Morita H*, Nishino H*, Nakajima Y*, Kakubari Y*, Nakata A*, Deguchi J*, Nugroho AE*, Hirasawa Y*, Kaneda T*, Kawasaki Y, Goda Y: Oxomollugin, a potential inhibitor of lipopolysaccharide-induced nitric oxide production including nuclear factor kappa B signals.

J Nat Med. 2015;69:608-11.

Mollugin, a naphthoquinone derivative, was reported to possess various biological activities such as antiinflammatory and anti-tumor activity. Mollugin isolated from Rubia tinctorum roots inhibited lipopolysaccharide induced nitric oxide (NO) production in RAW264.7 macrophages. However, mollugin synthesized for further investigation of its anti-inflammatory mechanism showed weak activity in addition to unstable assay results. From the result of analysis on a degradation product of mollugin, oxomollugin was found to be the main active substance of mollugin degradation, showing a potent inhibitory activity on NO-production including nuclear factor kappa B signals.

Keywords: oxomollugin, iNOS, inflammation

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Nishino H*, Nakajima Y*, Kakubari Y*, Asami N*, Deguchi J*, Nugroho AE*, Hirasawa Y*, Kaneda T*, Kawasaki Y, Goda Y, Morita H*: Syntheses and anti-inflammatory activity of azamollugin derivatives.

Bioorganic & Medicinal Chemistry Letters 2016;26:524-5.

Oxomollugin (2) is a degradation product of mollugin and a potent inhibitor of NO-production including nuclear factor kappa B signals. In our endeavor to develop a potent anti-inflammatory compound, we synthesized several aza-derivatives of oxomollugin and evaluated their NO-production inhibitory activity. Azamollugin showed a potent inhibitory activity, and its activity (IC_{50} 0.34 M) was proved to be more potent than that of oxomollugin (IC_{50} 1.3 μM).

Keywords: Azamollugin, Anti-inflammation, NO-production inhibitor

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伏見直子*1,2，安食菜穂子*3，伏見裕利*4，御影雅幸*5，合田幸広，川原信夫*3：分光測色計を用いた生薬滑石の識別に関する研究.

生薬学雑誌 2016;70:10-6.

“Kasseki” is defined as “a mineral substance, mainly composed of aluminum silicate hydrate and silicon dioxide. It is not the same substance with the mineralogical talc” in The Japanese Pharmacopoeia (JP). While, “Huashi” is defined as “mainly hydrated magnesium silicate” in Pharmacopoeia of The People’s Republic of China (CP). Thus, the origins of Kasseki defined in JP (JP Kasseki) and Huashi defined in CP (CP Huashi) are different from each other even though they are highly similar on morphological characteristics. The Kasseki distributed in the market and used in Japan is almost completely imported from China. Previously, we have found that Japanese and Chinese markets have at least six (one is in Japan and five are in China) types of these mineral crude drugs (Types A-F) by using X-ray diffraction method. These facts suggest that mineral crude drugs other than JP Kasseki may be mistakenly imported as Kasseki from the Chinese market to Japan in the future responding to increased demand. In this paper, for development a new simple and easy method for discrimination of the six types of this mineral crude drugs, we investigated their color by using spectrophotometer about L^*, a^*, b^* values as defined CIE 1976 $L^*a^*b^*$ Color system. Among six types, it was difficult to distinguish each other because we observed only slight difference of value among them, and the value of some parameters differed widely. On the other hand, the result suggested that it would be possible to discriminate Type A (JP Kasseki) from Type D (CP Huashi) by using this method. Furthermore, we examined ten samples of Type A and Type D, and the significant difference were recognized on each L^*, a^* and b^* values. These data suggested that the method by using spectrophotometer is valid for the discrimination JP Kasseki from CP Huashi.

Keywords: Kasseki, Huashi, Pharmacopoeia

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Shibata H, Izutsu K, Yomota C, Okuda H, Goda Y: Investigation of factors affecting in vitro doxorubicin release from PEGylated liposomal doxorubicin for the development of in vitro release testing conditions.
Drug Dev Ind Pharm. 2015;41:1376-86.

Establishing appropriate drug release testing methods of liposomal products for assuring quality and performance requires the determination of factors affecting in vitro drug release. In this study, we investigated the effects of test conditions (human plasma lot, pH/salt concentration in the test media, dilution factor, temperature, ultrasound irradiation, etc.), and liposomal preparation conditions (pH/concentration of ammonium sulfate solution), on doxorubicin (DXR) release from PEGylated liposomal DXR. Higher temperature and lower pH significantly increased DXR release. The evaluation of DXR solubility indicated that the high DXR release induced by low pH may be attributed to the high solubility of DXR at low pH. Ultrasound irradiation induced rapid DXR release in an amplitude-dependent manner. The salt concentration in the test solution, human plasma lot, and dilution factor had a limited impact on DXR-release. Variations in the ammonium sulfate concentration used in solutions for the formation/hydration of liposomes significantly affected DXR release behavior, whereas differences in pH did not. In addition, heating condition in phosphate-buffered saline at lower pH (<6.5) exhibited higher discriminative ability for the release profiles from various liposomes with different concentrations of ammonium sulfate than did ultrasound irradiation. These results are expected to be helpful in the process of establishing appropriate drug release testing methods for PEGylated liposomal DXR.

Keywords: doxorubicin, in vitro drug release, liposome

Yoshida H, Kuwana A, Shibata H, Izutsu K, Goda Y: Particle image velocimetry evaluation of fluid flow profiles in USP 4 flow-through dissolution cells.
Pharm Res. 2015;32:2950-9.

PURPOSE: To evaluate fluid flow profiles in the flow-through cell (FTC, USP apparatus 4) system with pulsatile and non-pulsatile pumps. METHODS: Instantaneous velocity vectors in the dissolution cells were obtained from images sequentially captured by a particle image velocimetry (PIV) system. The data were sorted to follow the pump pulse cycle. RESULTS: The analysis showed changes in the flow profiles during a pump pulse (0.5 s) at a 0.025-s interval in two sizes of cells installed in the FTC system. Supplying a slow flow from the pulsatile pump induced instantaneous downward (inner layer) and upward (outer layer) flow in the larger cell during the suction phase. Analysis at varied medium and cell temperatures strongly suggested a contribution of natural convection to the complex flow caused by relatively high cell temperature. Uniform upward flow was observed in other cells and flow rate conditions. The time-averaged vertical velocities in the cells were similar in the pulsatile and non-pulsatile pump systems. CONCLUSIONS: The PIV analysis provides information on how flow rate and pump pulse affect fluid flow profiles at multiple points in flow-through dissolution cells. An appropriate temperature control should reduce the complex flow of the medium in the FTC system.

Keywords: flow-through cell dissolution testing, hydrodynamics, pulsatile pump

Fujii K*1,2, Izutsu K, Kume M*1, Yoshino T*1, Yoshihashi Y*1, Sugano K*1, Terada K*1: Physical characterization of meso-erythritol as a crystalline bulking agent for freeze-dried formulations.
Chem Pharm Bull. 2015;63:311-7.

The purpose of this study was to identify and characterize new crystalline bulking agents applicable to freeze-dried pharmaceuticals. Thermal analysis of heat-melt sugar and sugar alcohol solids as well as their frozen aqueous solutions showed high crystallization propensity of meso-erythritol and D-mannitol. Experimental freeze-drying of the aqueous meso-erythritol solutions after their cooling by two different methods (shelf-ramp cooling and immersion of vials into liquid nitrogen) resulted in cylindrical crystalline solids that varied in appearance and microscopic structure. Powder X-ray diffraction and thermal analysis indicated different crystallization processes of meso-erythritol depending on the extent of cooling. Cooling

of the frozen meso-erythritol solutions at temperatures lower than their Tg' (glass transition temperature of maximally freeze-concentrated phase, -59.7 ° C) induced a greater number of nuclei in the highly concentrated solute phase. Growth of multiple meso-erythritol anhydride crystals at around -40° C explains the powder-like fine surface texture of the solids dried after their immersion in liquid nitrogen. Contrarily, shelf-ramp cooling of the frozen solution down to -40° C induced an extensive growth of the solute crystal from a small number of nuclei, leading to scale-like patterns in the dried solids. An early transition of the freezing step into primary drying induced collapse of the non-crystalline region in the cakes. Appropriate process control should enable the use of meso-erythritol as an alternative crystalline bulking agent in freeze-dried formulations.

Keywords: crystallization, freeze-drying, thermal analysis

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Shibata H, Yoshida H, Izutsu K, Haishima Y, Kawanishi T, Okuda H, Goda Y: Interaction kinetics of serum proteins with liposomes and their effect on phospholipase-induced liposomal drug release.
Int J Pharm. 2015;495:827-39.

We used surface plasmon resonance (SPR) to measure the affinity and kinetics of the interaction between serum proteins and both conventional and PEGylated liposomes. The effect of the interactions on secretory phospholipase A2 (sPLA2)-induced release of a model drug from liposomes was also assessed. SPR analysis of 12 serum proteins revealed that the mode of interaction between serum proteins and liposomes greatly varies depending on the type of protein. For example, albumin bound to liposomes at slower association/dissociation rates with higher affinity and prevented sPLA2-induced drug release from PEGylated liposomes. Conversely, fibronectin bound at faster association/dissociation rates with lower affinity and demonstrated little impact on the drug release. These results indicate that the effect of serum proteins on sPLA2 phospholipid hydrolysis varies with the mode of interaction between proteins and liposomes. Understanding how the proteins interact with liposomes and impact sPLA2 phospholipid hydrolysis should aid the rational design of therapeutic liposomal formulations.

Keywords: liposomes, surface plasmon resonance, phospholipase A2

Sasakura D*1, Nakayama K*2, Sakamoto T, Chikuma T*1: Strategic development of a multivariate calibration model for the uniformity testing of tablets by transmission NIR analysis.
Pharmazie 2015;70:289-95.

The use of transmission near infrared spectroscopy (TNIRS) is of particular interest in the pharmaceutical industry. This is because TNIRS does not require sample preparation and can analyze several tens of tablet samples in an hour. It has the capability to measure all relevant information from a tablet, while still on the production line. However, TNIRS has a narrow spectrum range and overtone vibrations often overlap. To perform content uniformity testing in tablets by TNIRS, various properties in the tableting process need to be analyzed by a multivariate prediction model, such as a Partial Least Square Regression modeling. One issue is that typical approaches require several hundred reference samples to act as the basis of the method rather than a strategically designed method. This means that many batches are needed to prepare the reference samples; this requires time and is not cost effective. Our group investigated the concentration dependence of the calibration model with a strategic design. Consequently, we developed a more effective approach to the TNIRS calibration model than the existing methodology.

Keywords: content uniformity testing, tablet analyses, near Infrared spectroscopy

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Yamamoto Y*1, Fukami T*2, Koide T, Onuki Y*3, Suzuki T*4, Katori N, Tomono K*4: Studies on Uniformity of the Active Ingredients in Acetaminophen Suppositories Re-solidified after Melting under High Temperature Conditions.
Chem Pharm Bull. 2015;63:263–72.

