ct-OATP1B3) has been identified as a cancer-specific transcript in various solid cancers, including colorectal cancer. Given its excellent cancer-specific expression profile, we hypothesized that Ct-OATP1B3 could represent a promising target for cancer-specific expression of the suicide gene, herpes simplex virus 1 thymidine kinase (HSV-tk), via a spliceosome-mediated RNA trans-splicing (SMaRT) approach. SMaRT technology is used to recombine two RNA molecules to generate a chimeric transcript. In this study, we engineered an RNA trans-splicing molecule carrying a translation-defective HSV-tk sequence (RTM44), which was capable of inducing its own trans-splicing to the desired Ct-OATP1B3 pre-mRNA target. RTM44 expression in LS180 cells resulted in generation of Ct-OATP1B3/HSV-tk fusion mRNA. A functional translation start site contributed by the target pre-

mRNA restored HSV-tk protein expression, rendering LS180 cells sensitive to ganciclovir treatment in vitro and in xenografted mice. The observed effects are ascribed to accurate and efficient trans-splicing, as they were absent in cells carrying a splicing-deficient mutant of RTM44. Collectively, our data highlights Ct-OATP1B3 as an ideal target for the HSV-tk SMaRT suicide system, which opens up new translational avenues for Ct-OATP1B3-targeted cancer therapy.

Keywords: Ct-SLCO1B3, suicide gene therapy, spliceosome-mediated RNA *trans*-splicing

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Aoki H*1,2, Ito N*2,3, Kaniwa N, Saito Y, Wada Y*2, Nakajima K*2, Sago H*2, Murashima A*2, Okamoto A*1, Ito S*4: Low levels of amlodipine in breast milk and plasma.

*Breastfeed Med*. 2018;13:622-626.

Few clinical reports have addressed the use of the antihypertensive drug amlodipine during breastfeeding. The objective of this study is to characterize concentration-time profiles of amlodipine in maternal and infant plasma, and milk. Plasma and breast milk samples were obtained from eight nursing mothers and their nine newborn nursing infants (median postnatal age: 6.5 days, range 5-7 days). Participants were recruited from February 2009 to June 2009. Multiple blood and milk samples were obtained from the mothers over a 24 hours dosing interval. The blood of infants was also obtained at before and 8 hours after nursing. Amlodipine concentrations were determined by high-performance liquid chromatography. Relative infant dose (RID) was calculated by dividing the infant's dose via milk in mg/kg/day by the maternal dose in mg/kg/day, assuming that a daily intake of milk is 150mL/kg/day in the infants. Maximal amlodipine concentrations in mothers ranged from 4.4 to 14.7ng/mL in plasma, and 6.5 to 19.7ng/mL in milk (Average milk/plasma ratio: 1.4). RID was 3.4% of the maternal weight-adjusted dose. All plasma concentrations in infants were under the quantitation limit (0.4ng/mL). Infant exposure to amlodipine in breast milk appears very small, suggesting that amlodipine can be used with little influence on infants during breastfeeding.

Keywords: amlodipine, breast milk, pharmacokinetics

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*Clin Pharmacol Ther*. 2019;105:112-120.

Specific ethnic genetic backgrounds are associated with the risk of Stevens-Johnson syndrome / toxic epidermal necrolysis (SJS/TEN) especially in Asians. However, there have been no large cohort, multiple-country epidemiological studies of medication risk related to SJS/TEN in Asian populations. Thus, we analyzed the registration databases from multiple Asian countries who were treated during 1998-2017. A total 1,028 SJS/TEN cases were identified with the algorithm of drug causality for epidermal necrolysis. Furthermore, those medications labeled by the US Food and Drug Administration (FDA) as carrying a risk of SJS/TEN were also compared with the common causes of SJS/TEN in Asian countries. Oxcarbazepine, sulfasalazine, COX-II inhibitors, and strontium ranelate were identified as new potential causes. In addition to sulfa drugs and beta-lactam antibiotics, quinolones were also a common cause. Only one acetaminophen-induced SJS was identified, while several medications (e.g., oseltamivir, terbinafine, isotretinoin, and sorafenib) labeled as carrying a risk of SJS/TEN by the FDA were not found to have caused any of the cases in the Asian countries investigated in this study.

Keywords: severe cutaneous adverse reactions, drug label, US FDA

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Imatoh T, Sai K, Takeyama M*1, Hori K*2, Karayama M*2, Furuhashi K*2, Segawa K, Kimura M*2, Kawakami J*2, Saito Y: Identification of risk factors and development of detection algorithm for denosumab-induced hypocalcaemia.
*J Clin Pharm Ther*. 2019;44:62-68.

This study used electronic medical records to identify risk factors and establish a detection algorithm for denosumab-induced hypocalcaemia. We identified 201 patients with cancer who were initially prescribed denosumab. Hypocalcaemia was defined as an adjusted serum calcium level of ≤2.13 mmol/L. A diagnosis of denosumab-induced hypocalcaemia was confirmed by two physicians after reviewing patient medical records. We evaluated patient characteristics as potential screening factors. Moreover, a retrospective cohort study was conducted to identify risk factors for denosumab-induced hypocalcaemia. Odds ratios (ORs) were estimated using logistic regression analysis. We analysed 164 patients with a low risk of hypocalcaemia. Among these, 29 (17.7%) patients were suspected to have denosumab-induced hypocalcaemia. The times to onset of definitive hypocalcaemia were shorter among these patients than among patients with non-denosumab-induced hypocalcaemia. Based on receiver operating characteristic curve analysis, we used time to onset of hypocalcaemia of ≤90 days as a second screening factor. The positive predictive value of this factor was 87.5%. In the retrospective cohort study, a significant difference was observed among patients with serum alkaline phosphatase (ALP) levels of >5.95 μkat/L before initial prescription ($P < 0.01$). Patients with higher serum ALP levels had a 6.63 times higher risk of developing hypocalcaemia than those without increased serum ALP levels (OR: 6.63, 95% confidence interval [CI]: 1.79-29.31). The same results were observed in a sensitivity analysis using another database. We developed a detection algorithm for denosumab-induced hypocalcaemia based on calcium levels and time to onset of hypocalcaemia. We also identified elevated ALP levels as a risk factor for hypocalcaemia. Clinicians should carefully monitor initial serum calcium levels and screen for signs of hypocalcaemia in patients receiving denosumab who demonstrate elevated serum ALP levels.
Keywords: denosumab, denosumab-induced hypocalcaemia, risk factor

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Su SC*1, Chen CB*1,2, Chang WC*1, Wang CW*1,2, Fan WL*1, Lu LY*1,2, Nakamura R, Saito Y, Ueta M*3, Kinoshita S*3, Sukasem C*4,5, Yampayon K*4,6, Kijsanayotin P*6, Nakkam N*7, Saksit N*7,8, Tassaneeyakul W*7, Aihara M*9, Lin YJ*2, Chang CJ*2, Wu T*1, Hung SI*10, Chung WH*1,2: HLA Alleles and CYP2C9*3 as Predictors of Phenytoin Hypersensitivity in East Asians.
*Clin Pharmacol Ther*. 2019;105:476-485.

To develop a pre-emptive genetic test that comprises multiple predisposing alleles for the prevention of phenytoin-related severe cutaneous adverse reactions (SCARs), three sets of patients with phenytoin-SCAR and drug-tolerant controls from Taiwan, Thailand, and Japan, were enrolled for this study. In addition to cytochrome P450 (CYP)2C9*3, we found that HLA-B*13:01, HLA-B*15:02, and HLA-B*51:01 were significantly associated with phenytoin hypersensitivity with distinct phenotypic specificities. Strikingly, we showed an increase in predictive sensitivity of concurrently testing CYP2C9*3/HLA-B*13:01/HLA-B*15:02/HLA-B*51:01 from 30.5-71.9% for

selecting the individuals with the risk of developing phenytoin-SCAR in Taiwanese cohorts, accompanied by a specificity of 77.7% (combined sensitivity, 64.7%; specificity, 71.9% for three Asian populations). Meta-analysis of the four combined risk alleles showed significant associations with phenytoin-SCAR in three Asian populations. In conclusion, combining the assessment of risk alleles of HLA and CYP2C9 potentiated the usefulness of predictive genetic tests to prevent phenytoin hypersensitivity in Asians.

Keywords: severe cutaneous adverse reactions, phenytoin, HLA

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Okiyama Y, Watanabe C*1, Fukuzawa K*2, Mochizuki Y*3, Nakano T, Tanaka S*4: Fragment molecular orbital calculations with implicit solvent based on the Poisson–Boltzmann equation: II. Protein and its ligand-binding system studies.
*J. Phys. Chem. B* 2019;123:957–73.

The electronic properties of bioactive proteins were analyzed in solution using a fragment molecular orbital methodology coupled with an implicit solvent model based on the Poisson－Boltzmann surface area. We investigated the solvent effects on practical and heterogeneous target like ubiquitin protein. For ligand-binding complexes of estrogen receptor alpha, we also found the binding free energies evaluated by this methodology correlate well with the experimental binding affinities of bioactive compounds, even though they have different charges.

Keywords: fragment molecular orbital (FMO) method, Poisson–Boltzmann implicit solvent model, estrogen receptor alpha

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Yokota S, Oshio S*: A simple and robust quantitative analysis of retinol and retinyl palmitate using a liquid chromatographic isocratic method.
*J Food Drug Anal.* 2018;26(2):504-11.

Vitamin A is a vital nutritional substances that regulates biological activities including development, but is also associated with disease onset. The extent of vitamin A intake influences the retinoid content in the liver, the most important organ for the storage of vitamin A. Measurement of endogenous retinoid in biological samples is important to understand retinoid homeostasis. Here we present a reliable, highly sensitive, and robust method for the quantification of retinol and retinyl palmitate using a reverse-phase HPLC/UV isocratic method. We determined the impact of chronic dietary vitamin A on retinoid levels in livers of mice fed an AIN-93G semi-purified diet (4 IU/g) compared with an excess vitamin A diet (1000 IU/g) over a period from birth to 10 weeks of age. Coefficients of variation for intra-assays for both retinoids were less than 5%, suggesting a higher reproducibility than any other HPLC/UV gradient method. Limits of detection and quantification for retinol were 0.08 pmol, and 0.27 pmol, respectively, which are remarkably higher than previous results. Supplementation with higher doses of vitamin A over the study period significantly increased liver retinol and retinyl palmitate concentrations in adult mice. The assays described here provide a sensitive and rigorous quantification of endogenous retinol and retinyl palmitate, which can be used to help determine retinoid homeostasis in disease states, such as toxic hepatitis and liver cancer.

Keywords: vitamin A excess, retinol, retinyl palmitate, liver

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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*. 2018 Dec 5; pii: S0168-0102(18)30388-2.

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 14Hz, 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 14Hz. Our results highlight usefulness of the operant conditioning method in measurement of the mouse visual temporal resolution.

Keywords: touchscreen, operant behavior, critical flicker-fusion frequency

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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.

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, spermatogonial stem cells, retinoid, mice

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Ono R, Yasuhiko Y, Aisaki KI, Kitajima S, Kanno J*, Hirabayashi Y: Exosome-mediated horizontal gene transfer occurs in double-strand break repair during genome editing.

*Communications Biology*. 2019 Feb 8;2:57.

The CRISPR-Cas9 system has been successfully applied in many organisms as a powerful genome-editing tool. Undoubtedly, it will soon be applied to human genome editing, including gene therapy. We have previously reported that unintentional DNA sequences derived from retrotransposons, genomic DNA, mRNA and vectors are captured at double-strand breaks (DSBs) sites when DSBs are introduced by the CRISPR-Cas9 system. Therefore, it is possible that unintentional insertions associated with DSB repair represent a potential risk for human genome editing gene therapies. To address this possibility, comprehensive sequencing of DSB sites was performed. Here, we report that exosome-mediated horizontal gene transfer occurs in DSB repair during genome editing. Exosomes are present in all fluids from living animals, including seawater and breathing mammals, suggesting that exosome-mediated horizontal gene transfer is the driving force behind

mammalian genome evolution. The findings of this study highlight an emerging new risk for this leading-edge technology.

Keyword: exosome

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Tanabe S, Ono R. The gene and microRNA networks of stem cells and reprogramming, AIMS Cell and *Tissue Engineering*. 2018;2(4): 238-245

The molecular interactions and regulations are dynamically changed in stem cells and reprogramming. This review article mainly focuses on the networks of molecules and epigenetic regulations including microRNA. The stem cells have molecular networks related to the stemness and the reprogramming of differentiated cells include the signaling networks consist of the transcriptional and post-transcriptional regulation of the genes and the protein modification. The gene expression is regulated by the binding of microRNAs towards the regulating regions of the coding genes. The molecular network pathways in stem cells include Wnt/$\beta$-catenin signaling and MAPK signaling, Shh signaling and Hippo signaling pathway. The epigenetic regulation of the genes included in the signaling pathways related to stem cells is mediated by the transcription factors and microRNAs consist of 18–25 nucleotides. Molecular interactions of the signaling proteins in stem cells is at least three factors including the quantity of the molecules partly regulated by the gene transcription and protein synthesis, the modification of the proteins such as phosphorylation, and localization of the molecules. In the epigenetic regulation level, the methylation and acetylation of genomes are critical for the regulation of the transcription. The binding sites and the combination of microRNAs, and regulated genes related to the stem cells and reprogramming are discussed in this review.

Mishima M[*1], Hoffmann D [*2], Ichihara G[*3], Kitajima S., Shibutani M[*4], Furukawa S[*5], Hirose A: Derivation of acceptable daily exposure value for alanine, N,N-bis (carboxymethyl)-, trisodium salt.

*Fund Toxicol Sci*. 2018;5:167-170.

Use of a non-phosphate detergent builder, alanine, N,N-bis(carboxymethyl)-trisodium salt (ABCT), has been expanded to wide range of washing and cleaning products for consumer uses and industrial applications including cleaning agents in food or pharmaceutical factories. Therefore, determination of acceptable daily exposure (ADE) of ABCT by oral, parenteral or inhalation route based on updated toxicity database could provide valuable information on the risk management for protection of consumers, patients and workers. Here, we proposed the ADEs based on the toxicological information of various *in vivo* and *in vitro* non-human studies. Because the full report of each toxicity study was not disclosed, derivation of the ADE was done based on available information mainly from ECHA database. ABCT exhibited renal toxicity as a main effect; however, ABCT did not exhibit carcinogenicity, genotoxicity, reproductive toxicity, irritation, and sensitization. Applying modification factors to the NOAEL of the animal study of longest treatment period, oral ADE was determined as 260 mg/person/day. Taking the oral bioavailability into the consideration of conversion to other routes, parenteral and inhalation ADEs were determined as 50 mg/person/day.

Keywords: alanine, N,N-bis (carboxymethyl), detergent builder, chelate, cleaning agent, aDE

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Tsuboi I*, Harada T*, Hirabayashi Y and Aizawa S*: Senescence-accelerated mice (SAMP1/TA-1) treated repeatedly with lipopolysaccharide develop a condition that resembles hemophagocytic lymphohistiocytosis.