The target of the present pharmaceutical study was the antipyretic analgesic, acetaminophen;

its suppositoryform is usually split when used in pediatric patients. We focused on the active ingredient uniformity in these products, which were re-solidified after melting under high temperature condition. When sections of the cut surfaces of the seven acetaminophen suppository products (SUP-A–G) commercially available in Japan were visualized by polarized microscopy, acetaminophen crystals that were dispersed in the base were identified. The results of the quantitative determination of agent concentration for each cut portion (mg/g) suggested uniform dispersion of these crystals in the base of each product. The agent concentration in each portion of the suppositories that was re-solidified after melting at high temperatures was measured. Segregation of the active ingredient was observed in four products at a temperature of 40 ° C for 1 h, while active ingredient uniformity was maintained in the other three products (SUP-C, SUP-F and SUP-G). The latter three products also showed high viscosity at 40 ° C. At 50 ° C for 4 h, only the uniformity of the active ingredient in SUP-C was maintained. These results suggest that the uniformity of the active ingredient is lost in some acetaminophen suppositories that were re-solidified after melting under high temperature conditions. The degree of loss varies depending on the product.

Keywords: Suppository, Acetaminophen, Uniformity

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Onuki Y*1, Funatani C*1, Yokawa T*2, Yamamoto Y*3, Fukami T*4, Koide T, Obata Y*1, Takayama K*1: Magnetic resonance imaging of the phase separation in mixed preparations of moisturizing cream and steroid ointment after centrifugation.

Chem Pharm Bull. 2015;63:377–83.

A mixed preparation consisting of a water-in-oil emulsion-type moisturizing cream and a steroid ointment is frequently prescribed for the treatment of atopic dermatitis. We have investigated the compatibility of moisturizing creams and ointments because there are concerns regarding the physical stability of these mixed preparations. The key technology used in this study was magnetic resonance imaging (MRI). A commercial moisturizing cream and white petrolatum or clobetasone butyrate (CLB) ointment samples were mixed in a weight ratio of 1 : 1. A centrifugation test protocol (20000×g for 3 min) was implemented to accelerate the destabilization processes in the samples. After centrifugation, the mixed preparations separated into three distinct layers (upper, middle, and lower), while no phase separation was observed using moisturizing cream alone. The phase separation was monitored using chemical shift selective images of water and oil and quantitative T2 maps. In addition, MR and near-infrared spectroscopy were employed for component analysis of each phase-separated layer. Collectively, it was confirmed that the lower layer contained water, oils, and organic solvent, while the upper and middle layers were composed solely of oils. Furthermore, this study investigated the distribution of CLB in the phase-separated samples and showed that a heterogeneous distribution existed. From our results, it was confirmed that the mixed preparation became unstable because of the incompatibility of the moisturizing cream and ointment.

Keywords: Mixed external preparation, Magnetic resonance spectroscopy, MR imaging

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Koide T, Yamamoto Y*1, Fukami T*2, Katori N, Okuda H, Hiyama Y: Analysis of Distribution of Ingredients in Commercially Available Clarithromycin Tablets Using Near-Infrared Chemical Imaging with Principal Component Analysis and Partial Least Squares.

Chem Pharm Bull. 2015;63:663–8.

The aim of this study was to evaluate pharmaceuticals using a near-infrared chemical imaging(NIR-CI) technique for visualizing the distribution of ingredients in solid dosage forms of commercially available clarithromycin tablets. The cross section of a tablet was measured using the NIR-CI system for evaluating the distribution of ingredients in the tablet. The chemical images were generated by performing multivariate analysis methods: principal component analysis (PCA) and partial least squares

(PLS) with normalized near-infrared(NIR) spectral data. We gained spectral and distributional information related to clarithromycin, cornstarch, and magnesium stearate by using PCA analysis. On the basis of this information, the distribution images of these ingredients were generated using PLS analysis. The results of PCA analysis enabled us to analyze individual components by using PLS even if sufficient information on the products was not available. However, some ingredients such as binder could not be detected using NIR-CI, because their particle sizes were smaller than the pixel size (approximately 25×25×50 μm) and they were present in low concentrations. The combined analysis using both PCA and PLS with NIR-CI was useful to analyze the distribution of ingredients in a commercially available pharmaceutical even when sufficient information on the product is not available.

Keywords: near-infrared, chemical imaging, clarithromycin

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Hisada H*1, Inoue M*1, Koide T, Carriere J*2, Heyler R*2, Fukami T*1: Direct High-Resolution Imaging of Crystalline Components in Pharmaceutical Dosage Forms Using Low-Frequency Raman Spectroscopy.
Org Process Res Dev. 2015;19:1796–8.

Crystalline forms of active pharmaceutical ingredients need to be clearly understood and characterized by the pharmaceutical industry to ensure the correct dosage is produced. In this study, we evaluated the crystalline form of two different pharmaceutical cocrystals and a physical mixture consisting of caff eine and 4-hydroxybenzoic acid using a Raman microscopy system equipped with a measurement module to access the low-frequency region. We also demonstrated the differences between a low-frequency Raman spectroscopy image of a cocrystal and its physical mixture in a pharmaceutical dosage form. The measured pharmaceutical dosage forms were: a prepared pharmaceutical cocrystal, a physical mixture, and microcrystalline cellulose. The spectral patterns of the cocrystal and physical mixture were easily distinguished in the low-frequency region of the Raman spectrum. Based on the spectrum of the cocrystal and physical mixture, two different crystalline forms in the pharmaceutical dosage form were visualized using Raman microscopy. We concluded that low-frequency Raman spectroscopy is able to directly visualize the crystalline form of active pharmaceutical ingredients in pharmaceutical dosage forms without any pretreatment.

Keywords: Cocrystal, Low-frequency Raman microscopy, Imaging

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Fukami T*1, Koide T, Hisada H*1, Inoue M*1, Yamamoto Y*2, Suzuki T*3, Tomono K*3: Pharmaceutical evaluation of atorvastatin calcium tablets available on the Internet: a preliminary investigation of substandard medicines in Japan.
J Drug Deliv Sci Tec. 2016;31:35-40.

Substandard medicine is a type of substandard/spurious/falsely labeled/falsified/counterfeit (SSFFC) drug as defined by the WHO that has permeated the distribution of drugs on the Internet, and is accessible without prescription. An influx of substandard medicines is thus a serious matter in many developed countries. Here, Lipitor and its generic drugs containing atorvastatin calcium (ATC), used for the treatment of hyperlipidemia worldwide, were selected as a model prescription drug. Six brands of ATC tablets were purchased from four Japanese-language web sites. Raman spectroscopy and powder X-ray diffraction (PXRD) were employed to determine ATC and ingredients in the tablets. Although PXRD measurements showed no diffraction peaks of ATC because of its low content, a handheld Raman spectrometer detected ATC in unmodified tablets (without crushing). The tablets were assayed for drug content and dissolution profile according to the Japanese Pharmacopoeia, and one product showed an obviously slower drug release. X-ray computed tomography (CT) showed the interior of the tablet in detail and suggested that massive agglomerations caused slow disintegration of the tablet. This is the first report applying X-ray CT to tablets obtained on the Internet and indicates that unqualified prescription drugs are easily distributed on the Internet without any quality assurance.

Keywords: Internet drugs, Atorvastatin tablets, X-ray

computed tomography

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Imazato-Hirano M*1, Taniguchi Y*2, Kakehi M*3, Kuze Y*3, Nakamura T*4, Minamide Y*5, Miya K*6, Hosogi J*7, Katashima M*8, Maekawa K*9, Okuda H, Niimi S, Kawasaki N, Ishii-Watabe A, Katori N: Japanese bioanalytical method validation guideline: the world's first regulatory guideline dedicated to ligand-binding assays.
Bioanalysis 2015;7:1151-6.

After almost one and a half years of thorough discussion, 'The Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development' was issued on 1 April 2014 from the Ministry of Health, Labour and Welfare (MHLW) of Japan [1]. This Guideline, hereinafter referred to as the 'MHLW LBA Guideline,' is the world's first regulatory guideline solely dedicated to ligand-binding assays (LBA) and became effective on 1 April 2015.

To develop the MHLW LBA Guideline, its supplemental Q&A Document [2] and their English translation [3], the authors have worked in the Study Group of MHLW and its affiliated LBA Working Group, represent- ing the regulatory agency and industries. This manuscript provides an overview of the developing process of the MHLW LBA Guideline and the highlights of key issues.

Keywords: LBA, BMV Guideline, Regulated Bioanalysis

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*4 Shin Nippon Biomedical Laboratories
*5 Shimadzu Techno-Research
*6 Chugai Pharmaceutical
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Welink J*1, Fluhler E*2, Hughes N*3, Arnold M*4, Garofolo F*5, Bustard M*6, Coppola L*7, Dhodda R*8, Evans C*9, Gleason C*10, Haidar S*11, Hayes R*12, Heinig K*13, Katori N, Le Blaye O*14, Li W*15, Liu G*4, Santos MGL*16, Meng M*17, Nicholson B*18, Savoie N*19, Skelly M*11, Sojo L*20, Tampal N*6, van de Merbel N*21, Verhaeghe T*22, Vinter S*23, Wickremsinhe E*24, Whale E*23, Wilson A*25, Witte B*26, Woolf E*27: 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 1 – small molecules by LCMS).
Bioanalysis 2015;7:2913-25.

The 2015 9th Workshop on Recent Issues in Bioanalysis (9th WRIB) took place in Miami, Florida with participation of over 600 professionals from pharmaceutical and biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. It is once again a 5-day week long event – a full immersion bioanalytical week – specifically designed to facilitate sharing, reviewing, discussing and agreeing on approaches to address the most current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS, LBA approaches including the focus on biomarkers and immunogenicity. This 2015 White Paper encompasses recommendations that emerged from the extensive discussions held during the workshop, and is aimed to provide the bioanalytical community with key information and practical solutions on topics and issues addressed, in an effort to advance scientific excellence, improve quality and deliver better regulatory compliance. Due to its length, the 2015 edition of this comprehensive White Paper has been divided into three parts. Part 1 covers the recommendations for small molecule bioanalysis using LCMS. Part 2 (hybrid LBA/LCMS and regulatory agencies' inputs) and Part 3 (large molecule bioanalysis using LBA, biomarkers and immunogenicity) will also be published in volume 7 of Bioanalysis, issues 23 and 24, respectively.

Keywords: LC-MS, BMV Guideline, Regulated Bioanalysis

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*10 Bristol-Myers Squibb
*11 US FDA
*12 MPI Research, Mattawan
*13 F. Hoffmann-La Roche Ltd.
*14 France ANSM
*15 Novartis
*16 Brazil Anvisa
*17 Covance
*18 PPD
*19 CFABS
*20 Xenon Pharmaceuticals Inc.
*21 PRA Health Sciences
*22 Janssen Research & Development, Beerse, Belgium
*23 UK MHRA, London
*24 Eli Lilly & Company
*25 AstraZeneca
*26 Germany BfArM
*27 Merck Research Labs

Ackermann B*1, Neubert H*2, Hughes N*3, Garofolo F*4, Abberley L*5, Alley SC*6, Brown-Augsburger P*1, Bustard M*7, Chen L*8, Heinrich J*9, Katori N, Kaur S*10, Kirkovsky L*11, Laterza OF*12, Le Blaye O*13, Lévesque A*14, Mendes G, Santos GML*15, Olah T*16, Savoie N*17, Skelly M*18, Spitz S*19, Szapacs M*5, Tampal N*18, Wang J*16, Welink J*20, Wieling J*21, Haidar S*18, Vinter S*22, Whale E*22, Witte B*23: 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 2 – hybrid LBA/LCMS and input from regulatory agencies).
Bioanalysis 2015;7:3019-34.

The 2015 9th Workshop on Recent Issues in Bioanalysis (9th WRIB) took place in Miami, Florida with participation of over 600 professionals from pharmaceutical and biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. It is once again a 5-day week long event – a full immersion bioanalytical week – specifically designed to facilitate sharing, reviewing, discussing and agreeing on approaches to address the most current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS, LBA approaches including the focus on biomarkers and immunogenicity. This 2015 White Paper encompasses recommendations that emerged from the extensive discussions held during the workshop, and is aimed at providing the bioanalytical community with key information and practical solutions on topics and issues addressed, in an effort to advance scientific excellence, improve quality and deliver better regulatory compliance. Due to its length, the 2015 edition of this comprehensive White Paper has been divided into three parts. Part 2 covers the recommendations for hybrid LBA/LCMS and regulatory agencies' inputs. Part 1 (small molecule bioanalysis using LCMS) and Part 3 (large molecule bioanalysis using LBA, biomarkers and immunogenicity) will be published in volume 7 of Bioanalysis, issues 22 and 24, respectively.

Keywords: Hybrid LBA/LC-MS, BMV Guideline, Regulated Bioanalysis

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*10 Genentech
*11 Pfizer, San Diego
*12 Merck & Co., Inc.
*13 France ANSM
*14 inVentiv Health Clinical
*15 Brazil ANVISA
*16 Bristol-Myers Squibb
*17 CFABS
*18 US FDA
*19 MedImmune
*20 Dutch MEB
*21 Antaeus Biopharma
*22 UK MHRA
*23 Germany BfArM

Amaravadi L*1, Song A*2, Myler H*3, Thway T*4, Kirshner S*5, Devanarayan V*6, Ni Y G*3, Garofolo F*7, Birnboeck H*8, Richards S*9, Gupta S*4, Luo L*3,

Kingsley C[*10], Salazar-Fontana L[*9], Fraser S[*11], Gorovits B[*12], Allinson J[*10], Barger T[*4], Chilewski S[*13], Fjording M S[*14], Haidar S[*5], Islam R[*15], Jaitner B[*16], Kamerud J[*17], Katori N, Krinos-Fiorotti C[*12], Lanham D[*18], Ma M[*4], McNally J[*12], Morimoto A[*2], Mytych D[*4], da Costa A N[*19], Papadimitriou A[*20], Pillutla R[*3], Ray S[*1], Safavi A[*21], Savoie N[*22], Schaefer M[*20], Shih J[*4], Smeraglia J[*19], Skelly M F[*5], Spond J[*23], Staack R F[*20], Stouffer B[*3], Tampal N[*5], Torri A[*24], Welink J[*25], Yang T-Y[*26], Zoghbi J[*9]: 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 3 – LBA, biomarkers and immunogenicity).

Bioanalysis 2015;7:3107-24.

The 2015 9th Workshop on Recent Issues in Bioanalysis (9th WRIB) took place in Miami, Florida with participation of 600 professionals from pharmaceutical and biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. WRIB was once again a 5 day, week- long event – A Full Immersion Bioanalytical Week – specifically designed to facilitate sharing, reviewing, discussing and agreeing on approaches to address the most current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS and LBA approaches, including the focus on biomarkers and immunogenicity. This 2015 White Paper encompasses recommendations emerging from the extensive discussions held during the workshop, and is aimed to provide the bioanalytical community with key information and practical solutions on topics and issues addressed, in an effort to enable advances in scientific excellence, improved quality and better regulatory compliance. Due to its length, the 2015 edition of this comprehensive White Paper has been divided into three parts. Part 3 discusses the recommendations for large molecule bioanalysis using LBA, biomarkers and immunogenicity. Part 1 (small molecule bioanalysis using LCMS) and Part 2 (hybrid LBA/LCMS and regulatory inputs from major global health authorities) have been published in volume 7, issues 22 and 23 of Bioanalysis, respectively.

Keywords: LBA, BMV Guideline, Regulated Bioanalysis

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[*7] Angelini Pharma, Pomezia
[*8] Roche Pharma Research and Early Development, Roche Innovation Center
[*9] Sanofi
[*10] LGC
[*11] Pfizer, Groton
[*12] Pfizer, Andover
[*13] Bristol-Myers Squibb
[*14] Novo Nordisk A/S
[*15] Celerion, Lincoln
[*16] Novartis Pharma
[*17] Eurofins Bioanalytical Services
[*18] Eurofins Bioanalytical Services
[*19] UCB Biopharma
[*20] Roche Pharma Research and Early Development, Roche Innovation Center
[*21] Bioagilytix Labs
[*22] CFABS
[*23] Merck
[*24] Regeneron Pharmaceuticals
[*25] Dutch MEB
[*26] Janssen R&D

Sakai-Kato K, Nanjo K, Kusuhara H[*1], Nishiyama N[*2], Kataoka K[*3], Kawanishi T, Okuda H, Goda Y: Effect of knockout of Mdr1a and Mdr1b ABCB1 genes on the systemic exposure of a doxorubicin-conjugated block copolymer in mice.

Mol Pharm. 2015;12:3175-83.

We previously elucidated that ATP-binding cassette subfamily B member 1 (ABCB1) mediates the efflux of doxorubicin-conjugated block copolymers from HeLa cells. Here, we investigated the role of ABCB1 in the in vivo behavior of a doxorubicin-conjugated polymer in Mdr1a/1b(-/-) mice. The area under the curve for intravenously administered polymer in Mdr1a/1b(-/-) mice was 2.2-fold greater than that in wild-type mice. The polymer was mostly distributed in the liver followed by spleen and less so in the brain, heart, kidney, and lung. The amount of polymer excreted in the urine was significantly decreased in Mdr1a/1b(-/-) mice. The amounts of polymers excreted in the feces were similar in both groups despite the

higher systemic exposure in Mdr1a/1b(-/-) mice. Confocal microscopy images showed polymer localized in CD68(+) macrophages in the liver. These results show that knockout of ABCB1 prolonged systemic exposure of the doxorubicin-conjugated polymer in mice. Our results suggest that ABCB1 mediated the excretion of doxorubicin-conjugated polymer in urine and feces. Our results provide valuable information about the behavior of block copolymers in vivo, which is important for evaluating the pharmacokinetics of active substances conjugated to block copolymers or the accumulation of block copolymers in vivo.

Keywords: ABCB1, Block copolymer micelles, Clearance

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Sakai-Kato K, Nishiyama N*1, Kozaki M*2, Nakanishi T*3, Matsuda Y*4, Hirano M*4, Hanada H*5, Hisada S*6, Onodera H*4, Harashima H*7, Matsumura Y*8, Kataoka K*9, Goda Y, Okuda H, Kawanishi T: General considerations regarding the in vitro and in vivo properties of block copolymer micelle products and their evaluation.

J Control Release. 2015;210:76-83.

Block copolymer micelles are nanoparticles formed from block copolymers that comprise a hydrophilic polymer such as poly(ethylene glycol) and a poorly soluble polymer such as poly(amino acids). The design of block copolymer micelles is intended to regulate the in vivo pharmacokinetics, stability, and distribution profiles of an entrapped or block copolymer-linked active substance. Several block copolymer micelle products are currently undergoing clinical development; however, a major challenge in the development and evaluation of such products is identification of the physicochemical properties that affect the properties of the drug product in vivo. Here we review the overall in vitro and in vivo characteristics of block copolymer micelle products with a focus on the products currently under clinical investigation. We present examples of methods suitable for the evaluation of the physicochemical properties, non-clinical pharmacokinetics, and safety of block copolymer micelle products.

Keywords: Block copolymer micelle, Nanotechnology, Regulatory affairs

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Sakai-Kato K, Nanjo K, Kawanishi T, Okuda H, Goda Y: Effects of lipid composition on the properties of doxorubicin-loaded liposomes.

Ther Deliv. 2015;6:785-94.

The liposomal lipid composition of doxorubicin-loaded liposome likely will influence its pharmacological activity. We prepared 18 formulations of doxorubicin-loaded liposomes in which the lipid composition was varied. It was indicated that the intracellular uptake of doxorubicin is the primary property of doxorubicin-loaded liposome that affects its cytotoxicity in vitro. Furthermore, the release rate of doxorubicin from liposome and the biological activity of the lipid itself also affected the cytotoxicity. These findings provide an insight into how lipid composition influences the cytotoxicity of the doxorubicin-loaded liposomes. Our results provide valuable information that should help to enhance the therapeutic efficacy of liposomal anticancer drug products by optimizing their formulations.

Keywords: liposome, lipid composition, Physicochemical properties

Sakai-Kato K, Nanjo K, Kawanishi T, Okuda H, Goda Y: Size Exclusion Chromatography Coupled with Multi-Angle Light Scattering Analysis of Physicochemical Properties of Block Copolymer

Micelles.

Chromatography 2015;36:29-32.

In this study, we report the physicochemical properties of three types of block copolymer micelles as measured by means of size exclusion chromatography coupled with multi-angle light scattering (SEC–MALS). Micelles were prepared from doxorubicin (Dox)-, Nile Red-, and 4-(N,N-dimethylsulfamoyl)-2,1,3-benzoxadiazole (DBD)-conjugated block copolymers. The molar mass and radius of gyration of the block copolymer micelles were measured, and the association number of the polymers was calculated from the measured molar mass. When the chemical structure of the block copolymers was varied (i.e., by varying the poly(ethylene glycol) chain length or by varying the polymerization degree of poly(aspartic acid)), the physicochemical properties of the resultant micelles differed markedly. The association number of the Dox-conjugated polymeric micelles was about 2.3 and 2.7 times as high as those of DBD- and Nile Red-conjugated polymeric micelles, respectively. The radius calculated from SEC–MALS was compared with that calculated from dynamic light scattering, and the ratio of the two radii provided information about the conformation of the resultant micelles.

Keywords: SEC-MALS, Block copolymer micelle, Physicochemical properties

Nagano K[*1], Imai S[*1], Zhao X[*1], Yamashita T[*1], Yoshioka Y[*2], Abe Y, Mukai Y[*1], Kamada H[*1,2], Nakagawa S[*2], Tsutsumi Y[*1,2], Tsunoda S[*1,2]:

Identification and evaluation of metastasis-related proteins, oxysterol binding protein-like 5 and calumenin, in lung tumors.