*Haematologica*. 2019 doi: 10.3324/haematol.2018.209551. [Epub ahead of print]

Hemophagocytic lymphohistiocytosis is a life-threatening systemic hyperinflammatory disorder with primary and secondary forms. Primary hemophagocytic lymphohistiocytosis is associated with inherited defects in various genes that affect the immunological cytolytic

pathway. Secondary hemophagocytic lymphohistiocytosis is not inherited, but complicates various medical conditions including infection, autoinflammatory/autoimmune disease, and malignant disease. When senescence-accelerated mice (SAMP1/TA-1) with latent deterioration of immunological function and senescence-resistant control mice (SAMR1) were treated repeatedly with lipopolysaccharide, SAMP1/TA-1 mice displayed the clinicopathological features of hemophagocytic lymphohistiocytosis such as hepatosplenomegaly, pancytopenia, hypofibrinogenemia, hyperferritinemia, and hemophagocytosis. SAMR1 mice showed no features of hemophagocytic lymphohistiocytosis. Lipopolysaccharide induced up-regulation of proinflammatory cytokines such as interleukin-1beta, interleukin-6, tumor necrosis factor-alpha, and interferon-gamma, and interferon-gamma-inducible chemokines such as c-x-c motif chemokine ligand 9 and 10 in the liver and spleen in both SAMP1/TA-1 and SAMR1 mice. However, up-regulation of proinflammatory cytokines and interferon-gamma-inducible chemokines in the liver of SAMP1/TA-1 mice was prolonged compared with that in SAMR1 mice. In addition, the magnitude of up-regulation of interferon-gamma in the liver and spleen after lipopolysaccharide treatment was greater in SAMP1/TA-1 mice than in SAMR1 mice. Furthermore, lipopolysaccharide treatment led to a prolonged increase in the proportion of peritoneal M1 macrophages and simultaneously to a decrease in the proportion of M2 macrophages in SAMP1/TA-1 mice compared with SAMR1 mice. Lipopolysaccharide appeared to induce a hyperinflammatory reaction and prolonged inflammation in SAMP1/TA-1 mice, resulting in the features of secondary hemophagocytic lymphohistiocytosis. Thus, SAMP1/TA-1 mice represent a useful mouse model to investigate the pathogenesis of bacterial infection-associated secondary hemophagocytic lymphohistiocytosis.

Keywords: disseminated intravascular coagulation (DIC), hematopoiesis, infectious disorders

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Abdelgied M[*1], El-Gazzar AM[*1], Alexander DB[*1], Alexander WT[*1], Numano T[*1], Iigou M[*1], Naiki-Ito A[*1], Takase H[*1], Abdou KA[*1], Hirose A, Taquahashi Y, Kanno J[*2], Abdelhamid M[*1], 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 Feb 13;909-920.

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-n$TiO_2$, r-n$TiO_2$) 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-n$TiO_2$ 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-n$TiO_2$ 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-n$TiO_2$-treated groups, none of the measured parameters were different from the controls.

Keywords: intra-tracheal intra-pulmonary spraying, potassium octatitanate fibers, titanium dioxide nanoparticles

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Otsuka K[*1], Yamada K[*1], Taquahashi Y, Arakaki R[*1], Ushio A[*1], Saito M[*1], Yamada A[*1], Tsunematsu T[*1], Kudo Y[*1], Kanno J[*2], Ishimaru N: Long-term

polarization of alveolar macrophages to a profibrotic phenotype after inhalation exposure to multi-wall carbon nanotubes.

*PLoS One*. 2018 Oct 29;13(10):e0205702.

Nanomaterials are widely used in various fields. Although the toxicity of carbon nanotubes (CNTs) in pulmonary tissues has been demonstrated, the toxicological effect of CNTs on the immune system in the lung remains unclear. In this study, exposure to Taquann-treated multi-walled CNTs (T-CNTs) was performed using aerosols generated in an inhalation chamber. At 12 months after T-CNT exposure, alveolar inflammation with macrophage accumulation and hypertrophy of the alveolar walls were observed. In addition, fibrotic lesions were enhanced by T-CNT exposure. The macrophages in the bronchoalveolar lavage fluid of T-CNT-exposed mice were not largely shifted to any particular population, and were a mixed phenotype with M1 and M2 polarization. Moreover, the alveolar macrophages of T-CNT-exposed mice produced matrix metalloprotinase-12. These results suggest that T-CNT exposure promoted chronic inflammation and fibrotic lesion formation in profibrotic macrophages for prolonged periods.

Keywords: CNT, inhalation toxicity, macrophage

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Abdelgied M*1, El-Gazzar AM*1, Alexander DB*1, Alexander WT*1, Numano T*1, Iigou M*1, Naiki-Ito A*1, Takase H*1, Abdou KA*1, Hirose A, Taquahashi Y, Kanno J*2, Tsuda H*1, Takahashi S*1: Potassium octatitanate fibers induce persistent lung and pleural injury and are possibly carcinogenic in male Fischer 344 rats.

*Cancer Sci*. 2018 Jul;109(7):2164-2177.

Potassium octatitanate fibers ($K_2O \cdot 8TiO_2$, POT fibers) are widely used as an alternative to asbestos. We investigated the pulmonary and pleural toxicity of POT fibers with reference to 2 non-fibrous titanium dioxide nanoparticles ($nTiO_2$), photoreactive anatase (a-$nTiO_2$) and inert rutile (r-$nTiO_2$). Ten-week-old male F344 rats were given 0.5 mL of 250 μg/mL suspensions of POT fibers, a-$nTiO_2$, or r-$nTiO_2$, 8 times (1 mg/rat) over a 15-day period by trans-tracheal intrapulmonary spraying (TIPS). Rats were killed at 6 hours and at 4 weeks after the last TIPS dose. Alveolar macrophages were significantly increased in all treatment groups at 6 hours and at 4 weeks. At week 4, a-$nTiO_2$ and r-$nTiO_2$ were largely cleared from the lung whereas a major fraction of POT fibers were not cleared. In the bronchoalveolar lavage, alkaline phosphatase activity was elevated in all treatment groups, and lactate dehydrogenase (LDH) activity was elevated in the a-$nTiO_2$ and POT groups. In lung tissue, oxidative stress index and proliferating cell nuclear antigen (PCNA) index were elevated in the a-$nTiO_2$ and POT groups, and there was a significant elevation in C-C motif chemokine ligand 2 (CCL2) mRNA and protein in the POT group. In pleural cavity lavage, total protein was elevated in all 3 treatment groups, and LDH activity was elevated in the a-$nTiO_2$ and POT groups. Importantly, the PCNA index of the visceral mesothelium was increased in the POT group. Overall, POT fibers had greater biopersistence, induced higher expression of CCL2, and provoked a stronger tissue response than a-$nTiO_2$ or r-$nTiO_2$.

Keywords: inhalation toxicity, intra-tracheal intra-pulmonary spraying, potassium octatitanate fibers

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Shigemoto-Mogami Y, Hoshikawa K, Sato K: Activated Microglia Disrupt the Blood-Brain Barrier and Induce Chemokines and Cytokines in a Rat in vitro Model.

*Front Cell Neurosci* 2018;12:494

Severe neuroinflammation is associated with blood brain barrier (BBB) disruption in CNS diseases. Although microglial activation and the subsequent changes in cytokine/chemokine (C/C) concentrations are thought to be key steps in the development of neuroinflammation, little data are available concerning the interaction of microglia with BBB cells. In this study, we investigated this interaction by adding LPS-activated microglia (LPS-MG) to the abluminal side of a BBB model composed of endothelial cells (EC), pericytes (Peri) and astrocytes (Ast). We then examined the abluminal concentrations of 27 C/Cs and the interactions between the LPS-MG and BBB cells. LPS-MG caused collapse of the BBB, revealed by decreases in the trans-endothelial electrical resistance (TEER) and by changes in the expression levels of

tight junction（TJ）proteins. Under these conditions, 19 C/Cs were markedly increased on the abluminal side. Unexpectedly, although LPS-MG alone released 10 of the 19 C/Cs, their concentrations were much lower than those detected on the abluminal side of the BBB model supplemented with LPS-MG. Co-culture of LPS-MG with Ast caused marked increases in 12 of the 19 C/Cs, while co-culture of LPS-MG with EC and Peri resulted in a significant increase in only 1 of the 19 C/Cs（fractalkine）. These results suggest that C/C dynamics in this system are not only caused by activated microglia but also are due to the interaction between activated microglia and astrocytes.

Keywords: BBB disruption, inflammation, microglia

Yamazaki D, Kitaguchi T[*1], Ishimura M[*2], Taniguchi T[*3], Yamanishi A[*4], Saji D[*5], Takahashi E[*6], Oguchi M[*7], Moriyama Y[*2], Maeda S[*3], Miyamoto K[*6], Morimura K[*6], Ohnaka H[*5], Tashibu H[*7], Sekino Y[*8], Miyamoto N[*3], Kanda Y: Proarrhythmia risk prediction using human induced pluripotent stem cell-derived cardiomyocytes.

*J Pharmacol Sci*. 2018;136:249-256.

Human induced pluripotent stem cell-derived cardiomyocytes（hiPSC-CMs）are expected to become a useful tool for proarrhythmia risk prediction in the non-clinical drug development phase. Several features including electrophysiological properties, ion channel expression profile and drug responses were investigated using commercially available hiPSC-CMs, such as iCell-CMs and Cor.4U-CMs. Although drug-induced arrhythmia has been extensively examined by microelectrode array（MEA）assays in iCell-CMs, it has not been fully understood an availability of Cor.4U-CMs for proarrhythmia risk. Here, we evaluated the predictivity of proarrhythmia risk using Cor.4U-CMs. MEA assay revealed linear regression between inter-spike interval and field potential duration（FPD）. The hERG inhibitor E-4031 induced reverse-use dependent FPD prolongation. We next evaluated the proarrhythmia risk prediction by a two-dimensional map, which we have previously proposed. We determined the relative torsade de pointes risk score, based on the extent of FPD with Fridericia's correction（FPDcF）change and early afterdepolarization occurrence, and calculated the margins normalized to free effective therapeutic plasma concentrations. The drugs were classified into three risk groups using the two-dimensional map. This risk-categorization system showed high concordance with the torsadogenic information obtained by a public database CredibleMeds. Taken together, these results indicate that Cor.4U-CMs can be used for drug-induced proarrhythmia risk prediction.

Keywords: microelectrode array, proarrhythmia, hiPSC-CMs

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Kobayashi H[*1], Misawa T, Oba M[*2], Hirata N, Kanda Y, Tanaka M[*2], Matsuno K[*1], Demizu Y: Structural Development of Cell-Penetrating Peptides Containing Cationic Proline Derivatives.

*Chemical and Pharmaceutical Bulletin*. 2018; 66: 575-580.

We designed and synthesized a series of cell-penetrating peptides containing cationic proline derivatives（$Pro^{Gu}$）that exhibited responsive changes in their secondary structures to the cellular environment. Effects of the peptide length and steric arrangement of the side chain in cationic proline derivatives [$Pro^{4SGu}$ and $Pro^{4RGu}$] on their secondary structures and cell membrane permeability were investigated. Moreover, peptides 3 and 8 exhibited efficient intracellular delivery of plasmid DNA.

Keywords: cationic proline derivative, cell-penetrating peptide, drug delivery system（DDS）carrier

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Yamada S[*], Yamazaki D, Kanda Y: 5-Fluorouracil inhibits neural differentiation via Mfn1/2 reduction in human induced pluripotent stem cells.

*The Journal of Toxicological Sciences*. 2018;43:727-734.

5-fluorouracil (5-FU) has been widely used for the treatment of tumors. Regardless of its widespread use as an anti-cancer drug, 5-FU therapy can cause several side effects, including developmental toxicity and neurotoxicity. However, the potential action of 5-FU at the early fetal stage has not yet been completely elucidated. In the present study, we investigated the effect of 5-FU exposure on neural induction, using human induced pluripotent stem cells (iPSCs) as a model of human fetal stage. 5-FU exposure reduced the expression of several neural differentiation marker genes, such as OTX2, in iPSCs. Since the neural differentiation process requires ATP as a source of energy, we next examined intracellular ATP content using iPSCs. We found that 5-FU decreased intracellular ATP levels in iPSCs. We further focused on the effects of 5-FU on mitochondrial dynamics, which plays a role of ATP production. We found that 5-FU induced mitochondrial fragmentation and reduced the level of mitochondrial fusion proteins, mitofusin 1 and 2 (Mfn1/2). Double knockdown of Mfn1/2 genes in iPSCs downregulated the gene expression of OTX2, suggesting that Mfn mediates neural differentiation in iPSCs. Taken together, these results indicate that 5-FU has a neurotoxicity via Mfn-mediated mitochondria dynamics in iPSCs. Thus, mitochondrial dysfunction in iPSCs could be used as a possible marker for cytotoxic effects of drugs.

Keywords: 5-FU, induced pluripotent stem cells neural induction

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Yamada S*[1], Kubo Y, Yamazaki D, Sekino Y*[2], Nomura Y*[3], Yoshida S*[4], Kanda Y: Tributyltin Inhibits Neural Induction of Human Induced Pluripotent Stem Cells.
*Sci Rep*. 2018;8:12155.

Tributyltin (TBT), one of the organotin compounds, is a well-known environmental pollutant. In our recent study, we reported that TBT induces mitochondrial dysfunction, in human-induced pluripotent stem cells (iPSCs) through the degradation of mitofusin1 (Mfn1), which is a mitochondrial fusion factor. However, the effect of TBT toxicity on the developmental process of iPSCs was not clear. The present study examined the effect of TBT on the differentiation of iPSCs into the ectodermal, mesodermal, and endodermal germ layers. We found that exposure to nanomolar concentration of TBT (50nM) selectively inhibited the induction of iPSCs into the ectoderm, which is the first step in neurogenesis. We further assessed the effect of TBT on neural differentiation and found that it reduced the expression of several neural differentiation marker genes, which were also downregulated by Mfn1 knockdown in iPSCs. Taken together, these results indicate that TBT induces developmental neurotoxicity via Mfn1-mediated mitochondrial dysfunction in iPSCs.

Keywords: TBT, induced pluripotent stem cell, neural induction

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Yamada S*, Yamazaki D, Kanda Y: Silver nanoparticles inhibit neural induction in human induced pluripotent stem cells.
*Nanotoxicology*. 2018;12:836-846.

Silver nanoparticles (AgNPs) have been widely used as consumer products due to their antibacterial activities. Despite their extensive use, AgNPs have been reported to cause various types of cytotoxicity, including neurotoxicity. However, the potential action of AgNPs on early fetal development has not been elucidated. This study determined the effects of AgNPs on neural induction in human induced pluripotent stem cells (iPSCs), used as a model for human fetal stage development. It was observed that exposure to AgNPs reduced the expression of several neural differentiation marker genes, including OTX2, an early biomarker for neurogenesis in iPSCs. Since neural differentiation requires ATP as a source of energy, the intracellular ATP content was also measured. It was observed that AgNPs decreased intracellular ATP levels in iPSCs. Since AgNPs suppressed energy production, a critical mitochondrial function, the effects of AgNPs on mitochondrial dynamics were

further studied. The results revealed that AgNPs induced mitochondrial fragmentation and reduced the level of mitochondrial fusion protein mitofusin 1 (Mfn1). Previously, we reported that knockdown of Mfn1 in iPSCs inhibited neural induction via OTX2 downregulation. This suggested that AgNPs could induce cytotoxicity, including neurodevelopmental toxicity, via Mfn1-mediated mitochondrial dysfunction in iPSCs. Thus, mitochondrial function in iPSCs can be used for assessing the cytotoxic effects associated with nanomaterials, including AgNPs.

Keywords: AgNP, induced pluripotent stem cells, neural induction

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Watari R*1, Kakiki M*1, Oshikata A*2, Takezawa T*2, Yamasaki C*3, Ishida Y*3, Tateno C*3, Kuroda Y, Ishida S, Kusano K*1: A long-term culture system based on a collagen vitrigel membrane chamber that supports liver-specific functions of hepatocytes isolated from mice with humanized livers.

*J Toxicol Sci.* 2018;43:521-529.

During drug discovery, in vitro models are used to predict the in vivo pharmacokinetic and toxicological properties of drug candidates in humans. However, the conventional method of culturing human hepatocytes as monolayers does not necessarily replicate biologic reactions and does not support liver-specific functions, such as cytochrome P450 (CYP) activities, for prolonged periods. To remedy these problems and thus increase and prolong hepatic functions, we developed a culture system comprising a collagen vitrigel membrane (CVM) chamber and PXB-cells®, fresh hepatocytes isolated from liver-humanized chimeric mice (PXB-mice®). The results indicate that our vitrigel culture method is superior to the conventional monolayer method in terms of diverse liver-specific functions, including CYP activity. Our findings suggest that the vitrigel culture method could be a powerful in vitro tool for predicting the pharmacokinetic and toxicological properties of drug candidates in humans.

Keywords: collagen vitrigel membrane (CVM), cytochrome P450 (CYP), PXB-cells

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*3 PhoenixBio Co., Ltd.