Int J Oncol. 2015;47:195-203.

Metastasis is an important prognosis factor in lung cancer, therefore, it is imperative to identify target molecules and elucidate molecular mechanism of metastasis for developing new therapeutics and diagnosis methods. We searched for metastasis-related proteins by utilizing a novel antibody proteome technology developed in our laboratory that facilitated efficient screening of useful target proteins. Two-dimensional differential in-gel electrophoresis analysis identified sixteen proteins, which were highly expressed in metastatic lung cancer cells, as protein candidates. Monoclonal single-chain variable fragments (scFvs) binding to candidates were isolated from a scFv-displaying phage library by affinity selection. Tissue microarray analysis of scFvs binding to candidates revealed that oxysterol binding protein-like 5 (OSBPL5) and calumenin (CALU) were expressed at a significantly higher levels in the lung tissues of metastasis-positive cases than that in the metastasis-negative cases. Furthermore, 80% of OSBPL5 and CALU double-positive cases were positive for lymph node metastasis. Consistent with these observations, overexpression of OSBPL5 and CALU promoted invasiveness of lung cancer cells. Conversely, knockdown of these proteins using respective siRNAs reversed the invasiveness of the lung cancer cells. Moreover, these proteins were expressed in lung tumor tissues, but not in normal lung tissues. In conclusion, OSBPL5 and CALU are related to metastatic potential of lung cancer cells, and they could be useful targets for cancer diagnosis and also for development of drugs against metastasis.

Keywords: metastasis, lung cancer, antibody proteomics

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Takechi-Haraya Y[*1], Nadai R[*2,3], Kimura H[*2,3], Nishitsuji K[*2], Uchimura K[*4], Sakai-Kato K, Kawakami K[*5], Shigenaga A[*2], Kawakami T[*6], Otaka A[*2], Hojo H[*6], Sakashita N[*2], Saito H[*3]:

Enthalpy-driven interactions with sulfated glycosaminoglycans promote cell membrane penetration of arginine peptides.

Biochim Biophys Acta. 2016;1858:1339-49.

Cell membrane penetration of arginine peptides is thought to occur via electrostatic interactions with glycosaminoglycans (GAGs) on the cell surface. However, the molecular interaction in relation to the cell membrane penetration still remains unclear. We demonstrated that the cell penetration efficiency of arginine peptides is correlated with the favorable enthalpy of binding to heparin of GAG.

Keywords: Arginine peptide, Lysine peptide, Heparin

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Yabuno K*1, Morise J*1, Kizuka Y*1, Hashii N, Kawasaki N, Takahashi S*2, Miyata S*3, Izumikawa T*3, Kitagawa H*3, Takematsu H*1, Oka S*1: A Sulfated Glycosaminoglycan Linkage Region Is a Novel Type of Human Natural Killer-1 (HNK-1) Epitope Expressed on Aggrecan in Perineuronal Nets.
PLOS One 2015;10:e0144560.

Human natural killer-1 (HNK-1) carbohydrate (HSO3-3GlcAβ1-3Galβ1-4GlcNAc-R) is highly expressed in the brain and required for learning and neural plasticity. We previously demonstrated that expression of the HNK-1 epitope is mostly abolished in knockout mice for GlcAT-P (B3gat1), a major glucuronyltransferase required for HNK-1 biosynthesis, but remained in specific regions such as perineuronal nets (PNNs) in these mutant mice. Considering PNNs are mainly composed of chondroitin sulfate proteoglycans (CSPGs) and regulate neural plasticity, GlcAT-P-independent expression of HNK-1 in PNNs is suggested to play a role in neural plasticity. However, the function, structure, carrier glycoprotein and biosynthetic pathway for GlcAT-P-irrelevant HNK-1 epitope remain unclear. In this study, we identified a unique HNK-1 structure on aggrecan in PNNs. To determine the biosynthetic pathway for the novel HNK-1, we generated knockout mice for GlcAT-S (B3gat2), the other glucuronyltransferase required for HNK-1 biosynthesis. However, GlcAT-P and GlcAT-S double-knockout mice did not exhibit reduced HNK-1 expression compared with single GlcAT-P-knockout mice, indicating an unusual biosynthetic pathway for the HNK-1 epitope in PNNs. Aggrecan was purified from cultured cells in which GlcAT-P and -S are not expressed and we determined the structure of the novel HNK-1 epitope using liquid chromatography/mass spectrometry (LC/MS) as a sulfated linkage region of glycosaminoglycans (GAGs), HSO3-GlcA-Gal-Gal-Xyl-R. Taken together, we propose a hypothetical model where GlcAT-I, the sole glucuronyltransferase required for synthesis of the GAG linkage, is also responsible for biosynthesis of the novel HNK-1 on aggrecan. These results could lead to discovery of new roles of the HNK-1 epitope in neural plasticity.

Keywords：Glycosaminoglycan, Human Natural Killer-1, Aggrecan

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Miura Y*1, Hashii N, Tsumoto H*1, Takakura D, Ohta Y, Abe Y*2, Arai Y*2, Kawasaki N, Hirose N*2, Endo T*1: Change in N-Glycosylation of Plasma Proteins in Japanese Semisupercentenarians.
PLOS One 2015;10:e0142645.

An N-glycomic analysis of plasma proteins was performed in Japanese semisupercentenarians (SSCs) (mean 106.7 years), aged controls (mean 71.6 years), and young controls (mean 30.2 years) by liquid chromatography/mass spectrometry (LC/MS) using a graphitized carbon column. Characteristic N-glycans in SSCs were discriminated using a multivariate analysis; orthogonal projections to latent structures (O-PLS). The results obtained showed that multi-branched and highly sialylated N-glycans as well as agalacto- and/or bisecting N-glycans were increased in SSCs, while biantennary N-glycans were decreased. Since multi-branched and highly sialylated N-glycans have been implicated in anti-inflammatory activities, these changes may play a role in the enhanced chronic inflammation observed in SSCs. The levels of inflammatory proteins, such as CRP, adiponectin, IL-6, and TNF-α, were elevated in SSCs. These results suggested that responses to inflammation may play an important role in extreme longevity and healthy aging in humans. This is the first study to show that the N-glycans of plasma proteins were associated with extreme longevity and healthy aging in humans.

Keywords: N-glycosylation, Plasma Proteins, Semisupercentenarians

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Yoshitake H*1, Hashii N, Kawasaki N, Endo S*1, Takamori K*1, Hasegawa A*2, Fujiwara H*3, Araki Y*1: Chemical Characterization of N-Linked

Oligosaccharide As the Antigen Epitope Recognized by an Anti-Sperm Auto-Monoclonal Antibody, Ts4.
PLOS One 2015;62:1844-50.

Ts4, an anti-sperm auto-monoclonal antibody, possesses immunoreactivity to the acrosomal region of mouse epididymal spermatozoa. In addition, the mAb shows specific immunoreactivity to reproduction-related regions such as testicular germ cells and early embryo. Our qualitative study previously showed that the antigen epitope for Ts4 contained a N-linked common oligosaccharide (OS) chain on testicular glycoproteins as determined by Western blotting for testicular glycoproteins after treatment with several glycohydrolases. Since the distribution of the Ts4-epitope is unique, the OS chain in Ts4-epitope may have role(s) in the reproductive process. The aim of this study was to clarify the molecular structure of the Ts4-epitope, particularly its OS moiety. Using Ts4 immunoprecipitation combined with liquid chromatography and multiple-stage mass spectrometry, the candidate carbohydrate structure in the Ts4-epitope is proposed to be N-linked fucosylated agalacto-biantennary with bisecting N-acetylglucosamine (GlcNAc) or with N-acetylgalactosamine-GlcNAc motif. Further binding analyses using various lectins against the mouse testicular Ts4-immunoprecipitants revealed that Phaseolus vulgaris erythroagglutinin and Pisum sativum agglutinin showed positive staining of the bands corresponding to Ts4 reactive proteins. Moreover, the immunoreactivity of Ts4 against the testicular extract was completely abrogated after digestion with β-N-acetylglucosaminidase. These results show that the Ts4-epitope contains agalacto-biantennary N-glycan with bisecting GlcNAc carrying fucose residues.

Keywords: N-linked oligosaccharide, Anti-Sperm Auto-Monoclonal Antibody, Ts4-epitope

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Tsumoto H*1, Ogasawara D*2, Hashii N, Suzuki T*2, Akimoto Y*3, Endo T*1, Miura Y*1: Enrichment of O-GlcNAc-modified peptides using novel thiol-alkyne and thiol-disulfide exchange.
Bioorg Med Chem Lett. 2015;25:2645-9.

We have developed a selective method for the enrichment of O-linked β-N-acetylglucosamine (O-GlcNAc)-modified peptides, which uses a newly synthesized thiol-alkyne and a thiol-disulfide exchange. First, O-GlcNAc-modified peptides were enzymatically labeled with an azide-containing GalNAc analog. Then, the azide moiety was reacted with thiol-alkyne through a copper(I)-catalyzed azide-alkyne cycloaddition. The thiol-modified peptides were enriched with thiol-reactive resin through a thiol-disulfide exchange. At least 500fmol of O-GlcNAc-modified peptides was selectively isolated from α-crystallin tryptic peptides and detected by mass spectrometry. This novel enrichment strategy could be used for O-GlcNAcome analysis of biological samples.

Keywords: O-GlcNAc, Thiol-alkyne, Thiol-disulfide exchange

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Yagi H*1, Nakamura M*2, Yokoyama J*3, Zhang Y*4, Yamaguchi T*4, Kondo S*1,5, Kobayashi J*6, Kato T*7, Park EY*7, Nakazawa S*8, Hashii N, Kawasaki N, Kato K*1,4,5: Stable isotope labeling of glycoprotein expressed in silkworms using immunoglobulin G as a test molecule.
J Biomol NMR. 2015;62:157-67.

Silkworms serve as promising bioreactors for the production of recombinant proteins, including glycoproteins and membrane proteins, for structural and functional protein analyses. However, lack of methodology for stable isotope labeling has been a major deterrent to using this expression system for nuclear magnetic resonance (NMR) structural biology. Here we developed a metabolic isotope labeling technique using commercially available silkworm larvae. The fifth instar larvae were infected with baculoviruses for co-expression of recombinant human immunoglobulin G (IgG) as a test molecule, with calnexin as a chaperone. They were subsequently reared on an artificial diet containing (15)N-labeled yeast crude protein extract. We harvested 0.1 mg of IgG from larva with a (15)N-enrichment ratio of approximately 80%. This allowed us to compare NMR spectral data of the Fc fragment cleaved from the

silkworm-produced IgG with those of an authentic Fc glycoprotein derived from mammalian cells. Therefore, we successfully demonstrated that our method enables production of isotopically labeled glycoproteins for NMR studies.

Keywords: Silkworms, recombinant human immunoglobulin G, Stable isotope labeling

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Yagi H*1, Fukuzawa N*2, Tasaka Y*2, Matsuo K*2, Zhang Y*1,3, Yamaguchi T*1, Kondo S*1,4, Nakazawa S*5, Hashii N, Kawasaki N, Matsumura T*2, Kato K*1,3,4,6: NMR-based structural validation of therapeutic antibody produced in Nicotiana benthamiana.

Plant Cell Rep. 2015;34:959-68.

We successfully developed a method for metabolic isotope labeling of recombinant proteins produced in transgenic tobacco. This enabled assessment of structural integrity of plant-derived therapeutic antibodies by NMR analysis. A variety of expression vehicles have been developed for the production of promising biologics, including plants, fungi, bacteria, insects, and mammals. Glycoprotein biologics often experience altered folding and post-translational modifications that are typified by variant glycosylation patterns. These differences can dramatically affect their efficacy, as exemplified by therapeutic antibodies. However, it is generally difficult to validate the structural integrity of biologics produced using different expression vehicles. To address this issue, we have developed and applied a stable-isotope-assisted nuclear magnetic resonance (NMR) spectroscopy method for the conformational characterization of recombinant antibodies produced in plants. Nicotiana benthamiana used as a vehicle for the production of recombinant immunoglobulin G (IgG) was grown in a (15)N-enriched plant growth medium. The Fc fragment derived from the (15)N-labeled antibody thus prepared was subjected to heteronuclear two-dimensional (2D) NMR measurements. This approach enabled assessment of the structural integrity of the plant-derived therapeutic antibodies by comparing their NMR spectral properties with those of an authentic IgG-Fc derived from mammalian cells.

Keywords: stable-isotope-assisted nuclear magnetic resonance (NMR) spectroscopy, Nicotiana benthamiana, therapeutic antibody

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Suzuki T, Miyazaki C, Ishii-Watabe A, Tada M, Sakai-Kato K, Kawanishi T, Kawasaki N: A fluorescent imaging method for analyzing the biodistribution of therapeutic monoclonal antibodies that can distinguish intact antibodies from their breakdown products.

mAbs 2015;7:759-76.

Many monoclonal antibodies have been developed for therapy over the last two decades. In the development of therapeutic antibodies, the preclinical assessment of an antibody's biodistribution is important for the prediction of the antibody's efficacy and safety. For imaging analyses of such biodistributions, radioisotope (RI) labeling and fluorescence labeling methods are typically used, but the resulting data are limited because these methods cannot distinguish breakdown products from intact antibodies. To resolve this problem, we developed a novel method using fluorescent resonance energy transfer (FRET)-type labeling and a spectral unmixing tool. With FRET-type labeling (labeling with two species of fluorophore), different fluorescence properties of labeled intact antibodies and their breakdown products (the hydrolyzed/digested type of breakdown products) are made visible. With the spectral unmixing tool, the fluorescence of a solution containing the intact antibody and its breakdown products could be unmixed in proportion to their contents. Moreover, when labeled antibodies that targeted either human epidermal

growth factor receptor-2 or epidermal growth factor receptor were injected into nude mice implanted subcutaneously with tumor cells, the accumulation of the injected labeled antibodies and their breakdown products in the tumor could be separately analyzed by both whole-mouse imaging and a tumor homogenate analysis. These results suggest that our method using FRET-type labeling and a spectral unmixing tool could be useful in distinguishing breakdown products from intact antibodies.

Keywords: Fluorescence imaging, Biodistribution, Antibody

Ishii-Watabe A, Hirose A, Katori N, Hashii N, Arai S[*1], Awatsu H[*2], Eiza A[*3], Hara Y[*4], Hattori H[*5], Inoue T[*6], Isono T[*7], Iwakura M[*8], Kajihara D[*9], Kasahara N[*10], Matsuda H[*11], Murakami S[*12], Nakagawa T[*13], Okumura T[*14], Omasa T[*15], Takuma S[*7], Terashima I[*16], Tsukahara M[*13], Tsutsui M[*17], Yano T[*18], Kawasaki N[*19]: Approaches to Quality Risk Management When Using Single-Use Systems in the Manufacture of Biologics.

AAPS Pharm Sci Tech. 2015;16:993-1001.

Biologics manufacturing technology has made great progress in the last decade. One of the most promising new technologies is the single-use system, which has improved the efficiency of biologics manufacturing processes. To ensure safety of biologics when employing such single-use systems in the manufacturing process, various issues need to be considered including possible extractables/leachables and particles arising from the components used in single-use systems. Japanese pharmaceutical manufacturers, together with single-use suppliers, members of the academia and regulatory authorities have discussed the risks of using single-use systems and established control strategies for the quality assurance of biologics. In this study, we describe approaches for quality risk management when employing single-use systems in the manufacturing of biologics. We consider the potential impact of impurities related to single-use components on drug safety and the potential impact of the single-use system on other critical quality attributes as well as the stable supply of biologics. We also suggest a risk-mitigating strategy combining multiple control methods which includes the selection of appropriate single-use components, their inspections upon receipt and before releasing for use and qualification of single-use systems. Communication between suppliers of single-use systems and the users, as well as change controls in the facilities both of suppliers and users, are also important in risk-mitigating strategies. Implementing these control strategies can mitigate the risks attributed to the use of single-use systems. This study will be useful in promoting the development of biologics as well as in ensuring their safety, quality and stable supply.

Keywords: Biologics, Single-use syetem, Risk management

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Jose M. M. Caaveiro[*], Kiyoshi M, Tsumoto K[*]: Structural analysis of Fc/FccR complexes: a blueprint for antibody design

Immunological Reviews 2015;268:201-21.

The number of studies and the quality of the structural data of Fcγ receptors (FcγRs) has rapidly increased in the last few years. Upon critical examination of the literature, we have extracted general conclusions that could explain differences in affinity and selectivity of FcγRs for immunoglobulin G (IgG) based on structural considerations. FcγRs employ a little conserved asymmetric surface of domain D2 composed of two distinct subsites to recognize the well-

conserved lower hinge region of IgG1-Fc. The extent of the contact interface with the antibody in subsite 1 of the receptor (but not in subsite 2), the geometrical complementarity between antibody and receptor, and the number of polar interactions contribute decisively toward strengthening the binding affinity of the antibody for the receptor. In addition, the uncertain role of the N-linked glycan of IgG for the binding and effector responses elicited by FcγRs is discussed. The available data suggest that not only the non-covalent interactions between IgG and FcγRs but also their dynamic features are essential for the immune response elicited through these receptors. We believe that the integration ofstructural, thermodynamic, and kinetic data will be critical for the design and validation of the next generation of therapeutic antibodies with enhanced effector capabilities.

Keywords: immunoglobulin G gamma receptor, X-ray crystallography, therapeutic antibodies

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Yamashita M*[1], Iida M*[1], Tada M, Shirasago Y*[2], Fukasawa M*[2], Nagase S*[1], Watari A*[1], Ishii-Watabe A, Yagi K*[1], Kondoh M*[1]: Discovery of anti-claudin-1 antibodies as candidate therapeutics against hepatitis C virus.

J Pharmacol Exp Ther. 2015;353:112-8.

Claudin-1 (CLDN1), a known host factor for hepatitis C virus (HCV) entry and cell-to-cell transmission, is a target molecule for inhibiting HCV infection. We previously developed four clones of mouse anti-CLDN1 monoclonal antibody (mAb) that prevented HCV infection in vitro. Two of these mAbs showed the highest antiviral activity. Here, we optimized the anti-CLDN1 mAbs as candidates for therapeutics by protein engineering. Although Fab fragments of the mAbs prevented in vitro HCV infection, their inhibitory effects were much weaker than those of the whole mAbs. In contrast, human chimeric IgG1 mAbs generated by grafting the variable domains of the mouse mAb light and heavy chains inhibited in vitro HCV infection as efficiently as the parental mouse mAbs. However, the chimeric IgG1 mAbs activated Fcγ receptor, suggesting that cytotoxicity against mAb-bound CLDN1-expressing cells occurred through the induction of antibody-dependent cellular cytotoxicity (ADCC). To avoid ADCC-induced side effects, we prepared human chimeric IgG4 mAbs. The chimeric IgG4 mAbs did not activate Fcγ receptor or induce ADCC, but they prevented in vitro HCV infection as efficiently as did the parental mouse mAbs. These findings indicate that the IgG4 form of human chimeric anti-CLDN1 mAb may be a candidate molecule for clinically applicable HCV therapy.

Keywords: Claudin-1, monoclonal antibody, HCV

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Tada M, Tatematsu K*, Ishii-Watabe A, Harazono A, Takakura D, Hashii N, Sezutsu H*, Kawasaki N: Characterization of anti-CD20 monoclonal antibody produced by transgenic silkworms (Bombyx mori).

mAbs 2015;7:1138-50.

In response to the successful use of monoclonal antibodies (mAbs) in the treatment of various diseases, systems for expressing recombinant mAbs using transgenic animals or plants have been widely developed. The silkworm (Bombyx mori) is a highly domesticated insect that has recently been used for the production of recombinant proteins. Because of their cost-effective breeding and relatively easy production scale-up, transgenic silkworms show great promise as a novel production system for mAbs. In this study, we established a transgenic silkworm stably expressing a human-mouse chimeric anti-CD20 mAb having the same amino acid sequence as rituximab, and compared its characteristics with rituximab produced by Chinese hamster ovary (CHO) cells (MabThera®). The anti-CD20 mAb produced in the transgenic silkworm showed a similar antigen-binding property, but stronger antibody-dependent cell-mediated cytotoxicity (ADCC) and weaker complement-dependent cytotoxicity (CDC) compared to MabThera. Post-translational modification analysis was performed by peptide mapping using liquid chromatography/mass spectrometry. There was a significant difference in the N-glycosylation profile between the CHO- and the silkworm-derived mAbs, but not in other post-translational modifications including oxidation and deamidation. The mass spectra of the N-glycosylated peptide revealed that the observed biological properties were attributable to the characteristic

N-glycan structures of the anti-CD20 mAbs produced in the transgenic silkworms, i.e., the lack of the core-fucose and galactose at the non-reducing terminal. These results suggest that the transgenic silkworm may be a promising expression system for the tumor-targeting mAbs with higher ADCC activity.

Keywords: monoclonal antibody, ADCC, transgenic silkworm

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Takakura D*, Tada M, Kawasaki N*: Membrane glycoproteomics of fetal lung fibroblasts using LC/MS.

Proteomics 2016;16:47-59.

Some aberrant N-glycosylations are being used as tumor markers, and glycoproteomics is expected to provide novel diagnosis markers and targets of drug developments. However, one has trouble in mass spectrometric glycoproteomics of membrane fraction because of lower intensity of glycopeptides in the existence of surfactants. Previously, we developed a glycopeptide enrichment method by acetone precipitation, and it was successfully applied to human serum glycoproteomics. In this study, we confirmed that this method is useful to remove the surfactants and applicable to membrane glycoproteomics. The glycoproteomic approach to the human fetal lung fibroblasts membrane fraction resulted in the identification of over 272 glycoforms on 63 sites of the 44 glycoproteins. According to the existing databases, the structural features on 41 sites are previously unreported. The most frequently occurring forms at N-glycosylation site were high-mannose type containing nine mannose residues (M9) and monosialo-fucosylated biantennary oligosaccharides. Several unexpected N-glycans, such as fucosylated complex-type and fucosylated high-mannose and/or fucosylated pauci-mannose types were found in ER and lysosome proteins. Our method provides new insights into transport, biosynthesis, and degradation of glycoproteins.