Horiuchi S, Kuroda Y, Fujii R, Kim SR, Ishida S: Deactivation of Hepatic Stellate Cells by Culturing on VECELL Inserts.

*AATEX.* 2018;23:53-62.

Hepatic stellate cells play a cardinal role in the development of liver fibrosis. Quiescent hepatic stellate cells isolated from normal liver are activated by plating on a plastic culture dish. Therefore, a culture method that maintains hepatic stellate cells in a quiescent state is required for studies of fibrosis. We attempted to deactivate human hepatic stellate cells by culturing on VECELL® culture inserts (Preset VECELL). Cryopreserved human hepatic stellate cells and LI90 cells, which is a cell line established from an outgrowth of a human hepatic mesenchymal tumor, were cultured on Preset VECELL. The results suggest that human hepatic stellate cells were deactivated by VECELL cultivation, which could provide a model system for the analysis of deactivated human hepatic stellate cells. Thus, Preset VECELL will be a useful in vitro tool for the clarification of underlying mechanisms and the development of drugs to treat liver fibrosis. This study will contribute to provide alternative methods to animal tests that have been mainly carried out in studies of hepatic stellate cell, liver fibrosis, and liver cirrhosis.

Keywords: preset VECELL®, hepatic stellate cells, liver fibrosis.

Inamura K*, Komizu Y*, Yamakuchi M*, Ishida S, Matsumoto Y*, Matsushita T*: Inhibitory effect of hybrid liposomes on the growth of liver cancer stem cells.

*Biochem Biophys Res Commun.* 2019;509:268-274.

Cancer stem cells (CSCs) are involved in tumor progression, metastasis, and drug resistance. Hybrid liposomes (HLs) are nano-sized liposomal particles that can be easily prepared by ultrasonicating a mixture of vesicular and micellar molecules in buffer solutions. In this study, we investigated the inhibitory effects of HL on the growth of CSC subpopulations in liver cancer cells (HepG2) in vitro. HLs selectively inhibited liver cancer cell growth without affecting normal hepatocytes. Additionally, HLs induced apoptosis of HepG2 cells by activating caspase-3. Notably,

the CD133(+)/EpCAM(+) CSC sub-population of liver cancer cells treated with HLs was reduced. Furthermore, HLs markedly decreased the number of colony-forming cells. These results suggest that HLs are a novel nanomedical therapeutic agent for targeting CSCs in liver cancer therapy.

Keywords: cancer stem cell, doxorubicin, hybrid liposomes

* Sojo University

Watari R*1, Kakiki M*1, Yamasaki C*2, Ishida Y*2, Tateno C*2, Kuroda Y, Ishida S, Kusano K*1: Prediction of human hepatic clearance for cytochrome P450 substrates via a new culture method using the collagen vitrigel membrane chamber and fresh hepatocytes isolated from liver humanized mice.

*Biol. Pharm. Bull.* 2019;42:348-53

In drug discovery, hepatocytes have been widely utilized as in vitro tools for predicting the in vivo hepatic clearance (CL) of drug candidates. However, conventional hepatocyte models do not always reproduce in vivo physiological function, and CYP activities in particular decrease quite rapidly during culture. In order to accurately predict hepatic CL of candidate drugs, a new method of culturing hepatocytes that activates their functional properties, including CYP activities, is in high demand. In this study, the vitrigel culture method was applied to predictions of hepatic CL for 22 CYP typical substrates with low to middle CL, and the prediction accuracy by this method was assessed by comparing CL data between predicted and observed values. The results suggest that the new culture method using the CVM chamber and PXB-cells is a promising in vitro system for predicting human hepatic CL with high accuracy for CYP substrates, including metabolically stable drug candidates.

Keywords: CYP, PXB-cell, in vitro in vivo correlation (IVIVC)

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Blinova K*1, Dang Q*1, Millard D*2, Smith G*3, Pierson J*4, Guo L*5, Brock M*6, Lu HR*7, Kraushaar U*8, Zeng H*9, Shi H*10, Zhang X*11, Sawada K*12, Osada T*13, Kanda Y, Sekino Y*14, Pang L*1, Feaster TK*15, Kettenhofen R*16, Stockbridge N*1, Strauss DG*1, Gintant G*17: International Multisite Study of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Drug Proarrhythmic Potential Assessment.

*Cell Rep.* 2018;24:3582-3592.

To assess the utility of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) as an in vitro proarrhythmia model, we evaluated the concentration dependence and sources of variability of electrophysiologic responses to 28 drugs linked to low, intermediate, and high torsades de pointes (TdP) risk categories using two commercial cell lines and standardized protocols in a blinded multisite study using multielectrode array or voltage-sensing optical approaches. Logistical and ordinal linear regression models were constructed using drug responses as predictors and TdP risk categories as outcomes. Three of seven predictors (drug-induced arrhythmia-like events and prolongation of repolarization at either maximum tested or maximal clinical exposures) categorized drugs with reasonable accuracy (area under the curve values of receiver operator curves ~ 0.8). hiPSC-CM line, test site, and platform had minimal influence on drug categorization. These results demonstrate the utility of hiPSC-CMs to detect drug-induced proarrhythmic effects as part of the evolving Comprehensive In Vitro Proarrhythmia Assay paradigm.

Keywords: human-induced pluripotent stem cell-derived cardiomycotes, multi-electrode array, proarrhythmia

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Izumi-Nakaseko H[*1], Hagiwara-Nagasawa M[*1], Naito AT[*1], Goto A[*1], Chiba K[*1], Sekino Y[*2], Kanda Y, Sugiyama A[*1]: Application of human induced pluripotent stem cell-derived cardiomyocytes sheets with microelectrode array system to estimate antiarrhythmic properties of multi-ion channel blockers.
*J Pharmacol Sci*. 2018;137:372-380.

Wexamined electrophysiological indices of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) sheets in order to quantitatively estimate $Na^+$, $K^+$ and $Ca^{2+}$ channel blocking actions of bepridil and amiodarone using microelectrode array system in comparison with that of E-4031. We analyzed the field potential duration, effective refractory period, current threshold and conduction property using a programmed electrical stimulation protocol to obtain the post repolarization refractoriness and coefficient a of the relationship between the pacing cycle length and field potential duration. Electropharmacological profile of each drug was successfully characterized; namely, 1) the changes in the current threshold and conduction property provided basic information of $Na^+$ channel blocking kinetics, 2) the relationship between pacing cycle length and field potential duration reflected drug-induced inhibition of human ether-à-go-go-related gene (hERG) $K^+$ channel, 3) the post repolarization refractoriness indicated the relative contribution of these drugs to $Na^+$ and $K^+$ channel blockade, and 4) L-type $Ca^{2+}$ channel blocking action was more obvious in the field potential waveform of the hiPSC-CMs sheets than that expected in the electrocardiogram in humans. Thus, this information may help to better utilize the hiPSC-CMs sheets for grasping the properties and net effects of drug-induced $Na^+$, $Ca^{2+}$ and $K^+$ channel blockade.
Keywords: antiarrhythmic property, human induced pluripotent stem cell-derived cardiomyocytes, multichannel blocker

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Toyoda T, Matsushita K, Morikawa T, Yamada T, Miyoshi N[*], Ogawa K: Distinct differences in the mechanisms of mucosal damage and γ-H2AX formation in the rat urinary bladder treated with *o*-toluidine and *o*-anisidine.
*Arch Toxicol*. 2019;93:753-62.

Although aromatic amines are widely used as raw materials for dyes, some of them have been concerned about carcinogenicity in the urinary bladder. We examined early changes in histopathology and the formation of γ-H2AX, a biomarker of DNA damage, in the urinary bladder of rats to investigate the mechanisms of mucosal damage induced by monocyclic aromatic amines. Six-week-old male F344 rats were administered 0.4% or 0.8% *o*-toluidine, 0.3% or 1.0% *o*-anisidine, 0.4% 2,4-xylidine, 0.2% *p*-toluidine, or 0.6% aniline in the diet for 4 weeks. Animals were sequentially sacrificed from day 2 to after 2 weeks of recovery, and histopathological and immunohistochemical analyses were performed. In the 0.8% *o*-toluidine group, there was sequential progression of bladder lesions, characterized by edematous changes and intramucosal hemorrhage at day 2 and formation of granulation tissue with mononuclear cell infiltration at week 1, followed by diffuse hyperplasia at weeks 2 and 4. In the 1.0% *o*-anisidine group, simple hyperplasia only with slight inflammation was detected from week 1. Whereas γ-H2AX-positive bladder epithelial cells in the 1.0% *o*-anisidine group were significantly increased in a time-dependent manner, transient increases in γ-H2AX-positive cells were detected at day 2 and week 1 in the 0.8% *o*-toluidine group. No apparent bladder lesions or increases in γ-H2AX formation were observed in any other groups. These results revealed different mechanisms of bladder mucosal damage associated with *o*-toluidine and *o*-anisidine. Moreover, immunohistochemical analysis for γ-H2AX suggested that both compounds may induce DNA damage in epithelial cells, mainly basal cells, of the bladder mucosa.
Keywords: urinary bladder, γ-H 2 AX, aromatic amine

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Akagi J, Cho YM, Mizuta Y, Toyoda T, Ogawa K: Subchronic toxicity evaluation of 5-hexenyl isothiocyanate, a nature identical flavoring substance from *Wasabia japonica*, in F344/DuCrj rats.
*Food Chem Toxicol*. 2018;122:80-6.

5-Hexenyl isothiocyanate (5-HeITC) is a naturally derived flavoring substance from *Wasabia japonica*. To clarify the toxicological profile of 5-HeITC, we

performed a subchronic toxicity study of 5-HeITC with intragastric administration at daily doses of 0, 3, 12, or 48 mg/kg body weight (BW) to 6-week-old male and female F344/DuCrj rats for 13 weeks. Body weight gain was decreased in the male 48 mg/kg BW group. Decreased triglycerides were observed in the male over 12 mg/kg BW and female 48 mg/kg BW groups. Decreased total cholesterol was observed in the male 48 mg/kg BW group. Increases in relative liver weights were observed in the male 48 mg/kg BW and female over 12 mg/kg BW groups. Increases in absolute and relative heart weights were observed in the female over 12 mg/kg BW groups. Simple hyperplasia in the urinary bladder was found in the male and female 12 mg/kg BW groups, and nodular hyperplasia was found in the female 48 mg/kg BW group. Based on these findings, the target organs of 5-HeITC were determined to be the urinary bladder, heart, and liver. The no-observed-adverse-effect level of 5-HeITC for both sexes was estimated to be 3 mg/kg BW.

Keywords: 5-hexenyl isothiocyanate, food additive, urinary bladder

Yang Q[*1], Yasuda T[*2], Choi E[*1], Toyoda T, Roland JT[*1], Uchida E[*3], Yoshida H[*3], Seto Y[*2], Goldenring JR[*1], Nomura S[*2]: MEK inhibitor reverses metaplasia and allows re-emergence of normal lineages in *Helicobacter pylori*-infected gerbils.

*Gastroenterology*. 2019;156:577-81.

Recent studies in mice suggest that activation of Ras drives the development and progression of metaplasia in the stomach. MEK inhibitor treatment may inhibit this process and allow re-establishment of normal lineages in the stomach. Mongolian gerbils with one year of *H. pylori* infection treated with Selumetinib, a MEK inhibitor, for 4 weeks showed regression of metaplasia and recrudescence of normal gastric lineages in the stomach. The studies were performed in gerbils with continuing *H. pylori* infection. Selumetinib treatment could ameliorate metaplasia even with continued *H. pylori* infection. Selumetinib treatment might reduce gastric cancer risk in patients such as those who receive endoscopic resection of a Stage I cancer.

Keywords: MEK inhibitor, *Helicobacter pylori*, stomach

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Toyoda T, Totsuka Y[*1], Matsushita K, Morikawa T, Miyoshi N[*2], Wakabayashi K[*2], Ogawa K: γ-H2AX formation in the urinary bladder of rats treated with two norharman derivatives obtained from *o*-toluidine and aniline.

*J Appl Toxicol*. 2018;38:537-43.

Aminomethylphenylnorharman (AMPNH) and aminophenylnorharman (APNH) are mutagenic norharman derivatives obtained from *o*-toluidine and aniline, respectively. APNH is carcinogenic to the urinary bladder of rats and present in urine samples of healthy volunteers, indicating that norharman derivatives may be associated with cancer development in the urinary bladder of humans. To evaluate the possible role of AMPNH and APNH in bladder carcinogenesis, we examined the formation of γ-H2AX, a DNA damage response marker, in the urinary bladder of rats. Seven-week-old male F344 rats were treated with 400 ppm AMPNH or 40 ppm APNH in the diet for 4 weeks. Animals were sacrificed at the end of administration or after 2 weeks of recovery, and immunohistochemistry for γ-H2AX and Ki67, a cell proliferation marker, was performed. At week 4, γ-H2AX formation in bladder epithelial cells was significantly increased by APNH treatment as compared with that in controls. AMPNH also induced upregulation of γ-H2AX formation, although there was no statistical significance. After the recovery period, γ-H2AX-positive cells were reduced but remained significantly higher in AMPNH and APNH groups than in the control group. Ki67-positive cells were significantly increased by AMPNH and APNH at week 4 and reduced to the same level as the control after 2 weeks of recovery. Expression of KRT14, a bladder stem cell marker, was also increased in the basal layer by the two norharman derivatives. Thus, AMPNH and APNH showed *in vivo* genotoxicity in the bladder epithelium of rats, and APNH may be a potent causative agent of bladder carcinogenesis.

Keywords: urinary bladder, γ-H 2 AX, norharman

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Tanoue Y[*1], Toyoda T, Sun J[*1], Mustofa MK[*1], Tateishi C[*1], Endo S[*1], Motoyama N[*2], Araki K[*1], Wu D[*3], Okuno Y[*1], Tsukamoto T[*4], Takeya M[*1], Ihn H[*1], Vaziri C[*3], Tateishi S[*1]: Differential roles of Rad18 and Chk2 in genome maintenance and skin carcinogenesis following UV exposure.

*J Invest Dermatol*. 2018;138:2550-7.

Defects in DNA polymerase Eta (Polη) cause the sunlight-sensitivity and skin cancer-propensity disorder xeroderma pigmentosum variant (XP-V). The extent to which Polη function depends on the upstream E3 ubiquitin ligase Rad18 is controversial and has not been investigated using mouse models. Therefore, we tested the role of Rad18 in UV-inducible skin tumorigenesis. Because Rad18-deficiency leads to compensatory DNA damage signaling by Chk2, we also investigated genetic interactions between Rad18 and Chk2 *in vivo*. *Chk2*$^{-/-}$*Rad18*$^{-/-}$ mice were prone to spontaneous lymphomagenesis. *Chk2*$^{-/-}$ and *Chk2*$^{-/-}$*Rad18*$^{-/-}$ mice were both prone to UV-B irradiation-induced skin tumorigenesis when compared to wild-type (WT) animals but unexpectedly *Rad18*$^{-/-}$ mice did not recapitulate the skin tumor-propensity of Polη mutants. UV-irradiated *Rad18*$^{-/-}$ cells were more susceptible to G1/S arrest and apoptosis than WT cultures. Chk2-deficiency alleviated both UV-induced G1/S-phase arrest and apoptosis of WT and *Rad18*$^{-/-}$ cells, but led to increased genomic instability. Taken together, our results demonstrate that the tumor-suppressive role of Polη in UV-treated skin is Rad18-independent. We also define a role for Chk2 in suppressing UV-induced skin carcinogenesis *in vivo*. This study identifies Chk2 dysfunction as a new potential risk factor for sunlight-induced skin tumorigenesis in humans.

Keywords: carcinogenesis, UV radiation, DNA repair

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Funahashi S[*], Okazaki Y[*], Nagai H[*], Chew SH[*], Ogawa K, Toyoda T, Cho YM, Toyokuni S[*]: Twist1 was detected in mesenchymal cells of mammary fibroadenoma and invasive components of breast carcinoma in rats.

*J Toxicol Pathol*. 2019;32:19-26.