Keywords: Glycoproteomics, LC/MS, Membrane glycoproteins

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Nishmura Y*1, Hyuga S*2, Takiguchi S*3, Hyuga M, Itoh K*4, Hanawa T*2: Ephedrae herba stimulates hepatocyte growth factor-induced MET endocytosis and downregulation via early/late endocytic pathways in gefitinib-resistant human lung cancer cells.

Int J Oncol. 2016;48:1895-906.

The MET tyrosine kinase receptor and its ligand, hepatocyte growth factor (HGF), are known to be overexpressed in a variety of malignant tumor cells, and are implicated in the development of gefitinib-resistance in human non-small cell lung cancer (NSCLC) cells. Ephedrae herba was previously reported to prevent HGF-induced cancer cell motility by directly suppressing HGF/MET signaling through the inhibition of MET tyrosine kinase, and treatment with its extract also considerably reduced MET protein levels. To further investigate the mechanism underlying the Ephedrae herba-induced inhibition of MET phosphorylation as well as its degradation and subsequent disappearance, we examined the effect of Ephedrae herba on HGF-stimulated MET endocytosis and downregulation via early/late endocytic pathways in an NSCLC cell line. Using immunofluorescence microscopy, we found that pretreatment of cells with Ephedrae herba extract dramatically changed the intracellular distribution of plasma membrane-associated MET, and that the resultant MET staining was distributed throughout the cytoplasm. Pretreatment of the cells with Ephedrae herba extract also led to the rapid loss of MET and phosphorylated (p)-MET in HGF-stimulated cells. In contrast, inefficient endocytic delivery of MET and p-MET from early to late endosomes was observed in the absence of Ephedrae herba extract, since considerable amounts of the internalized MET accumulated in the early endosomes and were not delivered to lysosomes up to 1 h after HGF-stimulation. Furthermore, large amounts of MET and p-MET that had accumulated in late endosomes of Ephedrae herba-pretreated cells after HGF stimulation were observed along with bafilomycin A1. Therefore, we inferred that degradation of MET occurred in the late endosome/lysosome pathway. Moreover, western blot analysis revealed the accelerated degradation of MET and p-MET proceeds in cells pretreated with Ephedrae herba extract. Collectively, our results suggest that some components

of Ephedrae herba have a novel role in promoting HGF-stimulated MET and p-MET endocytosis followed by its downregulation, likely mediated by the early/late endocytic pathways.

Keywords: Ephedrae herba, hepatocyte growth factor, MET

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Kammoto T*1,2, Yomura K*2, Nakamura Y*2, Kikuchi Y*2, Hirakura K*2, Nishimura H*2, Hakamatsuka T, Goda Y, Kawahara N*3, Kiuchi F*1: Discrimination between Kan-jio and Juku-jio by TLC.

Shoyakugaku Zasshi 2015;69:41-7.

Rehmannia Root is an important crude drug used in Kampo products. The Japanese Pharmacopoeia (JP) defines Rehmannia root as the root of Rehmannia glutinosa Liboschitz var. purprea Makino or Rehmannia glutinosa Liboschitz (Scrophulariaceae), with the application of steaming (prepared one: Juku-jio) or without it (non-prepared one: Kan-jio). Aiming at establishing identification tests to distinguish these two types (Juku-jio and Kan-jio) distributed in the Japanese market, we compared the constituents of Juku-jio and those of Kan-jio by TLC and found differences in the sugar components between them. Based on this finding, we established identification tests for Kan-jio and Juku-jio, which are adopted in the monograph of JP from supplement I to JP16.

Keywords: Rehmannia Root, identification test, TLC

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Horii H*, Okonogi R*, Kamakura H, Hakamatsuka T, Goda Y: Studies on Bioequivalence of Kakkonto Decoction and Its Extract Preparation (II).

Shoyakugaku Zasshi 2015;69:59-65.

In a previous report (Horii, C. *et al.*, *Shoyakugaku Zasshi*, 68(1), 9-12, 2014), a crossover study was performed in order to obtain basic information about the bio-equivalence between the Kakkonto decoction and its extract product. The result suggested that ephedrine and pseudoephed-rine may be used as the marker compounds for the bio-equivalence judgment between preparations. In this study, we selected 3 components of the Kakkonto formula, Puerariae Radix, Glycyrrhizae Radix and Paeoniae Radix. The change in concentration of the five marker compounds, puerarin, daidzein, glycyrrhizic acid, liquiritin and paeoniflorin, in human blood plasma was observed after their oral administration. As a result, the dispersion of those plasma levels in the decoction and the product were observed at various sampling times. Variance analysis of the maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve ($AUC_{0\text{-}4}$) for the five marker compounds revealed no significant differences between the decoction and the product or between the administration days. The statistical power (1-β) was determined to be insufficient (less than 80%) for both C_{max} and $AUC_{0\text{-}4}$ on the five marker compounds. However, assuming that the standard deviation was the same as our result for puerarin and daidzein, when the number of the study participants is increased to 8 and 16 it is revealed that the statistical power become sufficient (more than 80%) for both C_{max} and $AUC_{0\text{-}4}$ on puerarin and daidzein. Since puerarin and daidzein are known to be important biological active components in the Kakkonto formula, these results suggest that both compounds may be used as markers for a bio-equivalence judgment between preparations, although further study is needed to clarify this issue.

Keywords: bio-equivalence, Kakkonto, blood plasma level

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Oshima N, Masada S, Suzuki R, Yagi H, Matsufuji Y*1, Takahashi Y*2, Yahagi T*3, Watanabe M*4, Yahara S*4, Iida O*5, Kawahara N*5, Maruyama T, Goda Y, Hakamatsuka T: Identification of new diterpenes as marker compounds distinguishing Agnus Castus Fruit (Chaste Tree) from Shrub Chaste Tree Fruit (Viticis Fructus).

Planta Medica 2016;82:147-53.

We tried to identify putative marker compounds that distinguish between Agnus Castus Fruit

(ACF) and Shrub Chaste Tree Fruit (SCTF). We analyzed extracts of each crude drug by LC-MS, and performed differential analysis by comparison of each chromatogram to find one or more peaks characteristic of ACF. A peak was isolated and identified as an equilibrium mixture of new compounds named chastol **(1)** and epichastol **(1a)**. The planar structures of **1** and **1a** were determined spectroscopically.

Keywords: Vitex products, Western herb, marker compound

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Yahagi T*1, Masada S, Oshima N, Suzuki R, Matsufuji Y*2, Takahashi Y*3, Watanabe M*4, Yahara S*4, Iida O*5, Kawahara N*5, Maruyama T, Goda Y, Hakamatsuka T: Determination and identification of specific marker compound for discriminating Shrub Chaste Tree Fruit from Agnus Castus Fruit based on the LC/MS metabolic analysis.
Chem Pharm Bull. 2016;64:1-6.

To ensure the efficacy and safety of both Shrub Chaste Tree Fruit (SCTF) and Agnus Castus Fruit (ACF) products, it is important to authenticate their botanical origins precisely and to distinguish between SCTF and ACF clearly. Therefore, we tried to identify SCTF-specific marker compounds based on LC/MS metabolic analysis. The multivariate analysis of LC/MS data from SCTF and ACF samples furnished the candidate marker compounds of SCTF. An SCTF-specific marker was isolated from SCTF crude drugs and identified as 3-*O*-*trans*-feruloyl tormentic acid on the basis of spectroscopic data from NMR and MS.

Keywords: Vitex products, Western herb, multivariate analysis

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Sato-Masumoto N, Masada S, Takahashi S*, Terasaki S*, Yokota Y*, Hakamatsuka T, Goda Y: Disintegration test of health food products containing *Ginkgo Biloba* L. or *Vitex Agnus-Castus* L. in the Japanese market.
Medicines 2015;2:47-54.

For many years now, a number of Western herbs have been widely used in health food products in Japan and as pharmaceuticals in Europe. There are few or no mandated criteria concerning the quality of these herbal health food products, thus clarification is warranted. Here, we performed disintegration tests of 26 pharmaceutical and health food products containing the Western herbs ginkgo leaf and chaste tree fruit, in accord with the Japanese Pharmacopoeia. All eight pharmaceutical herbal products found in the European market completely disintegrated within the defined test time, and 11 of the 18 tested herbal products distributed as health foods in Japan disintegrated. Among the incompatible products identified in the Pharmacopoeia test, some products remained intact after incubation in water for 60 min. To ensure the efficacy of Western herbal products sold as health food in Japan, quality control, including disintegration, is therefore recommended, even though these products are not regulated under the Pharmaceutical Affairs Law.

Keywords: OTC crude drug product, health food, disintegration test

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Uchiyama N, Kikura-Hanajiri R, Hakamatsuka T: A phenethylamine derivative 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(3,4-methylenedioxyphenyl)methyl]ethanamine (25I-NB34MD) and a piperazine derivative 1-(3,4-difluoromethylenedioxybenzyl)piperazine (DF-MDBP), newly detected in illicit products.
Forensic Toxicol. 2016;34:166-73.

Two new psychoactive substances (NPSs), a phenethylamine derivative 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(3,4-methylenedioxyphenyl)methyl]ethanamine (25I-NB34MD, **1**) and a piperazine derivative 1-(3,4-difluoromethylenedioxybenzyl)piperazine (DF-MDBP, **2**), were identified in illicit

products distributed from January to March 2015 in Japan. The identification was based on LC-MS, GC-MS, high-resolution MS and NMR analyses. Compound **1** has a 3,4-methylenedioxy benzyl moiety which is an analog of *N*-benzylmethoxy derivatives of 2,5-dimethoxyphenethylamines ("NBOMe"-compounds), e.g. 25I-NBOMe. Compound **2** is a difluoromethylenedioxy analog of a known designer drug 1-(3,4-methylenedioxybenzyl)piperazine (MDBP). To our knowledge, this is the first report on compounds **1** and **2** detected as NPSs in illicit products. Although there is no chemical or pharmaceutical information for compound **1**, a 2,3-methylenedioxy isomer of **1**, 25I-NBMD, is reported to have a binding affinity for 5-HT2A receptor. In the GC-MS and LC-MS analyses, compound 1 (25I-NB34MD) showed spectra that are very similar to those of the isomer 25I-NBMD. The structure of compound **1** was determined here by an NMR analysis. Considering these results, we should be careful when analyzing NPSs in illicit products to prevent their misidentification as isomers of other NPSs. It is important to directly compare an unknown substance with an authentic substance by using multiple instruments such as GC-MS and LC-MS.

Keywords: 2-(4-Iodo-2,5-dimethoxyphenyl)-*N*-[(3,4-methylenedioxyphenyl)methyl]ethanamine (25I-NB34MD), 1-(3,4-Difluoromethylenedioxybenzyl) piperazine (DF-MDBP), New psychoactive substance

Uchiyama N, Asakawa K*, Kikura-Hanajiri R, Tsutsumi T*, Hakamatsuka T: A new pyrazole-carboxamide type synthetic cannabinoid AB-CHFUPYCA [*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide] identified in illegal products.

Forensic Toxicol. 2015;33:367-73.

A new pyrazole-carboxamide type synthetic cannabinoid, AB-CHFUPYCA (**1**), was detected in illegal herbal products by our ongoing survey in Japan. The structure of **1** was identified by GC-MS, LC-MS, HR-LC-MS and NMR analyses. Compound **1** showed a molecular weight of 400, and accurate mass measurement using HR-LC-MS revealed its molecular formula to be $C_{22}H_{29}N_4O_2F$. The MS and NMR spectrometric data revealed that the structure of **1** is *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*pyrazole-5-carboxamide. Compound **1**, which is a new type of synthetic cannabinoid, has a 3-(4-fluorophenyl)-1*H*-pyrazole group in place of a 1*H*-indazole group of AB-CHMINACA. To our knowledge, data on the chemistry and pharmacology of compound **1** have never been reported, and we therefore named compound **1** "AB-CHFUPYCA."

Keywords: AB-CHFUPYCA [*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1*H*-pyrazole-5-carboxamide], Pyrazole-carboxamide derivative, synthetic cannabinoid

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Uchiyama N, Shimokawa Y, Kikura-Hanajiri R, Demizu Y, Goda Y, Hakamatsuka T: A synthetic cannabinoid FDU-NNEI, two 2H-indazole isomers of synthetic cannabinoids AB-CHMINACA and NNEI indazole analog (MN-18), a phenethylamine derivative N-OH-EDMA, and a cathinone derivative dimethoxy-α-PHP, newly identified in illegal products.

Forensic Toxicol. 2015;33:244-59.

Our continuous survey of illegal products in Japan revealed the new distribution of 15 designer drugs. We identified four synthetic cannabinoids, i.e., NNEI (**1**), 5-fluoro-NNEI (**2**), 5-chloro-NNEI (**3**) and NNEI indazole analog (**4**), and seven cathinone derivatives, i.e., MPHP (**5**), α-PHPP (**6**),α-POP (**7**), 3,4-dimethoxy-α-PVP (**8**), 4-fluoro-α-PVP (**9**),α-ethylaminopentiophenone (**10**) and N-ethyl-4-methylpentedrone (**11**). We also determined LY-2183240 (**12**) and its 2'-isomer (**13**), which were reported to inhibit endocannabinoid uptake, a methylphenidate analog, 3,4-dichloromethylphenidate (**14**), and an MDA analog, 5-APDB (**15**). No chemical and pharmaceutical data for compounds **3**, **4**, **6** and **7** had been reported, making this the first report on these compounds.

Keywords: NNEI indazole analog, 5-Chloro-NNEI, 3,4-Dichloromethylphenidate

Sato M*1, Yagishita F*2, Mino T*2, Uchiyama N, Patel A*3, Chooi Y-H*4, Goda Y, Xu W*4, Noguchi H*1, Yamamoto T*1, Hotta K*5, Houk KN*3, Tang Y*4, Watanabe K*1: Involvement of Lipocalin-like CghA in Decalin-Forming Stereoselective Intramolecular [4+2] Cycloaddition.

Chem Bio Chem. 2015;16:2294-8.

Understanding enzymatic Diels–Alder (DA) reactions that can form complex natural product scaffolds is of considerable interest. Sch 210972 **1**, a potential anti-HIV fungal natural product, contains a decalin core that is proposed to form through a DA reaction. We identified the gene cluster responsible for the biosynthesis of **1** and heterologously reconstituted the biosynthetic pathway in Aspergillus nidulans to characterize the enzymes involved. Most notably, deletion of cghA resulted in a loss of stereoselective decalin core formation, yielding both an endo (**1**) and a diastereomeric exo adduct of the proposed DA reaction. Complementation with cghA restored the sole formation of **1**. Density functional theory computation of the proposed DA reaction provided a plausible explanation of the observed pattern of product formation. Based on our study, we propose that lipocalin-like CghA is responsible for the stereoselective intramolecular [4+2] cycloaddition that forms the decalin core of **1**.

Keywords: cycloaddition, decalin, density functional calculations

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Toyo'oka T*, Kikura-Hanajiri R: A Reliable Method for the separation and detection of synthetic cannabinoids by supercritical fluid chromatography with mass spectrometry and its application to plant products.

Chem Pharm Bull. 2015;63:762-9.

A reliable method using supercritical fluid chromatography with mass spectrometry (SFC-MS) was developed for cannabinoids using compressed carbon dioxide (CO_2) and methanol as the mobile-phase. The cannabinoids, i.e., cannabicyclohexanol (CCH: cis-isomer), trans-CCH, 5-(1,1-dimethylheptyl)-2-[(1*R*,3*S*)-3-hydroxycyclohexyl]-phenol (CP-47497), 5-(1,1-dimethylheptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-phenol (CP-55940), 3-(1,1'-dimethylheptyl)-6a*R*,7,10,10a*R*-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol (HU-210), 2-[1*R*-3-methyl-6*R*-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol (CBD), (1-pentyl-1*H*-indol-3-yl)-1-naphthalenyl-methanone (JWH-018), (1-butyl-1*H*-indol-3-yl)-1-naphthalenyl-methanone (JWH-073) and 1-(1-pentyl-1*H*-indol-3-yl)-2-(2-methoxyphenyl)-ethanone (JWH-250), were determined within 12 min using a conventional column (2-EP) for SFC. Furthermore, two optical isomers of CCH and trans-CCH were completely and rapidly separated by a chiral stationary phase column (AMY1). A highly sensitive detection (0.002-3.75 ppb) was also obtained by these methods using 2-EP and AMY1 columns. These methods were applied to the qualitative and quantitative determination of cannabinoids in dried plant products. Although the concentration and species were different in the products, JWH-018, JWH-073 and CCH, including the cis-isomer, trans-isomer and the optical isomers, were detected in the products. Therefore, the proposed SFC-MS method seems to be useful as an alternative method to GC-MS and LC-MS for illegal drugs, such as cannabinoids.

Keywords: cannabinoid, supercritical fluid chromatography, mass spectrometry

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Fuchigami Y*, Fu X*, Ikeda R*, Kawakami S*, Wada M*, Kikura-Hanajiri R, Kuroda N*, Nakashima K*: Evaluation of the neurochemical effects of methoxetamine using brain microdialysis in mice.

Forensic Toxicol. 2015;33:374-9.

The ketamine analogue, 2-(3-methoxyphenyl)-2-(ethylamino)cyclohexanone (methoxetamine) has emerged as a drug of abuse. Both methoxetamine and ketamine are antagonists of glutamate *N*-methyl-*d*-asparate receptors, and several case reports show that methoxetamine produces similar schizophrenia-like symptoms and hallucinations to ketamine. Although methoxetamine is believed to change levels

of dopamine, glutamate, and serotonin in the brain, few studies thus far have examined these effects. We investigated the influence of methoxetamine on dopamine and serotonin concentrations using microdialysis and high performance liquid chromatography with electrochemical detection. To reveal the effects of methoxetamine, we monitored dopamine and serotonin concentrations in several brain areas [striatum, nucleus accumbens, and prefrontal cortex (mPFC)] after an administration of 20 mg/kg of methoxetamine. We compared the effects of methoxetamine with those of ketamine using two ketamine doses. Methoxetamine increased dopamine and serotonin concentrations most robustly in the mPFC. In addition, its effects were stronger than those of ketamine at the same molar dose, suggesting that methoxetamine causes schizophrenia-like symptoms and hallucinations by increasing the dopamine and serotonin concentrations. We conclude that consumption of methoxetamine may be more dangerous than consumption of ketamine.

Keywords: methoxetamine, ketamine, NMDA receptor antagonist

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Kudo K*, Usumoto Y*, Kikura-Hanajiri R, Sameshima N*, Tsuji A*, Ikeda N*: A fatal case of poisoning related to new cathinone designer drugs, 4-methoxy PV8, PV9 and 4-methoxy PV9, and a dissociative agent, diphenidine.

Legal Medicine 2015;17:421-6.

A woman in her thirties was found dead on a bed. Considerable amounts of "aroma liquid" and "bath salt" products and hypnotic drug tablets were scattered beside the bed. Autopsy showed pulmonary congestion and edema. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analyses of "aroma liquid" and "bath salt" products showed the presence of new cathinone designer drugs, 4-methoxy PV8 (4-methoxy PHPP), PV9 (α-POP), and 4-methoxy PV9 (4-methoxy α-POP), and a dissociative agent, diphenidine. Drug screening in stomach contents, blood and hydrolyzed urine of the woman by GC-MS and liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed the presence of the above 4 types of drugs and 3 types of benzodiazepines, triazolam, flunitrazepam, and nitrazepam, and their metabolites. The above 7 drugs and 3 benzodiazepine metabolites were simultaneously determined by LC-MS/MS after modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) extraction using diazepam-d5 as the internal standard. The concentrations of 4-methoxy PV8, PV9, 4-methoxy PV9, and diphenidine in the femoral blood were 2.69, 0.743, 0.261, and 1.38μg/ml, respectively, which were significantly higher than concentrations reported in previous cases. Alcohol concentration in the femoral blood was 1.52 mg/ml. Based on the pathological and toxicological findings, the cause of death was determined to be 3 types of cathinone drugs, 4-methoxy PV8, PV9 and 4-methoxy PV9, and diphenidine poisoning under the influence of 3 benzodiazepines and alcohol.

Keywords: synthetic cathinones, GC-MS, LC-MS/MS

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Piao YS*[1,2], Hall FS*[3], Moriya Y*[1], Ito M*[1], Ohara A*[1], Kikura-Hanajiri R, Goda Y, Lesch KP*[4], Murphy DL*[5], Uhl GR*[6], Sora I*[1,7]: Methylone-induced hyperthermia and lethal toxicity: role of the dopamine and serotonin transporters.

Behavioural Pharmacology 2015;26:345-52.