Fibroadenoma (FA) is a common mammary fibroepithelial tumor. Tumor size is increased by estrogen, progesterone, prolactin and pregnancy, whereas it decreases after menopause. These observations in humans indicate that FA is hormone-dependent. In rats, the most common mammary neoplasm is also FA. Expression levels of Twist1, a transcriptional regulator of epithelial-mesenchymal transition, were examined in paraffin-embedded tissue sections of an experimental rat breast model to find physiological alternations coincident with reproductive hormonal changes. Twenty-three Fischer 344/Brown Norway F1 hybrid rats were used as 14- to 16-week-old adolescent rats (n=3), pregnant rats (n=4), and lactating rats (n=6) in addition to rats over 100-weeks-old that exhibited aging (n=3) and FA (n=7). Seventy-six cases of chemically induced breast carcinoma and two cases of FA in Sprague-Dawley rats were also examined. Using tissue sections, we observed that Twist1-positive mesenchymal cells were predominantly located in the periductal region in adolescent and pregnant rats and in the terminal duct lobular unit in pregnant and elderly rats. Twist1 was also expressed diffusely in the mesenchymal cells of FA rats. Twist1-positive cancer-associated mesenchymal cells were found more frequently in the invasive components of breast carcinomas than in intraductal components. The expressions of Twist1 in mesenchymal cells were induced by physiological and pathological stimuli, suggesting the biological role of Twist1 in tissue structure. Further study may reveal the role of Twist1 in mesenchymal cells of mammary glands in rat.

Keywords: Twist1, fibroadenoma, rat

[*] Nagoya University

Tsuchiya T, Kijima A, Ishii Y, Takasu S, Yokoo Y, Nishikawa A, Yanai T[*], Umemura T: Mechanisms of oxidative stress-induced *in vivo* mutagenicity by potassium bromate and nitrofurantoin.

*J Toxicol Pathol*. 2018;31:179-88.

Oxidative stress is well known as a key factor of chemical carcinogenesis. However, the actual role of oxidative stress in carcinogenesis, such as oxidative stress-related *in vivo* mutagenicity, remains unclear. It has been reported that 8-hydroxydeoxyguanosine (8-OHdG), an oxidized DNA lesion, might contribute

to chemical carcinogenesis. Potassium bromate ($KBrO_3$) and nitrofurantoin (NFT) are known as renal carcinogens in rats. Our previous studies showed an increase in mutant frequencies accompanied by an increased level of 8-OHdG in the kidneys of rodents following $KBrO_3$ or NFT exposure. Furthermore, $KBrO_3$ and NFT induced different types of gene mutations. Thus, in the present study, we performed reporter gene mutation assays and 8-OHdG measurements following $KBrO_3$ or NFT exposure using *Nrf2*-proficient and *Nrf2*-deficient mice to clarify the relationship between $KBrO_3$- or NFT-induced oxidative stress and subsequent genotoxicity. Administration of 1,500 ppm of $KBrO_3$ in drinking water resulted in an increase in deletion mutations accompanied by an increase in 8-OHdG level, and administration of 2,500 ppm of NFT in diet induced an increase in guanine base substitution mutations without elevation of the 8-OHdG level in *Nrf2*-deficient mice. These results demonstrated that the formation of 8-OHdG, which resulted from the oxidizing potential of $KBrO_3$, was directly involved in the increase in deletion mutations, although factors related to oxidative stress other than 8-OHdG might be crucial for NFT-induced guanine base substitution mutations. The present study provides new insight into oxidative stress-related *in vivo* mutagenicity.
Keywords: DNA damage, NRF2, nitrofurantoin

* Gifu University

Tsuchiya T, Kijima A, Ishii Y, Takasu S, Yokoo Y, Nishikawa A, Yanai T*, Umemura T: Role of oxidative stress in the chemical structure-related genotoxicity of nitrofurantoin in *Nrf2*-deficient *gpt* delta mice.
*J Toxicol Pathol.* 2018;31:169-78.

Despite its antimicrobial activity, nitrofurantoin (NFT) is a renal carcinogen in rats. Oxidative stress induced by reduction of the nitro group of NFT may contribute to its genotoxicity. This is supported by our recent results indicating that the structure of the nitrofuran plays a key role in NFT-induced genotoxicity, and oxidative DNA damage is involved in renal carcinogenesis. Nuclear factor erythroid 2-related factor 2 (NRF2) regulates cellular responses to oxidative stress. To clarify the role of oxidative stress in the chemical structure-related genotoxic mechanism of NFT, we performed reporter gene mutation assays for NFT and 5-nitro-2-furaldehyde (NFA) using *Nrf2*-proficient and *Nrf2*-deficient *gpt* delta mice. NFT administration for 13 weeks resulted in a significant increase in 8-hydroxydeoxyguanosine (8-OHdG; a marker of oxidative stress) and *gpt* mutant frequency only in the kidneys of *Nrf2*$^{-/-}$ mice. The mutation spectrum, characterized by increased substitutions at guanine bases, suggested that oxidative stress is involved in NFT-induced genotoxicity. However, NFA did not increase the mutation frequency in the kidneys, despite the increased 8-OHdG in NFA-treated *Nrf2*$^{-/-}$ mice. Thus, it is unlikely that oxidative stress is involved in the genotoxic mechanism of NFA. These results imply that nitro reduction plays a key role in the genotoxicity of NFT, but the lack of a role of oxidative stress in the genotoxicity of NFA indicates a potential role of side chain interactions in oxidative stress caused by nitro reduction. These findings provide a basis for the development of safe nitrofurans.
Keywords: NRF2, nitrofurantoin, oxidative stress

* Gifu University

Hirata T, Cho YM, Suzuki I, Toyoda T, Akagi J, Nakamura Y*[1], Numasawa S*[2], Ogawa K: 4-Methylthio-3-butenyl isothiocyanate (MTBITC) induced apoptotic cell death and G2/M cell cycle arrest via ROS production in human esophageal epithelial cancer cells.
*J Toxicol Sci.* 2019;44:73-81.

To investigate the chemopreventive mechanisms of 4-methylthio-3-butenyl isothiocyanate (MTBITC), we analyzed cell viability, cell cycle distribution, and expression levels for cell cycle and apoptosis-related proteins in MTBITC-treated malignant esophageal KYSE510 cells, with and without the reactive oxygen species (ROS) scavenger *N*-acethyl-L-Cysteine (NAC). MTBITC dose-dependently reduced cell viability and Bcl2 protein expression, while it induced cleavages of caspase-3, caspase-9, and PARP-1, suggesting that reduced cell viability occurred through the mitochondrial apoptotic pathway in KYSE510 cells. In cell cycle distribution analysis, MTBITC (20 – 40 μM) induced cell cycle arrest at G2/M phase. Furthermore, MTBITC induced Chk1 and Akt phosphorylations and decreased p27 protein expression. Both apoptotic- and cell cycle-related changes induced by MTBITC

treatment were abolished by NAC. These results suggest that MTBITC has chemopreventive potential for esophageal carcinogenesis by elimination of cancer cells via induction of mitochondrial apoptotic cell death, G2/M cell cycle arrest, and ROS production.

Keywords: 4-methylthio-3-butenyl isothiocyanate, esophageal cancer, apoptosis

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Toyoda T, Cho YM, Akagi J, Mizuta Y, Matsushita K, Nishikawa A, Imaida K*, Ogawa K: A 13-week subchronic toxicity study of acetaminophen using an obese rat model.

*J Toxicol Sci.* 2018;43:423-33.

Although obesity is increasing worldwide, experimental studies examining the possible association between obesity and susceptibility to chemical toxicity are limited. In the present study, we performed a 13-week toxicity study for acetaminophen (APAP), a well known drug that exhibits hepatotoxicity as an adverse effect, using an obese rat model to investigate the differences in susceptibility between obese and normal individuals. Male F344 and obese Zucker (lean and fatty) rats were administered 0, 80, 253, 800, 2530, or 8000 ppm APAP in the diet for 13 weeks. No significant toxicity related to APAP treatment was observed in terms of clinical signs and hematology in all three strains. Body weight gain in F344 and lean rats was significantly decreased by 8000 ppm APAP treatment. Significant increases in serum total cholesterol level and relative liver weights were detected in F344 rats in the highest dose group. On histopathological assessment, centrilobular hepatocellular hypertrophy was observed in the 8000 ppm groups of F344 and lean rats, whereas no histopathological changes were induced by APAP in fatty rats. The no-observed-adverse-effect levels (NOAELs) of APAP were evaluated to be 2530 ppm in F344 and lean rats (142.1 and 152.8 mg/kg bw/day, respectively) and more than 8000 ppm in fatty rats (>539.9 mg/kg bw/day). These results suggested that obese Zucker rats may be less susceptible to APAP-dependent toxicity in the liver than their lean counterparts.

Keywords: subchronic toxicity, obesity, acetaminophen

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Akagi J, Cho YM, Mizuta Y, Tatebe C, Sato K, Ogawa K: Subchronic toxicity evaluation of isoeugenyl methyl ether in F344/DuCrj rats by 13-week oral administration.

*Regul Toxicol Pharmacol.* 2019;102:34-39.

Isoeugenyl methyl ether (CAS No. 93-16-3) is a food additive used as a nature identical flavoring agent. To determine the toxicity profile and the no-observed-adverse-effect level (NOAEL), we performed a subchronic toxicity test in male and female F344/DuCrj rats by intragastric administration of isoeugenyl methyl ether at doses of 8, 40, and 200 mg/kg body weight (BW)/day for 13 weeks. In this study, BW gain in the male 200 mg/kg BW/day group was decreased from week 9. In serum biochemistry, decreased triglycerides were observed in the male 200 mg/kg BW/day group. In organ weights, increases in both absolute and relative liver weights were observed in both sexes in the 200 mg/kg BW/day group. In histopathological examination, hepatocyte hypertrophy was observed in the male 200 mg/kg BW/day group. Based on these results, we concluded that the main target organ of isoeugenyl methyl ether was the liver and that the NOAEL of isoeugenyl methyl ether for both male and female F344/DuCrj rats was estimated to be 40 mg/kg BW/day.

Keywords: food additive, isoeugenyl methyl ether, subchronic toxicity

Matsushita K, Toyoda T, Morikawa T, Takahashi M, Inoue K, Ogawa K: A 13-week subchronic toxicity study of 2-ethylbutanal in F344 rats.

*Regul Toxicol Pharmacol.* 2018;100:118-26.

2-Ethylbutanal (2-EB) has been used as a flavoring agent. Here, we performed a 13-week subchronic toxicity study of 2-EB in F344 rats. 2- EB was given orally by gavage, using doses of 0, 50, 200 or 800 mg/kg BW/day. Reduced body weight gain was noted in both sexes at 800 mg/kg BW. Hematologic assessment showed a decrease in platelet counts in males at 200 mg/kg BW and both sexes at 800 mg/kg BW. Serum biochemistry demonstrated increases in inorganic phosphorus in both sexes at 200 and 800 mg/kg BW, increases in glucose in females at 200 and

800 mg/kg BW and increases in urea nitrogen in both sexes at 800 mg/kg BW. Regarding organ weights, increases in absolute and relative weights of the liver and kidney with toxicological significance were detected in both sexes at 200 and 800 mg/kg BW. Hepatocellular hypertrophy with eosinophilic granular cytoplasmic changes in the liver were observed in males at 200 mg/kg BW and in both sexes at 800 mg/kg BW. Necrosis/regeneration of proximal tubules in the kidney was detected in females at 800 mg/kg BW. Based on these results, the no-observed-adverse-effect level (NOAEL) of 2-EB was evaluated to be 50 mg/kg BW/day for both sexes.

Keywords: 2-ethylbutanal, flavoring agent, subchronic toxicity

Takasu S, Yokoo Y, Ishii Y, Kijima A, Ogawa K, Umemura T: Molecular pathological differences in global gene expression between two sustained proliferative lesions, nodular regenerative hepatocellular hyperplasia and hepatocellular adenoma, in mice.

*Toxicol Pathol.* 2019;47:44-52.

Long-term exposure to piperonyl butoxide (PBO) induces multiple nodular masses along with hepatocellular tumors in the liver of mice. The histopathological features of the nodules led to our diagnosis of nodular regenerative hepatocellular hyperplasia (NRH). However, because of the lack of data on the biological characteristics of NRH, whether this lesion is truly nonneoplastic remains unknown. In this study, the molecular characteristics of NRH were compared with those of hepatocellular adenoma (HCA) by global gene expression analysis. Six-week-old male ICR mice were fed a diet containing 6,000 ppm PBO for 43 weeks to induce NRH and HCA development. Complementary DNA microarray analysis was performed using messenger RNA extracted from NRH and HCA frozen sections collected by laser microdissection. Hierarchical cluster analysis showed that all NRH samples clustered together but were separate from the HCA cluster. Pathway analysis revealed activation of the cell cycle and Delta–Notch signaling in both lesions, but the latter was more upregulated in HCA. Downregulation of cytochrome p450 enzymes was observed in NRH, but not in HCA. These results imply that NRH differs from HCA in terms of not only morphological but also molecular characteristics.

Keywords: nodular regenerative hepatocellular hyperplasia, hepatocellular adenoma, piperonyl butoxide

Matsushita K, Takasu S, Kuroda K, Ishii Y, Kijima A, Ogawa K, Umemura T*: Mechanisms underlying exacerbation of osmotic nephrosis caused by pre-existing kidney injury.

*Toxicol Sci.* 2018;165:420-30.

Osmotic nephrosis, a disease caused by intravenous infusion of various fluids such as hypertonic sucrose and isotonic polysaccharide-based plasma volume expanders, exhibits specific histopathological features, including vacuolated and swollen proximal tubules, i.e., "clear tubules". Pre-existing kidney injury exacerbates this condition, resulting in major clinical problems. However, the underlying mechanisms are unclear. Animal models often yield results that are directly translatable to humans. Therefore, in this study, we performed detailed histopathological analyses of the formation of clear tubules in rats treated with gentamicin or ischemia/reperfusion (IR) operation followed by dextran administration. The results showed that clear tubules may originate from regenerative tubules. Additionally, we classified regenerative tubules into three categories based on their development, with a particular focus on the middle and late stages. Comprehensive microarray and real-time polymerase chain reaction analyses of mRNA extracted from regenerative tubules at each stage using laser microdissection revealed that regenerative tubules in the middle stage showed an imbalance between dextran absorption and metabolism, resulting in accumulation of dextran, particularly in the cytoplasm of the tubules. Overall, our findings demonstrated that clear tubules originated from regenerated tubules and that tubules at the middle stage became clear tubules because of an imbalance during their development. This could explain why osmotic nephrosis is exacerbated in the presence of kidney lesions.

Keywords: osmotic nephrosis, renal toxicity, rat

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Ambe K*1, Ishihara K*1, Ochibe T*1, Ohya K*1, Tamura S*1, Inoue K, Yoshida M*2, Tohkin M*1: *In silico* prediction of chemical-induced hepatocellular hypertrophy using molecular descriptors.

*Toxicol Sci.* 2018;162:667-75.

*In silico* prediction for toxicity of chemicals is required to reduce cost, time, and animal testing. However, predicting hepatocellular hypertrophy, which often affects the derivation of the No-Observed-Adverse-Effect Level in repeated dose toxicity studies, is difficult because pathological findings are diverse, mechanisms are largely unknown, and a wide variety of chemical structures exists. Therefore, a method for predicting the hepatocellular hypertrophy of diverse chemicals without complete understanding of their mechanisms is necessary. In this study, we developed predictive classification models of hepatocellular hypertrophy using machine learning-specifically, deep learning (DL), random forest (RF), and support vector machine (SVM). We extracted hepatocellular hypertrophy data on rats from two toxicological databases, our original database developed from risk assessment reports such as pesticides, and the Hazard Evaluation Support System Integrated Platform (HESS). Then, we constructed prediction models based on molecular descriptors and evaluated their performance using independent test chemicals datasets, which differed from the training chemicals datasets. Further, we defined the applicability domain (AD), which generally limits the application for chemicals, as structurally similar to the training chemicals dataset. The best model was found to be the SVM model using the HESS dataset, which was trained with 251 chemicals and predicted 214 test chemicals inside the AD. It afforded a prediction accuracy of 0.76, sensitivity of 0.90, and area under the curve of 0.81. These *in silico* predictive classification models could be reliable tools for hepatocellular hypertrophy assessments and can facilitate the development of *in silico* models for toxicity prediction.

Keywords: hepatocellular hypertrophy, *in silico* prediction, repeated dose toxicity

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Gi M*1, Fujioka M*1, Kakehashi A*1, Okuno T*1, Masumura K, Nohmi T, Matsumoto M*2, Omori M*3, Wanibuchi H*1, Fukushima S*2,3: *In vivo* positive mutagenicity of 1,4-dioxane and quantitative analysis of its mutagenicity and carcinogenicity in rats.

*Arch Toxicol* 2018; 92:3207-3221.

1,4-Dioxane is a widely used synthetic industrial chemical and its contamination of drinking water and food is a potential health concern. It induces liver tumors when administered in the drinking water to rats and mice. However, the mode of action (MOA) of the hepatocarcinogenicity of 1,4-dioxane remains unclear. Importantly, it is unknown if 1,4-dioxane is genotoxic, a key consideration for risk assessment. To determine the *in vivo* mutagenicity of 1,4-dioxane, *gpt* delta transgenic F344 rats were administered 1,4-dioxane at various doses in the drinking water for 16 weeks. The overall mutation frequency (MF) and A:T- to -G:C transitions and A:T- to -T:A transversions in the *gpt* transgene were significantly increased by administration of 5000 ppm 1,4-dioxane. A:T- to -T:A transversions were also significantly increased by administration of 1000 ppm 1,4-dioxane. Furthermore, the DNA repair enzyme MGMT was significantly induced at 5000 ppm 1,4-dioxane, implying that extensive genetic damage exceeded the repair capacity of the cells in the liver and consequently led to liver carcinogenesis. No evidence supporting other MOAs, including induction of oxidative stress, cytotoxicity, or nuclear receptor activation, that could contribute to the carcinogenic effects of 1,4-dioxane were found. These findings demonstrate that 1,4-dioxane is a genotoxic hepatocarcinogen and induces hepatocarcinogenesis through a mutagenic MOA in rats. Because our data indicate that 1,4-dioxane is a genotoxic carcinogen, we estimated the point of departure of the mutagenicity and carcinogenicity of 1,4-dioxane using the no-observed effect-level approach and the Benchmark dose approach to characterize its dose-response relationship at low doses.

Keywords: 1,4-dioxane, mutagenicity, *gpt* delta transgenic rat

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Kato M*1, Sugiyama K, Fukushima T*2, Miura Y*2, Awogi T*3, Hikosaka S*4, Kawakami K*5, Nakajima M*6, Nakamura M*7, Sui H*5, Watanabe K*8, Hakura A*9: Negative and positive control ranges in the bacterial

reverse mutation test: JEMS/BMS collaborative study.
*Genes Environ.* 2018; 40:1-13.

A large-scale study was conducted by multiple laboratories affiliated with the Japanese Environmental Mutagen Society and the Bacterial Mutagenicity Study Group to investigate possible proficiency indicators for the bacterial reverse mutation test with a preincubation procedure. Approximately 30 laboratories generated negative and positive control count data and dose-response curves of the positive control articles for the bacterial reverse mutation test, with assays conducted annually from 2013 to 2016. Overall, the majority of the negative and positive control counts for *Salmonella* Typhimurium strains TA100, TA1535, TA98, and TA1537, and *Escherichia coli* strain WP2*uvrA*, with and without S9 mix, were within the range of the means ±2× standard deviation. The negative counts were normally distributed (strains TA100, TA98, and WP2*uvrA*) or followed Poisson distribution (strains TA1535 and TA1537), and the positive control counts for all strains were approximately normally distributed. In addition, the distribution of the negative and positive control counts was relatively constant over the 4 years. The number of revertant colonies increased in a dose-dependent linear or exponential fashion up to the recommended doses for the respective positive control articles in Japan. These data are valuable for determining the acceptance criteria and an estimation of the laboratory proficiency for the bacterial reverse mutation test.

Keywords: bacterial reverse mutation test, negative control range, positive control range

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You X*, Ando T, Xi J*, Cao Y*, Liu W*, Zhang X*, Honma M, Masumura K, Luan Y*: Gene mutation and micronucleus assays in *gpt* delta mice treated with 2,2',4,4'-tetrabromodiphenyl ether.
*Mutagenesis.* 2018; 33:153-160.

Flame retardant polybrominated diphenyl ethers (PBDEs) are a class of persistent organic pollutants (POPs). 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) is a representative PBDE congener with widespread distribution and relatively high toxicity potential. Although it has been reported that BDE-47 can cause DNA damage in various *in vitro* systems, few studies have provided *in vivo* genotoxicity information. The aim of the present study was to investigate the genotoxicity of BDE-47 in mice. Male *gpt* delta mice were administered BDE-47 by gavage at 0, 0.0015, 1.5, 10 and 30 mg/kg/day, and 6 days per week for six consecutive weeks. Before the first treatment, and at 2.5 and 5 weeks after the first treatment, peripheral blood was collected from tails and the micronucleus assay and the *Pig-a* gene mutation assay were performed. After the last treatment, the mutant frequencies of the *gpt* gene in the liver and the germ cells from seminiferous tubules were determined. All these assays failed to produce positive results, suggesting that BDE-47 was neither clastogenic nor mutagenic in both target and non-target tissues in *gpt* delta mice.

Keywords: PBDEs, genotoxicity, *gpt* delta transgenic mouse

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Suzuki T, Matsumoto K*, Honma M, Nohmi T: Impact of DNA polymerase ζ mutations on genotoxic thresholds of oxidative mutagens.
*Mutat Res.* 2018; 828:10-14.

In regulatory genetic toxicology, it is an axiom that there is no threshold for genotoxicity of chemicals, such that genotoxic chemicals may impose carcinogenic risk on humans even at very low doses. This paradigm is counterintuitive, however, because humans possess a number of self-defense mechanisms that may suppress the genotoxicity at these low doses and therefore manifest a practical threshold. DNA polymerase zeta (Pol ζ) is a specialized Pol that plays an important role in DNA synthesis across DNA damage, thereby modulating cell survival and genotoxicity. In this study, we compared the sensitivity of three types of human cells: D2781N, L2618M, and their wild-type

(WT) cells, to the low dose effects of genotoxicity of the oxidizing agents, potassium bromate (KBrO3) and sodium dichromate (Na2Cr2O7). D2781N cells express a variant form of Pol ζ, whose activity is weaker than that of the WT enzyme. L2618M cells express another variant form of Pol ζ, whose fidelity of DNA replication is lower than that of the WT enzyme. D2781N exhibited the highest sensitivity for TK gene mutation and micronucleus (MN) formation and displayed the lowest practical threshold for MN induction by KBrO3. In contrast, L2618M exhibited the lowest practical threshold for sister-chromatid exchange (SCE) induction by both chemicals. These results suggest that Pol ζ mutations have significant impacts on practical thresholds of genotoxicity; the factors affecting the practical threshold can differ depending on the endpoint of genotoxicity. Roles of the variant forms of Pol ζ in genotoxicity by the oxidizing agents are discussed.
Keywords: DNA polymerase zeta, translesion DNA synthesis, practical threshold

* The Institute of Environmental Toxicology

Grúz P, Shimizu M*1, Yamada M*2, Sugiyama K, Honma M: Opposing roles of Y-family DNA polymerases in lipid peroxide mutagenesis at the hisG46 target in the Ames test.

*Mutat Res.* 2018; 829-830:43-49

DNA polymerases play a key role in mutagenesis by performing translesion DNA synthesis (TLS). The Y-family of DNA polymerases comprises several evolutionarily conserved families, specializing in TLS of different DNA adducts. Exocyclic etheno and propano DNA adducts are among the most common endogenous DNA lesions induced by lipid peroxidation reactions triggered by oxidative stress. We have investigated the participation of two enterobacterial representatives of the PolIV and PolV branches of Y-family DNA polymerases in mutagenesis by two model lipid peroxidation derived genotoxins, glyoxal and crotonaldehyde. Mutagenesis by the ethano adduct (glyoxal-derived) and the propano adduct (crontonaldehyde-derived) at the GC target in the Ames test depended exclusively on PolV type DNA polymerases such as PolRI. In contrast, PolIV suppressed glyoxal and, even more, crotonaldehyde mutagenesis, as detected by enzyme overexpression and gene knockout approaches. We propose that DNA polymerase IV, which is the mammalian DNA polymerase κ ortholog, acts as a housekeeper protecting the genome from lipoxidative stress.
Keywords: Y-family DNA polymerase, lipid peroxide, Ames test

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Myatt GJ*1, Ahlberg E*2, Akahori Y*3, Allen D*4, Amberg A*5, Anger LT*5, Aptula A*6, Auerbach S*7, Beilke L*8, Bellion P*9, Benigni R*10, Bercu J*11, Booth ED*12, Bower D*13, Brigo A*14, Burden N*15, Cammerer Z*16, Cronin MTD*17, Cross KP*13, Custer L*18, Dettwiler M*19, Dobo K*20, Ford KA*21, Fortin MC*22, Gad-McDonald SE*23, Gellatly N*15, Gervais V*24, Glover KP*25, Glowienke S*26, Van Gompel J*27, Gutsell S*6, Hardy B*28, Harvey JS*29, Hillegass J*18, Honma M, Hsieh JH*30, Hsu CW*31, Hughes K*32, Johnson C*13, Jolly R*33, Jones D*34, Kemper R*35, Kenyon MO*20, Kim MT*31, Kruhlak NL*31, Kulkarni SA*32, Kümmerer K*36, Leavitt P*18, Majer B*37, Masten S*7, Miller S*13, Moser J*38, Mumtaz M*39, Muster W*14, Neilson L*40, Oprea TI*41, Patlewicz G*42, Paulino A*43, Lo Piparo E*44, Powley M*31, Quigley DP*12, Reddy MV*45, Richarz AN*46, Ruiz P*39, Schilter B*44, Serafimova R*47, Simpson W*6, Stavitskaya L*31, Stidl R*37, Suarez-Rodriguez D*6, Szabo DT*48, Teasdale A*49, Trejo-Martin A*11, Valentin JP*50, Vuorinen A*9, Wall BA*51, Watts P*52, White AT*29, Wichard J*53, Witt KL*7, Woolley A*54, Woolley D*54, Zwickl C*55, Hasselgren C*13: *In silico* toxicology protocols.

*Regul Toxicol Pharmacol.* 2018; 96:1-17.

The present publication surveys several applications of in silico (i.e., computational) toxicology approaches across different industries and institutions. It highlights the need to develop standardized protocols when conducting toxicity-related predictions. This contribution articulates the information needed for protocols to support in silico predictions for major toxicological endpoints of concern (e.g., genetic toxicity, carcinogenicity, acute toxicity, reproductive toxicity, developmental toxicity) across several industries and

regulatory bodies. Such novel in silico toxicology (IST) protocols, when fully developed and implemented, will ensure in silico toxicological assessments are performed and evaluated in a consistent, reproducible, and well-documented manner across industries and regulatory bodies to support wider uptake and acceptance of the approaches. The development of IST protocols is an initiative developed through a collaboration among an international consortium to reflect the state-of-the-art in in silico toxicology for hazard identification and characterization. A general outline for describing the development of such protocols is included and it is based on in silico predictions and/or available experimental data for a defined series of relevant toxicological effects or mechanisms. The publication presents a novel approach for determining the reliability of in silico predictions alongside experimental data. In addition, we discuss how to determine the level of confidence in the assessment based on the relevance and reliability of the information.

Keywords: *in silico* toxicology, computational toxicology, QSAR

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*14 Roche Innovation Center Basel
*15 National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)
*16 Janssen Research & Development
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*21 Global Blood Therapeutics
*22 The State University of New Jersey
*23 Gad Consulting Services
*24 Biologie Servier
*25 Defense Threat Reduction Agency, Edgewood Chemical Biological Center
*26 Novartis Pharma AG
*27 Janssen Pharmaceutical Companies of Johnson & Johnson
*28 Douglas Connect GmbH
*29 GlaxoSmithKline Pre-Clinical Development
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*32 Existing Substances Risk Assessment Bureau, Health Canada
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Saha LK[*1], Kim S[*2], Kang H[*2], Akter S[*1], Choi K[*2], Sakuma T[*3], Yamamoto T[*3], Sasanuma H[*1], Hirota K[*4], Nakamura J[*5], Honma M, Takeda S[*1]:
Differential micronucleus frequency in isogenic human cells deficient in DNA repair pathways is a valuable indicator for evaluating genotoxic agents and their genotoxic mechanisms.
*Environ Mol Mutagen*. 2018; 59:529-538.

The micronucleus (MN) test has become an attractive tool both for evaluating the genotoxicity of test chemicals because of its ability to detect clastogenic and aneugenic events and for its convenience. As the MN assay has been mostly performed using only DNA repair-proficient mammalian cells, we believed that the comparison of the MN frequency between DNA repair-proficient and -deficient human cells may be an excellent indicator for detecting the genotoxic potential of test chemicals and for understanding their mode of action. To address this issue, the following five genes encoding DNA-damage-response (DDR) factors were disrupted in the TK6 B cell line, a human cell line widely used for the MN test: *FANCD2*, DNA polymerase ζ (*REV3*), *XRCC1*, *RAD54*, and/or *LIG4*. Using these isogenic TK6 cell lines, the MN test was conducted for four widely-used DNA-damaging agents: methyl methanesulfonate (MMS), hydrogen peroxide ($H_2O_2$), γ-rays, and mitomycin C (MMC). The frequency of micronuclei in the double strand break repair-deficient *RAD54*$^{-/-}$/*LIG4*$^{-/-}$ cells after exposure to γ-rays, $H_2O_2$, MMS and MMC was 6.2-7.5 times higher than that of parental wild-type TK6 cells. The percentages of cells exhibiting micronuclei in the base excision repair- and single strand break repair-deficient *XRCC1*$^{-/-}$ cells after exposure to $H_2O_2$, MMC and MMS were all ~5 times higher than those of wild-type cells. In summary, a supplementary MN assay using the combination of *RAD54*$^{-/-}$/*LIG4*$^{-/-}$, *XRCC1*$^{-/-}$ and wild-type TK6 cells is a promising method for detecting the genotoxic potential of test chemicals and their mode of action.

Keywords: *in vitro* micronucleus assay, DNA-repair-deficient TK6 cell, DNA-damaging agent

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Benfenati E[*1], Golbamaki A[*1], Raitano G[*1], Roncaglioni A[*1], Manganelli S[*1,2], Lemke F[*3], Norinder U[*4,5], Lo Piparo E[*4,5], Honma M, Manganaro A[*6], Gini G[*7]:
A large comparison of integrated SAR/QSAR models of the Ames test for mutagenicity.
*SAR QSAR Environ Res*. 2018; 29:591-611.

Results from the Ames test are the first outcome considered to assess the possible mutagenicity of substances. Many QSAR models and structural alerts are available to predict this endpoint. From a regulatory point of view, the recommendation from international authorities is to consider the predictions of more than one model and to combine results in order to develop conclusions about the mutagenicity risk posed by chemicals. However, the results of those models are often conflicting, and the existing inconsistency in the predictions requires intelligent strategies to integrate them. In our study, we evaluated different strategies for combining results of models for Ames mutagenicity, starting from a set of 10 diverse individual models, each built on a dataset of around 6000 compounds. The novelty of our study is that we collected a much larger set of about 18,000 compounds and used the new data to build a family of integrated models. These integrations used probabilistic approaches, decision theory, machine learning, and voting strategies in the integration scheme. Results are discussed considering balanced or conservative perspectives, regarding the possible uses for different purposes, including screening of large collection of substances for prioritization.

Keywords: Ames test, integrating SAR and QSAR, prediction of mutagenicity

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Kohara A*, Matsumoto M, Hirose A, Hayashi M, Honma M, Suzuki T:
Mutagenic properties of dimethylaniline

isomers in mice as evaluated by comet, micronucleus and transgenic mutation assays.

*Genes Environ.* 2018; 40:18.

The carcinogenic potential of dimethylaniline (DMA) isomers in rodents and humans has been previously reported, and there is sufficient evidence for the carcinogenicity of 2,6-DMA in experimental animals. The target organ of carcinogenesis of 2,6-DMA is the nasal cavity. In the current study, six DMA isomers, 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-DMA, were evaluated for mutagenic properties. Male ddY mice (3/group) were treated intragastrically (i.g.) with 200 mg/kg of one of the six DMAs, and a comet assay was performed on samples of bone marrow, kidney, liver and lung at 3 and 24 h after the treatment. Positive responses were observed in the kidney, liver and lungs of mice from all of the DMA treatment groups after 3 h and in the bone marrow of mice treated with either 3,4- or 3,5-DMA after 3 h; however, these effects were diminished at the 24 h time point. The micronucleus induction in the bone marrow was analysed in the same mouse at 24 h after the treatment. No induction of micronucleated polychromatic erythrocytes was observed after treatment with any of the DMAs.Male transgenic Muta™ mice (five/group) were treated i.g. with 2,5-, 2,6- or 3,5-DMA at 100 mg/kg bw weekly for 4 weeks, and the lacZ and the cII mutation frequencies were examined in the nasal cavity, liver and bone marrow at 7 days after the last treatment. Statistically significant increases in the mutation frequencies of the lacZ and/or cII genes were observed in the nasal cavity of 2,5-DMA or 2,6-DMA treated mice. Sequence analysis showed increased incidences of AT to GC and GC to TA mutations in the nasal tissues. These findings suggest that the carcinogenic activities of DMAs are associated with mutagenic events.

Keywords: comet assay, micronucleus assay, transgenic mutation assay

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Yamada M, Honma M: Summarized data of genotoxicity tests for designated food additives in Japan.

*Genes Environ.* 2018; 40:27.

The Ministry of Health, Labour and Welfare has carried out genotoxicity tests for food additives used in Japan in cooperation with the Japan Food Additives Association since 1979. Hayashi et al. summarized these data and published a list of 337 designated additives (Shitei-tenkabutsu in Japanese) with genotoxicity test data in 2000. Thereafter, 29 items were eliminated, and 146 items were newly added. Currently, 454 designated additives are allowed to be used as food additives in Japan. This report, based on the Hayashi report, covers the addition of newly derived genotoxicity test data. Routinely, the bacterial reverse mutation test (Ames test), mammalian cell chromosomal aberration test, and in vivo rodent bone marrow micronucleus test have been used for the evaluation of genotoxicity of food additives. In addition to the data from these tests being updated in this report, it newly includes results of transgenic rodent somatic and germ cell gene mutation assays (TGR assays), incorporated in the Organisation for Economic Co-operation and Development (OECD) test guidelines after 2000. We re-evaluated the genotoxicity of 13 designated food additives considering their TGR data.

Keywords: designated additives, food additives, genotoxicity test

Yatagai F[*1], Honma M, Dohmae N[*2], Ishioka N[*1]: Biological effects of space environmental factors: A possible interaction between space radiation and microgravity.

*Life Sci Space Res.* 2019; 20:113-123.

In the mid-1980s, space experiments began to examine if microgravity could alter the biological effects of space radiation. In the late 1990s, repair of DNA strand breaks was reported to not be influenced by microgravity using the pre-irradiated cells, because the exposure doses of space radiation were few due to the short spaceflight. There were, however, conflicting reports depending on the biological endpoints used in various systems. While almost no attempts were made to assess the possibility that the microgravity effects could be altered by space radiation. This was probably due to the general understanding that microgravity plays a major role in space and works independently from space radiation. Recent ground-based simulation studies focusing on DNA oxidative damage and signal transduction suggested that combined effects of microgravity and space radiation might exist. These studies also implicated the importance of research

focusing not only on chromosomal DNA but also on cytoplasm, especially mitochondria. Therefore, we propose a new model which accounts for the combined-effects through the window of cellular responses. In this model, the interactions between microgravity and space radiation might occur during the following cellular-responses; (A) damaging and signaling by ROS, (B) damage responses on DNA (repair, replication, transcription, etc.), and (C) expression of gene and protein (regulation by chromatin, epigenetic control, etc.).

Keywords: space radiation, combined effects, DNA oxidative damage

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Honma M, Kitazawa A, Cayley A*1, Williams RV*1, Barber C*1, Hanser T*1, Saiakhov R*2, Chakravarti S*2, Myatt GJ*3, Cross KP*3, Benfenati E*4, Raitano G*4, Mekenyan O*5, Petkov P*5, Bossa C*6, Benigni R*6, 7, Battistelli CL*6, Giuliani A*6, Tcheremenskaia O*6, DeMeo C*8, Norinder U*9, 10, Koga H*11, Jose C*11, Jeliazkova N*12, Kochev N*12,13, Paskaleva V*13, Yang C*14, Daga PR*15, Clark RD*15, Rathman J*14, 16: Improvement of quantitative structure-activity relationship (QSAR) tools for predicting Ames mutagenicity: outcomes of the Ames/QSAR International Challenge Project.
*Mutagenesis*. 2019; 34:3-16.

The International Conference on Harmonization (ICH) M7 guideline allows the use of in silico approaches for predicting Ames mutagenicity for the initial assessment of impurities in pharmaceuticals. This is the first international guideline that addresses the use of quantitative structure-activity relationship (QSAR) models in lieu of actual toxicological studies for human health assessment. Therefore, QSAR models for Ames mutagenicity now require higher predictive power for identifying mutagenic chemicals. To increase the predictive power of QSAR models, larger experimental datasets from reliable sources are required. The Division of Genetics and Mutagenesis, National Institute of Health Sciences (DGM/NIHS) of Japan recently established a unique proprietary Ames mutagenicity database containing 12140 new chemicals that have not been previously used for developing QSAR models. The DGM/NIHS provided this Ames database to QSAR vendors to validate and improve their QSAR tools. The Ames/QSAR International Challenge Project was initiated in 2014 with 12 QSAR vendors testing 17 QSAR tools against these compounds in three phases. We now present the final results. All tools were considerably improved by participation in this project. Most tools achieved >50% sensitivity (positive prediction among all Ames positives) and predictive power (accuracy) was as high as 80%, almost equivalent to the inter-laboratory reproducibility of Ames tests. To further increase the predictive power of QSAR tools, accumulation of additional Ames test data is required as well as re-evaluation of some previous Ames test results. Indeed, some Ames-positive or Ames-negative chemicals may have previously been incorrectly classified because of methodological weakness, resulting in false-positive or false-negative predictions by QSAR tools. These incorrect data hamper prediction and are a source of noise in the development of QSAR models. It is thus essential to establish a large benchmark database consisting only of well-validated Ames test results to build more accurate QSAR models.

Keywords: quantitative structure-activity relationship, mutagenic effect, datasets

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Fukuchi J*1, Kitazawa A, Hirabayashi K*2, Honma M: A practice of expert review by read-across using QSAR Toolbox.
*Mutagenesis*. 2019; 34:49-54.

The International Council for Harmonisation of Technical Requirement for Pharmaceuticals for Human Use (ICH) M7 guideline on 'Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk' provides the application of two types of quantitative structure-activity relationship (QSAR) systems (rule- and statistics-based) as an alternative to the Ames test for evaluating the mutagenicity of impurities in pharmaceuticals. M7 guideline also states that the expert reviews can be applied when the outcomes of the two QSAR analyses show any conflicting or inconclusive prediction. However, the guideline does not provide any information of how to conduct expert reviews. Therefore, a conservative approach was chosen in this study, which is based on the intention to capture any mutagenic chemical substances. The 36 chemical substances, which are the model chemical substances in which positive mutagenicity was not observed according to the two types of QSAR analyses (i.e. the results are either conflicting or both negative), were selected from the list of chemical substances with strong mutagenicity known as the reported chemicals under the Industrial Safety and Health Act in Japan. The QSAR Toolbox was used in this study to rationally determine the positive mutagenicity of the 36 model chemical substances by applying a read-across method, a technique to evaluate the endpoint of the model chemical substances using the endpoint information of chemicals that are structurally similar to the model chemical substances. Resulting from the expert review by the read-across method, the 23 model chemical substances (63.8%) were rationally concluded as positive. In addition, 9 out of 11 model chemical substances that were assessed as negative for mutagenicity by both of the QSAR systems had positive analogues, supporting their mutagenicity. These results suggested that the read-across is a useful method, when conducting a conservative approach intended to capture any mutagenic chemical substances.

Keywords: quantitative structure-activity relationship, safety, mutagenic effect

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Amberg A*1, Anger LT*1, Bercu J*2, Bower D*3, Cross KP*3, Custer L*4, Harvey JS*5, Hasselgren C*6, Honma M, Johnson C*3, Jolly R*7, Kenyon MO*8, Kruhlak NL*9, Leavitt P*4, Quigley DP*3, Miller S*3, Snodin D*10, Stavitskaya L*9, Teasdale A*11, Trejo-Martin A*11, White AT*5, Wichard J*12, Myatt GJ*3: Extending (Q)SARs to incorporate proprietary knowledge for regulatory purposes: is aromatic N-oxide a structural alert for predicting DNA-reactive mutagenicity?
*Mutagenesis*. 2019; 34:67-82.

(Quantitative) structure-activity relationship or (Q)SAR predictions of DNA-reactive mutagenicity are important to support both the design of new chemicals and the assessment of impurities, degradants, metabolites, extractables and leachables, as well as existing chemicals. Aromatic N-oxides represent a class of compounds that are often considered alerting for mutagenicity yet the scientific rationale of this structural alert is not clear and has been questioned. Because aromatic N-oxide-containing compounds may be encountered as impurities, degradants and metabolites, it is important to accurately predict mutagenicity of this chemical class. This article analysed a series of publicly available aromatic N-oxide data in search of supporting information. The article also used a previously developed structure-activity relationship (SAR) fingerprint methodology where a series of aromatic N-oxide substructures was generated and matched against public and proprietary databases, including pharmaceutical data. An assessment of the number of mutagenic and non-mutagenic compounds matching each substructure across all sources was used to understand whether the general class or any specific subclasses appear to lead to mutagenicity. This analysis resulted in a downgrade of the general

aromatic N-oxide alert. However, it was determined there were enough public and proprietary data to assign the quindioxin and related chemicals as well as benzo [c][1,2,5]oxadiazole 1-oxide subclasses as alerts. The overall results of this analysis were incorporated into Leadscope's expert-rule-based model to enhance its predictive accuracy.

Keywords: oxides, fingerprints, mutagenic effect

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Petkov PI*1, Schultz TW*1,2, Honma M, Yamada T, Kaloyanova E*1, Mekenyan OG*1: Validation of the performance of TIMES genotoxicity models with EFSA pesticide data.
*Mutagenesis*. 2019; 34:83-90.

This study validates the performance of the TIssue MEtabolism Simulator (TIMES) genotoxicity models with data on pesticide chemicals included in a recently released European Food Safety Authority (EFSA) genotoxicity database. The EFSA database is biased towards negative chemicals. A comparison of substances included in the EFSA database and TIMES genotoxicity databases showed that the majority of the EFSA pesticides is not included in the TIMES genotoxicity databases and, thus, out of the applicability domains of the current TIMES models. However, the EFSA genotoxicity database provides an opportunity to expand the TIMES models. Where there is overlap of substances, consistency between EFSA and TIMES databases for the chemicals with documented data is found to be high (>80%) with respect to the Ames data and lower than the Ames data with respect to chromosomal aberration (CA) and mouse lymphoma assay (MLA) data. No conclusion for consistency with respect to micronucleus test and comet genotoxicity data can be provided due to the limited number of overlapping substances. Specificity of the models is important, given the prevalence of negative genotoxicity data in the EFSA database. High specificity (>80%) is obtained for prediction of the EFSA pesticides with Ames data. Moreover, this high specificity of the TIMES Ames models is not dependant on pesticides being within the domains. Specificity of the TIMES CA and MLA models is lower (>40%) to pesticides for out of domain. Sensitivity of TIMES in vitro and in vivo models cannot be properly estimated due to the small number of positive chemicals in the EFSA database.

Keywords: genotoxicity, pesticides, simulators

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Morita T, Shigeta Y, Kawamura T, Fujita Y*, Honda H*, Honma M: *In silico* prediction of chromosome damage: comparison of three (Q)SAR models.
*Mutagenesis*. 2019; 34:91-100.

Two major endpoints for genotoxicity tests are gene mutation and chromosome damage (CD), which includes clastogenicity and aneugenicity detected by chromosomal aberration (CA) test or micronucleus (MN) test. Many in silico prediction systems for bacterial mutagenicity (i.e. Ames test results) have been developed and marketed. They show good performance for prediction of Ames mutagenicity. On the other hand, it seems that in silico prediction of CD does not progress as much as Ames prediction. Reasons for this include different mechanisms and detection methods, many false positives and conflicting test results. However, some (quantitative) structure-activity relationship ((Q)SAR) models (e.g. Derek Nexus [Derek], ADMEWorks [AWorks] and CASE Ultra [MCase]) can predict CA test results. Therefore, performances of the three (Q)SAR models were compared using the expanded Carcinogenicity Genotoxicity eXperience (CGX) dataset for understanding current situations and future

development. The constructed dataset contained 440 chemicals (325 carcinogens and 115 non-carcinogens). Sensitivity, specificity, accuracy or applicability of each model were 56.0, 86.9, 68.6 or 89.1% in Derek, 67.7, 61.5, 65.2 or 99.3% in AWorks, and 91.0, 64.9, 80.5 or 97.7% in MCase, respectively. The performances (sensitivity and accuracy) of MCase were higher than those of Derek or AWorks. Analysis of predictivity of (Q)SAR models of certain chemical classes revealed no remarkable differences among the models. The tendency of positive prediction by (Q)SAR models was observed in alkylating agents, aromatic amines or amides, aromatic nitro compounds, epoxides, halides and N-nitro or N-nitroso compounds. In an additional investigation, high sensitivity but low specificity was noted in *in vivo* MN prediction by MCase. Refinement of test data to be used for in silico system (e.g. consideration of cytotoxicity or re-evaluation of conflicting test results) will be needed to improve performance of CD prediction.

Keywords: structure-activity relationship, chromosome abnormality, mutation

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Tennant RE*, Guesné SJ*, Canipa S*, Cayley A*, Drewe WC*, Honma M, Masumura K, Morita T, Stalford SA*, Williams RV*: Extrapolation of *in vitro* structural alerts for mutagenicity to the *in vivo* endpoint.

*Mutagenesis*. 2019; 34:111-121.

As part of the hazard and risk assessment of chemicals in man, it is important to assess the ability of a chemical to induce mutations *in vivo*. Because of the commonalities in the molecular initiating event, mutagenicity *in vitro* can correlate well to the *in vivo* endpoint for certain compound classes; however, the difficulty lies in identifying when this correlation holds true. In silico alerts for *in vitro* mutagenicity may therefore be used as the basis for alerts for mutagenicity *in vivo* where an expert assessment is carried out to establish the relevance of the correlation. Taking this into account, a data set of publicly available transgenic rodent gene mutation assay data, provided by the National Institute of Health Sciences of Japan, was processed in the expert system Derek Nexus against the *in vitro* mutagenicity endpoint. The resulting predictivity was expertly reviewed to assess the validity of the observed correlations in activity and mechanism of action between the two endpoints to identify suitable *in vitro* alerts for extension to the *in vivo* endpoint. In total, 20 alerts were extended to predict *in vivo* mutagenicity, which has significantly improved the coverage of this endpoint in Derek Nexus against the data set provided. Updating the Derek Nexus knowledge base in this way led to an increase in sensitivity for this data set against this endpoint from 9% to 66% while maintaining a good specificity of 89%.

Keywords: pharmacokinetics, nexus rules, surrogate endpoints

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Aoki Y*[1], Nakajima D*[1], Matsumoto M*[1], Yagishita M*[1], Matsumoto M*[1], Yanagisawa R*[1], Goto S*[2], Masumura K, Nohmi T: Change over time of the mutagenicity in the lungs of *gpt* delta transgenic mice by extract of airborne particles collected from ambient air in the Tokyo metropolitan area.

*Genes Environ*. 2018; 40:25.

Background: Previously we found that DNA adducts were accumulated in the lungs of the rats exposed to ambient air in the Tokyo metropolitan area. To examine chronological change in *in vivo* mutagenicity of airborne particles, extracts produced from samples of total suspended particulates (TSP) collected from urban air in 1980, 1990, and 2010 in the Tokyo metropolitan area were intratracheally administered into the lungs of *gpt* delta mice, and differences in mutation and mutant frequency were determined by using the *gpt* assay. *In vivo* mutations induced by the extracts were characterized and mutation hotspots were identified by DNA sequencing of the mutated *gpt* gene.

Results: Administration of the 1990 extract at a dose of 0.3 mg/animal significantly elevated total mutant frequency to 3.3-times that in vehicle control, and the *in vivo* mutagenicity of the extract (induced mutation frequency per milligram extract) was estimated to be 2.0- and 2.4-times higher than that of the 2010 and 1980 extract, respectively. G-to-A transition was the most common base substitution in the vehicle control mice. However, administration of the 1990 extract increased the frequency of G-to-T transversion,

which is a landmark base substitution induced by oxidative stress; furthermore, when the extract was administered at a dose of 0.15 mg, the mutant and mutation frequencies of G-to-T transversion were significantly increased to frequencies comparable with those of G-to-A transition. Similar increases in the mutant and mutation frequencies of G-to-T transversion were observed after administration of the 2010 extract. Hotspots（mutation foci identified in three or more mice）of G-to-A transition mutations at nucleotides 64 and 110 were induced by the 1980, 1990, and 2010 extracts; a hotspot of G-to-T transversions at nucleotide 406 was also induced by the 2010 extract. Previously, we showed that diesel exhaust particles or their extract, as well as 1,6-dinitropyrene, administered to mice induced these hotspots of G-to-A transitions.

Conclusions: The results of the present study suggested that mutagenesis induced by extracts produced from TSP collected in the Tokyo metropolitan area induced *in vivo* mutagenicity via the same mechanism underlying the induction of *in vivo* mutagenicity by components of diesel exhaust.

Keywords: airborne particles, mutagenicity, *gpt* delta transgenic mouse

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Hori H*, Shimoyoshi S*, Tanaka Y*, Momonami A*, Masumura K, Yamada M, Fujii W*, Kitagawa Y*: Integration of micronucleus tests with a gene mutation assay in F344 *gpt* delta transgenic rats using benzo[*a*]pyrene.
*Mutat Res*. 2019; 837:1-7.

Reduction of the number of animals used in *in vivo* genotoxicity tests is encouraged. For this purpose, we conducted integrated toxicity tests combining gene mutation assays with multiple-organ micronucleus（MN）tests（peripheral blood, bone marrow, liver, and colon）in F344 *gpt* delta transgenic（Tg）rats. Seven-week-old male F344 *gpt* delta rats were orally administered 62.5 or 125 mg/kg/day benzo[*a*]pyrene（B[*a*]P）for 28 days. One day after the final day of treatment（day 29）and three days after the final treatment（day 31）, bone marrow, liver, and colon samples were collected, and mutation assays and MN tests were performed. The *gpt* mutant frequency（MF）significantly increased in bone marrow, liver and colon but MN induction was only significant in bone marrow but not in liver and colon. Similarly MN induction was only observed in bone marrow in non-Tg F344 rats. In peripheral blood obtained on day 4, 15, 29, 31, a time-dependent increase was observed in reticulocyte MN frequency during the treatment. Thus, our integrated method successfully detected both gene mutations and MN induction caused by B[*a*]P. In addition, no significant differences were observed between sampling times（day 29 versus 31）, suggesting that sampling on day 29 is also valid to evaluate gene mutations. On the other hand, MN results in bone marrow and peripheral blood were different depending on the sampling day. An appropriate sampling day should be designated according to which assays are integrated. We confirmed that integration of the MN test with a gene mutation assay using F344 *gpt* delta Tg rats is useful to evaluate different endpoints related to genotoxicity using the same animals and to reduce animal use.

Keywords: mutagenicity, micronucleus test, *gpt* delta transgenic rat

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Aoki Y*, Matsumoto M*, Matsumoto M*, Masumura K, Nohmi T: Mutant frequency is not increased in mice orally exposed to sodium dichromate.
*Food Safety*. 2019; 7:2-10.

The *in vivo* mutagenicity of hexavalent chromium in the small intestine, the target organ of tumorgenicity, was examined by means of a transgenic mouse gene mutation assay. Sodium dichromate dihydrate was administered orally in drinking water to male *gpt* delta mice at a dose of 85.7 or 257.4 mg/L for 28 days or at a dose of 8.6, 28.6 or 85.7 mg/L for 90 days. No significant increase in *gpt* mutant frequency relative to that in control mice was observed in the small intestine in either the 28- or 90-day study, whereas 28-day oral administration of potassium bromate, a positive control substance, increased mutant frequency.

Keywords: hexavalent chromium, mutagenicity, *gpt* delta transgenic mouse

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Beilke L[*4], Bercu J[*5], Bower D[*6], Brigo A[*7], Cammerer Z[*8], Cross KP[*6], Custer L[*9], Dobo K[*10], Gerets H[*11], Gervais V[*12], Glowienke S[*13], Gomez S[*14], Van Gompel J[*15], Harvey J[*16], Hasselgren C[*2], Honma M, Johnson C[*6], Jolly R[*17], Kemper R[*18], Kenyon M[*10], Kruhlak N[*19], Leavitt P[*9], Miller S[*6], Muster W[*7], Naven R[*20], Nicolette J[*21], Parenty A[*13], Powley M[*22], Quigley DP[*6], Reddy MV[*22], Sasaki JC[*2], Stavitskaya L[*19], Teasdale A[*23], Trejo-Martin A[*5], Weiner S[*8], Welch DS[*21], White A[*16], Wichard J[*24], Woolley D[*25], Myatt GJ[*26]: Principles and procedures for handling out-of-domain and indeterminate results as part of ICH M7 recommended (Q)SAR analyses.

*Regul Toxicol Pharmacol*. 2019; 102:53-64.

The International Council for Harmonization (ICH) M7 guideline describes a hazard assessment process for impurities that have the potential to be present in a drug substance or drug product. In the absence of adequate experimental bacterial mutagenicity data, (Q)SAR analysis may be used as a test to predict impurities' DNA reactive (mutagenic) potential. However, in certain situations, (Q)SAR software is unable to generate a positive or negative prediction either because of conflicting information or because the impurity is outside the applicability domain of the model. Such results present challenges in generating an overall mutagenicity prediction and highlight the importance of performing a thorough expert review. The following paper reviews pharmaceutical and regulatory experiences handling such situations. The paper also presents an analysis of proprietary data to help understand the likelihood of misclassifying a mutagenic impurity as non-mutagenic based on different combinations of (Q)SAR results. This information may be taken into consideration when supporting the (Q)SAR results with an expert review, especially when out-of-domain results are generated during a (Q)SAR evaluation.

Keywords: (Q) SAR, impurities, mutagenicity prediction

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*15 Janssen Pharmaceutical Companies of Johnson & Johnson
*16 GlaxoSmithKline
*17 Toxicology Division, Eli Lilly and Company
*18 Vertex Pharmaceuticals Inc.
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Tanabe S, Aoyagi K[*1], Yokozaki H[*2], Sasaki H[*1]: PTCH1 pathway network model in diffuse-type gastric cancer and epithelial-mesenchymal transition.

*Journal of Stem Cell Research and Medicine*. 2018;3:1-15

Patched 1 (*PTCH1*) gene plays an important role in the Hedgehog signalling in cancer. To reveal the role of PTCH1 and the network in epithelial-mesenchymal transition (EMT), gene expression and molecular network of the PTCH1 was investigated in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC). The *PTCH1* gene expression was up-regulated in diffuse-type GC compared to MSCs. PTCH1 network model was generated with the gene expression profiling of the molecules related to PTCH1 and EMT. The signalling and molecular network of PTCH1 was analyzed using several databases, including cBioPortal for Cancer Genomics, Kyoto Encyclopedia of Genes and Genomes (KEGG), BioGRID, VaProS and Ingenuity Pathways Analysis (IPA) databases. The PTCH1 model network contains cancer-related genes such as cadherin 1 (CDH1), catenin beta 1 (CTNNB1) and transforming growth factor beta receptor 3 (TGFBR3). The results revealed a PTCH1 pathway network model in cancer and stem cells.

Keywords: epithelial-mesenchymal transition, PTCH1,

stem cell

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Tanabe S, Aoyagi K*1, Yokozaki H*2, Sasaki H*1: Molecular network pathway of ERBB in diffuse-type gastric cancer, mesenchymal stem cells and epithelial-mesenchymal transition.

*Journal of Clinical Epigenetics*. 2018;4:2:13

Epithelial-mesenchymal transition (EMT) is related to malignancy and metastasis in cancer. The molecular networks including tyrosine kinases are altered in gastric cancer (GC). This study aims to reveal the role of erb-b2 receptor tyrosine kinases (ERBBs) in EMT, and generate the molecular network pathway of ERBBs in diffuse-type GC and mesenchymal stem cells (MSCs). The expression of ERBB genes was analyzed in MSCs and diffuse-type GC which has mesenchymal characteristics compared to intestinal-type GC. The signaling and molecular network of ERBB was analyzed using several databases, including cBioPortal for Cancer Genomics, Kyoto Encyclopedia of Genes and Genomes (KEGG), BioGRID and VaProS. ERBB2 and ERBB3 gene expression were up-regulated in diffuse-type GC compared to MSCs. The ERBB3 molecular network includes epidermal growth factor receptor (EGFR), cadherin 1 (CDH1), catenin beta 1 (CTNNB1) and EPH receptor A5 (EPHA5). These results demonstrate the importance of the ERBB network in cancer signaling, and revealed a ERBB3 network pathway model in diffuse-type GC and MSCs, and EMT.

Keywords: epithelial-mesenchymal transition, ERBB, gastric cancer

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Tanabe S, Aoyagi K*1, Yokozaki H*2, Sasaki H*1: Molecular pathway network of EFNA1 in cancer and mesenchymal stem cells.

*AIMS Cell and Tissue Engineering*. 2018;2:58-77

Abundant molecules are dynamically activated in cancer and stem cells. To investigate the role of ephrin A1 (EFNA1) in cancer and stem cell signaling pathways, we analyzed the gene expression and molecular network of EFNA1 in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC). Diffuse-type GC has more mesenchymal-like feature and malignant characteristics compared to intestinal-type GC. The signaling and molecular network of EFNA1 in cancer and stem cells were analyzed using several databases, including cBioPortal for Cancer Genomics, Kyoto Encyclopedia of Genes and Genomes (KEGG). The gene expression of EFNA1 was up-regulated in diffuse-type GC compared to MSCs. The molecular pathway network of EFNA1 includes cadherin 1 (CDH1), catenin beta 1 (CTNNB1), ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1), EPH receptor A5 (EPHA5), and the KRAS proto-oncogene, GTPase (KRAS). We summarized molecular pathway network of EFNA1 in cancer and stem cells. The results revealed a network model for EFNA1 in cancer and stem cells.

Keywords: EFNA1, epithelial-mesenchymal transition, gene expression

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Igarashi T, Serizawa H*1, Kobayashi K, Suzuki H, Matsumoto M, Iso T, Kawamura T, Inoue K, Ono A*2, Yamada T, Hirose A: Initial hazard assessment of 4-benzylphenol, a structural analog of bisphenol F: Genotoxicity tests *in vitro* and a 28-day repeated-dose toxicity study in rats.

*Regul Toxicol Pharmacol*. 2018;96:64-75

4-Benzylphenol (CAS No. 101-53-1), a structural analog of bisphenol F, has estrogenic activity *in vitro* and *in vivo*, as is the case with bisphenol F. 4-Benzylphenol is used in plastics and during organic synthesis. Since its safety is largely unknown, we conducted toxicity tests as part of screening risk assessment in an existing chemical safety survey program. Based on results of the Ames test and the chromosomal aberration test using Chinese hamster lung cells (OECD TG 471 and 473), 4-benzylphenol was determined to be non-genotoxic *in vitro*. In a 28-day repeated-dose toxicity study, Crl:CD (SD) rats were administrated 4-benzylphenol by gavage at 0, 30, 150, or 750 mg/kg/day (OECD TG 407). Consequently, body weight was lower in males at 750 mg/kg/day. In the liver, relative organ weights were increased in both sexes

at 750 mg/kg/day, and centrilobular hepatocellular hypertrophy was observed in males at 150 and 750 mg/kg/day. In the forestomach, hyperkeratosis and hyperplasia of squamous cells were observed in males at 150 and 750 mg/kg/day, and in females at 750 mg/kg/day. Based on these results, we identified the NOAEL for 4-benzylphenol as 30 mg/kg/day, with a hazard assessment value (D-value) of 0.05 mg/kg/day corresponding to hazard class 3.

Keywords: 4-benzylphenol, hazard class, repeated-dose toxicity

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Igarashi T, Takashima H*1, Takabe M*1, Suzuki H, Ushida K, Kawamura T, Matsumoto M, Iso T, Tanabe S, Inoue K, Ono A*2, Yamada T, Hirose A: Initial hazard assessment of benzyl salicylate: *In vitro* genotoxicity test and combined repeated-dose and reproductive/developmental toxicity screening test in rats.

*Regul. Toxicol. Pharmacol.* 2018;100:105-117

Benzyl salicylate is used as a fragrance ingredient and an ultraviolet light absorber, but its toxicity is unknown. Therefore, toxicity tests and hazard classification were conducted for screening assessment under the Japanese Chemical Substances Control Law. Benzyl salicylate was found to be non-genotoxic *in vitro* based on the chromosomal aberration test using Chinese hamster lung cells. However, the combined repeated-dose and reproductive/developmental screening toxicity test, in which male and female rats were administered benzyl salicylate by gavage at 0, 30, 100, or 300 mg/kg/day for 42 and 41–46 days, respectively, from 14 days before mating until postnatal Day 4, showed that repeated doses had major effects on the thymus, liver, epididymis, and femur at 100 and/or 300 mg/kg/day. Furthermore, although benzyl salicylate had no effect on the estrus cycle, fertility, corpus lutea, or implantation rate, embryonic resorption, offspring mortality, and neural tube defects were observed at 300 mg/kg/day, and the offspring had lower body weights at 30 and 100 mg/kg/day, suggesting teratogenicity similar to other salicylates. Based on the developmental toxicity, this chemical was classified as hazard class 2, with a lowest observed adverse effect level (LOAEL) of 30 mg/kg/day and a D-value of 0.003 mg/kg/day.

Keywords: embryonic resorption, low body weight, neural tube defect

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Imai K*1,2, Nakanishi I*2, Ohkubo K*2,3,4, Ohno A, Mizuno M*1, Fukuzumi S*4,5, Matsumoto K*2, Fukuhara K*1: Synthesis and Radical-Scavenging Activity of C-Methylated Fisetin Analogues.

*Bioorg. Med. Chem.* 2019;27(8);1720–1727

The radical-scavenging reaction of fisetin, a natural antioxidant found in strawberries, is known to proceed via hydrogen transfer to produce a fisetin radical intermediate. Thus, introduction of an electron-donating group into the fisetin molecule is expected to stabilize the radical, leading to enhanced radical-scavenging activity. In this study, fisetin derivatives in which methyl substituents were introduced at the ortho positions relative to the catechol hydroxyl groups were synthesized and their radical scavenging activities were evaluated and compared with that of the parent fisetin molecule. Among the methyl derivatives, 5′-methyl fisetin, in which the inherent planar structure of fisetin was retained, exhibited the strongest radical scavenging activity. Introduction of methyl substituents may be effective for the enhancement of various biological activities of antioxidants, particularly radical-scavenging activity.

Keywords: antioxidant, fisetin, radical-scavenging activity

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*J. Appl. Toxicol.* 2019 Feb;39(2):191-208

The amino acid derivative reactivity assay (ADRA) is an *in chemico* alternative to animal testing for skin sensitization that solves certain problems found in the use of the direct peptide reactivity assay (DPRA). During a recent validation study conducted at multiple laboratories as part of the process to include ADRA in an existing OECD test guideline, one of the nucleophilic reagents used in ADRA-N-(2-(1-naphthyl)acetyl)-l-cysteine (NAC)-was found to be susceptible to oxidation in much the same manner that the cysteine peptide used in DPRA was. Owing to this, we undertook a study to clarify the cause of the promotion of NAC oxidation. In general, cysteine and other chemicals that have thiol groups are known to oxidize in the presence of even minute quantities of metal ions. When metal ions were added to the ADRA reaction solution, Cu2+ promoted NAC oxidation significantly. When 0.25 μm of EDTA was added in the presence of Cu2+, NAC oxidation was suppressed. Based on this, we predicted that the addition of EDTA to the NAC stock solution would suppress NAC oxidation. Next, we tested 82 chemicals used in developing ADRA to determine whether EDTA affects ADRA's ability to predict sensitization. The results showed that the addition of EDTA has virtually no effect on the reactivity of NAC with a test chemical, yielding an accuracy of 87% for predictions of skin sensitization, which was roughly the same as ADRA.

Keywords: alternative to animal testing, OECD test guideline, skin sensitization

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Kimura Y[*1], Watanabe M[*2], Suzuki N[*3], Iwaki T[*4], Yamakage K[*2], Saito K[*3], Nakajima Y[*4], Fujimura C[*1], Ohmiya Y[*5], Omori T[*6], Kojima H, Aiba S[*1]: The performance of an *in vitro* skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA).

*J. Toxicol. Sci.* 2018;43(12):741-749

In all current *in vitro* skin sensitisation assays, DMSO is used to dissolve water-insoluble chemicals. However, our previous study suggested the superiority of the modified IL-8 Luc assay (mIL-8 Luc), in which X-VIVOTM 15 is used to dissolve chemicals, over the original assay using DMSO (oIL-8 Luc). In this study, to confirm the superiority of the mIL-8 Luc, we first increased the number of chemicals examined and demonstrated the superiority of the mIL-8 Luc, in which the mIL-8 Luc provided 87.6% of sensitivity, 74.2% of specificity, and 84.6% of accuracy. Next, to clarify the cause of false negative judgment by the mIL-8 Luc, we examined the effects of physical properties of chemicals on judgment. The results demonstrated that high molecular weight, high LogKo/w, or poor water solubility, did not cause false negative judgment. When it was accepted as an OECD test guideline, the criteria of the mIL-8 Luc to determine sensitisers were modified to further decrease false negative judgment by poor solubility. By applying the new criteria, the test guideline IL-8 Luc assay (tgIL-8 Luc) improved sensitivity but decreased specificity and increased the number of chemicals that cannot be judged. To overcome this problem, we examined a simple combination of the tgIL-8 Luc with direct peptide reactive assay (DPRA), which could improve specificity and decrease the number of the chemicals that cannot be judged. These data suggest that the tgIL-8 Luc is a promising *in vitro* skin sensitisation assay in combination with other *in vitro* or *in chemico* methods.

Keywords: alternative method, contact dermatitis, skin sensitisation

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Koyama S*1, Arakawa H*2, Itoh M*3, Masuda N*3, Yano K*1, Kojima H, Ogihara T*1,4: Evaluation of the metabolic capability of primary human hepatocytes in three-dimensional cultures on microstructural plates.

*Biopharm Drug Dispos*. 2018; Apr;39(4):187-195

The NanoCulture Plate (NCP) is a novel microstructural plate designed as a base for the three-dimensional culture of cells/tissues. This study examined whether or not the metabolic capability of human primary hepatocytes is well maintained during culture on NCPs. The hepatocytes formed aggregates after seeding and their ATP content was well maintained during culture for 21 days. Expression of CYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 mRNAs was detected throughout the 21-day culture period. Addition of CYP substrate drugs (midazolam, diclofenac, lamotrigine and acetaminophen) resulted in the formation of multiple metabolites with a corresponding decrease in the amounts of the unchanged compounds. The inducers omeprazole, phenobarbital and rifampicin increased the levels of CYP1A2, 2B6 and 3A4 mRNAs by 110-fold, 12.5-fold and 5.4-fold, respectively, at day 2, compared with control human hepatocytes. CYP activities were also increased at 2 days after inducer treatment (CYP1A2, 2.2-fold; CYP2B6, 20.6-fold; CYP3A4, 3.3-fold). The results indicate that the hepatocyte spheroids on NCP have detectable and inducible metabolic abilities during the 7-day culture period.

Keywords: 3D culture, drug metabolism, hepatocyte

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

*Regul. Toxicol. Pharmacol*. 2018 Jun;95:52-65

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

Keywords: alternative approaches, non-animal methods, skin sensitization testing

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Narita K*1,2, Ishii Y*1, Vo PTH*1, Nakagawa F*1, Ogata S*3, Yamashita K*4, Kojima H, Itagaki H*1: Improvement of human cell line activation test (h-CLAT) using short-time exposure methods for prevention of false-negative results.

*J Toxicol Sci.* 2018;43(3):229-240

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

Keywords: allergic contact dermatitis, h-CLAT, skin sensitization test

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Mitachi T*1,2, Kouzui M*1, Maruyama R*1, Yamashita K*2, Ogata S*3, Kojima H, Itagaki H*1: Some non-sensitizers upregulate CD54 expression by activation of the NLRP3 inflammasome in THP-1 cells.

*J. Toxicol. Sci.* 2019;44(3):213-224

The human cell line activation test (h-CLAT) is a skin sensitization test that measures the expression of cell surface proteins CD86 and CD54 to evaluate the skin sensitization potential of test chemicals. However, some skin irritants have been reported to induce dramatically high CD54 expression leading to false-positive h-CLAT results. Furthermore, CD54 expression is strongly induced by cytokines, such as interleukin (IL)-1$\beta$ and tumor necrosis factor (TNF)-$\alpha$, or danger signals that activate its signaling pathways. In this study, we focused on the relationship between CD54 expression and the Nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome, a protein complex that plays a pivotal role in intra-cellular inflammation. We observed the activation of caspase-1 and production of IL-1$\beta$ after exposure of THP-1 cells to 2,4-dinitrochlorobenzene (DNCB, sensitizer), octanoic acid (OA, non-sensitizer), and salicylic acid (SA, non-sensitizer), implying NLRP3 activation. These observations confirmed the activation of the inflammasome by CD54-only positive chemicals. CD54 expression, induced by OA and SA, was suppressed by potassium chloride, a typical inhibitor of NLRP3

inflammasome activation. These results suggested that the NLRP3 inflammasome may be activated in THP-1 cells resulting in the expression of CD54, and subsequently leading to false-positive results.

Keywords: h-CLAT, inflammasome, skin sensitization

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Hoffmann S*1, Kleinstreuer N*2, Alépée N*3, Allen D*4, Api AM*5, Ashikaga T, Clouet E*6, Cluzel M*7, Desprez B*8, Gellatly N*9, Goebel C*10, Kern PS*11, Klaric M*8, Kühnl J*12, Lalko JF*5, Martinozzi-Teissier S*3, Mewes K*13, Miyazawa M*14, Parakhia R*5, van Vliet E*15, Zang Q*4, Petersohn D*13: Non-animal methods to predict skin sensitization (I): the Cosmetics Europe database.

*Crit. Rev. Toxicol.* 2018 May;48(5):344-358

Cosmetics Europe, the European Trade Association for the cosmetics and personal care industry, is conducting a multi-phase program to develop regulatory accepted, animal-free testing strategies enabling the cosmetics industry to conduct safety assessments. Based on a systematic evaluation of test methods for skin sensitization, five non-animal test methods (DPRA (Direct Peptide Reactivity Assay), KeratinoSensTM, h-CLAT (human cell line activation test), U-SENSTM, SENS-IS) were selected for inclusion in a comprehensive database of 128 substances. Existing data were compiled and completed with newly generated data, the latter amounting to one-third of all data. The database was complemented with human and local lymph node assay (LLNA) reference data, physicochemical properties and use categories, and thoroughly curated. Focused on the availability of human data, the substance selection resulted nevertheless resulted in a high diversity of chemistries in terms of physico-chemical property ranges and use categories. Predictivities of skin sensitization potential and potency, where applicable, were calculated for the LLNA as compared to human data and for the individual test methods compared to both human and LLNA reference data. In addition, various aspects of applicability of the test methods were analyzed. Due to its high level of curation, comprehensiveness, and completeness, we propose our database as a point of reference for the evaluation and development of testing strategies, as done for example in the associated work of Kleinstreuer et al. We encourage the community to use it to meet the challenge of conducting skin sensitization safety assessment without generating new animal data.

Keywords: *in chemico*, *in vitro*, skin sensitization

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Kleinstreuer NC*1, Hoffmann S*2, Alépée N*3, Allen D*4, Ashikaga T, Casey W*1, Clouet E*6, Cluzel M*7, Desprez B*7, Gellatly N*8, Göbel C*9, Kern PS*10, Klaric M*7, Kühnl J*11, Martinozzi-Teissier S*3, Mewes K*12, Miyazawa M*13, Strickland J*4, van Vliet E*14, Zang Q*4, Petersohn D*13: Non-animal methods to predict skin sensitization (II): an assessment of defined approaches.

*Crit. Rev. Toxicol.* 2018 May;48(5):359-374

Skin sensitization is a toxicity endpoint of widespread concern, for which the mechanistic understanding and concurrent necessity for non-animal testing approaches have evolved to a critical juncture, with many available options for predicting sensitization without using animals. Cosmetics Europe and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods collaborated to analyze the performance of multiple non-animal data integration approaches for the skin sensitization safety

assessment of cosmetics ingredients. The Cosmetics Europe Skin Tolerance Task Force (STTF) collected and generated data on 128 substances in multiple *in vitro* and *in chemico* skin sensitization assays selected based on a systematic assessment by the STTF. These assays, together with certain *in silico* predictions, are key components of various non-animal testing strategies that have been submitted to the Organization for Economic Cooperation and Development as case studies for skin sensitization. Curated murine local lymph node assay (LLNA) and human skin sensitization data were used to evaluate the performance of six defined approaches, comprising eight non-animal testing strategies, for both hazard and potency characterization. Defined approaches examined included consensus methods, artificial neural networks, support vector machine models, Bayesian networks, and decision trees, most of which were reproduced using open source software tools. Multiple non-animal testing strategies incorporating *in vitro*, *in chemico*, and *in silico* inputs demonstrated equivalent or superior performance to the LLNA when compared to both animal and human data for skin sensitization.

Keywords: adverse outcome pathway, integrated testing strategy, skin sensitization

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Morita T, Shigeta Y, Kawamura T, Fujita Y*, Honda H*, Honma M: *In silico* prediction of chromosome damage: Comparison of three (Q)SAR models. *Mutagenesis*. 2019;34:91-100

The human cell line activation test (h-CLAT) is a skin two major endpoints for genotoxicity tests are gene mutation and chromosome damage (CD), which includes clastogenicity and aneugenicity detected by chromosomal aberration (CA) test or micronucleus (MN) test. Many *in silico* prediction systems for bacterial mutagenicity (i.e. Ames test results) have been developed and marketed. They show good performance for prediction of Ames mutagenicity. On the other hand, it seems that *in silico* prediction of CD does not progress as much as Ames prediction. Reasons for this include different mechanisms and detection methods, many false positives and conflicting test results. However, some (quantitative) structure–activity relationship ((Q)SAR) models (e.g. Derek Nexus [Derek], ADMEWorks [AWorks] and CASE Ultra [MCase]) can predict CA test results. Therefore, performances of the three (Q)SAR models were compared using the expanded Carcinogenicity Genotoxicity eXperience (CGX) dataset for understanding current situations and future development. The constructed dataset contained 440 chemicals (325 carcinogens and 115 non-carcinogens). Sensitivity, specificity, accuracy or applicability of each model were 56.0, 86.9, 68.6 or 89.1% in Derek, 67.7, 61.5, 65.2 or 99.3% in AWorks, and 91.0, 64.9, 80.5 or 97.7% in MCase, respectively. The performances (sensitivity and accuracy) of MCase were higher than those of Derek or AWorks. Analysis of predictivity of (Q)SAR models of certain chemical classes revealed no remarkable differences among the models. The tendency of positive prediction by (Q)SAR models was observed in alkylating agents, aromatic amines or amides, aromatic nitro compounds, epoxides, halides and N-nitro or N-nitroso compounds. In an additional investigation, high sensitivity but low specificity was noted in in vivo MN prediction by MCase. Refinement of test data to be used for *in silico* system (e.g. consideration of cytotoxicity or re-evaluation of conflicting test results) will be needed to improve performance of CD prediction.

Keywords: chromosomes, mutagenicity tests, structure-activity relationship

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Yamada T, Tanaka Y*, Hasegawa R*, Igarashi T, Hirose A: Male-specific prolongation of prothombin time by industrial chemicals.

*Fundam. Toxicol.* Sci. 2018;5:75-82

Prolongation of prothrombin time (PT) induced by industrial chemicals was characterized using a database of repeated dose toxicity studies, HESS DB. Of the 685 chemicals in the DB, 20 chemicals markedly prolonged the PT by more than 150% of that of vehicle control. Prolonged PTs were detected in males for 20 chemicals but no significant prolongation of PT was observed in females for 19/20 chemicals, indicating that males are apparently more susceptible to PT prolongation than females. The effective dose of the chemicals for males were relatively high, in the range of 100 to 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$, compared to the dose range of 60 to 100 $\mu g \cdot kg^{-1}, \cdot day^{-1}$, for warfarin, a typical anticoagulant. Since not all chemicals had severe hepatotoxic effects at these doses, the low protein synthesis capacity of the liver may not contribute to prolonged PT. The mechanism of PT prolongation by the chemicals was considered different from that of warfarin, which is a specific inhibitor of vitamin K epoxide reductase, because of large differences in their effective dose and lack of structural similarity between them. Herein, the possible mechanisms of PT prolongation by industrial chemicals in males are explored, with a focus on the action of estradiol and vitamin K.

Keywords: industrial chemicals, prothrombin time, toxicity database

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Petkov PI[*1], Schultz TW[*1,2], Honma M. Yamada T, Kaloyanova E[*1], Mekenyan OG[*1]: Validation of the performance of TIMES genotoxicity models with EFSA pesticide data.

*Mutagenesis.* 2019;34:83-90

This study validates the performance of the TIssue MEtabolism Simulator (TIMES) genotoxicity models with data on pesticide chemicals included in a recently released European Food Safety Authority (EFSA) genotoxicity database. The EFSA database is biased towards negative chemicals. A comparison of substances included in the EFSA database and TIMES genotoxicity databases showed that the majority of the EFSA pesticides is not included in the TIMES genotoxicity databases and, thus, out of the applicability domains of the current TIMES models. However, the EFSA genotoxicity database provides an opportunity to expand the TIMES models. Where there is overlap of substances, consistency between EFSA and TIMES databases for the chemicals with documented data is found to be high (>80%) with respect to the Ames data and lower than the Ames data with respect to chromosomal aberration (CA) and mouse lymphoma assay (MLA) data. No conclusion for consistency with respect to micronucleus test and comet genotoxicity data can be provided due to the limited number of overlapping substances. Specificity of the models is important, given the prevalence of negative genotoxicity data in the EFSA database. High specificity (>80%) is obtained for prediction of the EFSA pesticides with Ames data. Moreover, this high specificity of the TIMES Ames models is not dependant on pesticides being within the domains. Specificity of the TIMES CA and MLA models is lower (>40%) to pesticides for out of domain. Sensitivity of TIMES *in vitro* and *in vivo* models cannot be properly estimated due to the small number of positive chemicals in the EFSA database.

Keywords: genotoxicity, pesticide, (Q) SAR

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