Methylone (2-methylamino-1-[3,4-methylenedioxyphenyl]propan-1-one), an amphetamine analog, has emerged as a popular drug of abuse worldwide. Methylone induces hyperthermia, which is thought to contribute toward the lethal consequences of methylone overdose. Methylone has been assumed to induce hyperthermic effects through inhibition of serotonin and/or dopamine transporters (SERT and DAT, respectively). To examine the roles of each of these proteins in methylone-induced toxic effects, we used SERT and DAT knockout (KO) mice and assessed the hyperthermic and lethal effects caused by a single administration of methylone. Methylone produced higher rates of lethal toxicity compared with other amphetamine analogs in wild-type mice. Compared with wild-type mice, lethality was significantly lower in DAT KO mice, but not in SERT KO mice. By contrast, only a slight diminution in the hyperthermic effects of methylone was observed in DAT KO mice, whereas

a slight enhancement of these effects was observed in SERT KO mice. Administration of the selective D1 receptor antagonist SCH 23390 and the D2 receptor antagonist raclopride reduced methylone-induced hyperthermia, but these drugs also had hypothermic effects in saline-treated mice, albeit to a smaller extent than the effects observed in methylone-treated mice. In contradistinction to 3,4-methylenedioxymethamphetamine, which induces its toxicity through SERT and DAT, these data indicate that DAT, but not SERT, is strongly associated with the lethal toxicity produced by methylone, which did not seem to be dependent on the hyperthermic effects of methylone. DAT is therefore a strong candidate molecule for interventions aimed at preventing acute neurotoxic and lethal effects of methylone.

Keywords: methylone, serotonin and/or dopamine transporters, knockout mice

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Takatori Y*1,2, Shimizu K*1, Ogata J, Endo H*1, Ishimaru K*3, Okamoto S*1, Hashimoto F*1: Cloning of the Flavonoid 3'-hydroxylase gene of *Eustoma grandiflorum* (Raf.) Shinn. (*EgF3'H*) and complementation of an F3'H-deficient mutant of *Ipomoea nil* (L.) Roth. by heterologous expression of *EgF3'H*.

The Horticulture Journal 2015;84:131-9

A full-length cDNA of a putative flavonoid 3'-hydroxylase (*F3'H*) gene encoding a key enzyme in the production of cyanidin was cloned from a lisianthus (*Eustoma grandiflorum*) petal. Overexpression of lisianthus *F3'H* cDNA altered flower color from red to blue in the *I. nil* cultivar 'Violet', which lacks a functional *F3'H* gene. In addition, the transgenic 'Violet' plants accumulated cyanidin and peonidin at similar levels to wild-type *I. nil*. Taking these findings together, this study demonstrates that *EgF3'H* functions as a flavonoid 3'-hydroxylase with a role in the synthesis of cyanidin and peonidin pigments.

Keywords: anthocyanin, flower color, transformation

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Maeda Y*, Terasawa H*, Tanaka Y*, Mitsuura C*, Nakashima K*, Yusa K, Harada S*: Separate cellular localizations of human T-lymphotropic virus 1 (HTLV-1) Env and glucose transporter type 1 (GLUT1) are required for HTLV-1 Env-mediated fusion and infection.

J Virol. 2015;89:502-11.

Interaction of the envelope glycoprotein (Env) of human T-lymphotropic virus 1 (HTLV-1) with the glucose transporter type 1 (GLUT1) expressed in target cells is essential for viral entry. This study found that the expression level of GLUT1 in virus-producing 293T cells was inversely correlated with HTLV-1 Env-mediated fusion activity and infectivity. Chimeric studies between GLUT1 and GLUT3 indicated that the extracellular loop 6 (ECL6) of GLUT1 is important for the inhibition of cell-cell fusion mediated by Env. When GLUT1 was translocated into the plasma membrane from intracellular storage sites by bafilomycin A1 (BFLA1) treatment in 293T cells, HTLV-1 Env-mediated cell fusion and infection also were inhibited without the overexpression of GLUT1, indicating that the localization of GLUT1 in intracellular compartments rather than in the plasma membrane is crucial for the fusion activity of HTLV-1 Env. Immunoprecipitation and laser scanning confocal microscopic analyses indicated that under normal conditions, HTLV-1 Env and GLUT1 do not colocalize or interact. BFLA1 treatment induced this colocalization and interaction, indicating that GLUT1 normally accumulates in intracellular compartments separate from that of Env. Western blot analyses of FLAG-tagged HTLV-1 Env in virus-producing cells and the incorporation of HTLV-1 Env in virus-like particles (VLPs) indicate that the processing of Env is inhibited by either overexpression of GLUT1 or BFLA1 treatment in virus-producing 293T cells. This inhibition probably is due to the interaction of the Env with GLUT1 in intracellular compartments. Taken together, separate intracellular localizations of GLUT1

and HTLV-1 Env are required for the fusion activity and infectivity of HTLV-1 Env.

Keywords: HTLV-1, Env, GLUT1

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Kusakawa S, Yasuda S, Kuroda T, Kawamata S*, Sato Y: Ultra-sensitive detection of tumorigenic cellular impurities in human cell-processed therapeutic products by digital analysis of soft agar colony formation.

Sci Rep. 2015;5:17892.

Contamination with tumorigenic cellular impurities is one of the most pressing concerns for human cell-processed therapeutic products (hCTPs). The soft agar colony formation (SACF) assay, which is a well-known in vitro assay for the detection of malignant transformed cells, is applicable for the quality assessment of hCTPs. Here we established an image-based screening system for the SACF assay using a high-content cell analyzer termed the digital SACF assay. Dual fluorescence staining of formed colonies and the dissolution of soft agar led to accurate detection of transformed cells with the imaging cytometer. Partitioning a cell sample into multiple wells of culture plates enabled digital readout of the presence of colonies and elevated the sensitivity for their detection. In practice, the digital SACF assay detected impurity levels as low as 0.00001% of the hCTPs, i.e. only one HeLa cell contained in 10,000,000 human mesenchymal stem cells, within 30 days. The digital SACF assay saves time, is more sensitive than in vivo tumorigenicity tests, and would be useful for the quality control of hCTPs in the manufacturing process.

Keywords: Tumorigenicity test, soft agar colony formation assay, quality control

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Kuroda T, Yasuda S, Matsuyama S, Tano K, Kusakawa S, Sawa Y*[1], Kawamata S*[2], Sato Y: Highly sensitive droplet digital PCR method for detection of residual undifferentiated cells in cardiomyocytes derived from human pluripotent stem cells.

Regenerative Therapy 2015;2:17-23.

Human pluripotent stem cells (hPSCs), such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are leading candidate cells as raw materials for cell therapy products, because of their capacity for pluripotent differentiation and unlimited self-renewal. hPSC-derived products have already entered the scope of clinical application. However, the assessment and control of their tumorigenicity remains to be a critical challenge. Sensitive detection of the pluripotent cellular impurities is necessary for the safety and quality control of the hPSC-derived products. In the present study, we established a sensitive assay for detection of the residual undifferentiated hiPSCs in cardiomyocytes, using droplet digital PCR (ddPCR). The ddPCR method with a probe and primers for LIN28 significantly detected as low as 0.001% undifferentiated hiPSCs in primary cardiomyocytes, which is equivalent to the ratio of a single hiPSC to 1×10^5 cardiomyocytes. The ddPCR also showed that LIN28 expression is extremely low in human tissues including liver, heart, pancreas, kidney, spinal cord, corneal epithelium and lung. These results suggest that the ddPCR method targeting LIN28 transcripts is highly sensitive and useful for the quality assessment of various cell therapy products derived from hPSCs.

Keywords：再生医療，iPS細胞，造腫瘍性

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Hayakawa T*[1], Aoi T*[2], Umezawa A*[3], Ozawa K*[4], Sato Y, Sawa Y*[5], Matsuyama A*[6], Yamanaka S*[7], Yamato M*[8]: A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from the processing of autologous human somatic stem cells.

Regenerative Therapy 2015;2:57-69.

To make sure that novel human cell-based products contribute to human health care, it is essential that, based on sound science at present, suitable measures be taken by the manufacturers and regulatory authorities on applying these products to the treatment of patients by taking into account specificity of starting cell lines, the manufacturing process, products, administration procedures, diseases in question, and patient population. As part of such an

endeavor, we studied scientific principles, concepts, and basic technical elements to ensure the quality and safety of therapeutic products derived from autologous human somatic stem cells, taking into consideration scientific and technological advances, ethics, regulatory rationale, and international trends in human stem cell-derived products. This led to the development of the Japanese official Notification No. 0907-2, "Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Autologous Human Somatic Stem Cells," issued by Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan on September 7, 2012. The present paper describes the background information and the development of our study and the resulting guidance. For products derived from autologous somatic stem cells, major points to consider include 1) multipotency and self-replication ability of autologous human somatic stem cells and differences in cell characteristics of the final products from those of the starting cells; 2) a donor's infectious status; 3) the risk of proliferation/reactivation of viruses during the manufacturing processes; 4) robust process control to minimize unevenness of "custom-made" products; 5) a limited amount of samples for quality evaluation of products; and 6) robust application and function of the final products in a cell environment different from where the original cells were localized and were performing their natural endogenous functions. The ultimate goal of this guidance is to provide suitable medical opportunities as soon as possible to the patients.

Keywords: Guideline, Autologous Human Somatic Stem Cells, human cell-based products

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Hayakawa T*1, Aoi T*2, Umezawa A*3, Ozawa K*4, Sato Y, Sawa Y*5, Matsuyama A*6, Yamanaka S*7, Yamato M*8: A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from the processing of allogeneic human somatic stem cells.
Regenerative Therapy 2015;2:70-80.

As a series of endeavors to establish suitable measures for the sound development of regenerative medicine using human stem cell-based products, we studied scientific principles, concepts, and basic technical elements to ensure the quality and safety of therapeutic products derived from allogeneic human somatic stem cells, taking into consideration scientific and technological advances, ethics, regulatory rationale, and international trends in human stem cell-derived products. This led to the development of the Japanese official Notification No. 0907-3, "Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Allogeneic Human Somatic Stem Cells," issued by Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan, on September 7, 2012. The present paper describes the background information and development of our study and the resulting guidance. For products derived from allogeneic somatic stem cells, major points to consider include 1) history, the source, and derivation of starting cells; 2) donor screening/testing and donor eligibility, especially in relation to the presence of adventitious agents, potential occurrence of donor-derived diseases, and immunocompatibility; 3) clinical records of a donor; 4) multipotency and self-replication ability of allogeneic human somatic stem cells; 5) cell banking; 6) potential presence of viruses in the final product; 7) extensive characterization of the cells at critical stage(s) of manufacture; 8) robustness of the manufacturing process; 9) quality consistency of the products such as the final products and critical intermediate(s) if any; and 10) robust application and function of the final products in a cell environment different from where the original cells were localized and were performing their natural endogenous function. The ultimate goal of this guidance is to provide suitable medical opportunities as soon as possible to the patients with severe diseases that are difficult to treat with conventional modalities.

Keywords: Guideline, Allogeneic Human Somatic Stem Cells, human stem cell-based products

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Hayakawa T*1, Aoi T*2, Umezawa A*3, Ozawa K*4, Sato Y, Sawa Y*5, Matsuyama A*6, Yamanaka S*7, Yamato M*8: A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from processing of autologous human induced pluripotent stem(-like) cells.
Regenerative Therapy 2015;2:81-94.

As a series of endeavors to establish suitable measures for the sound development of regenerative medicine using human stem cell-based products, we studied scientific principles, concepts, and basic technical elements to ensure the quality and safety of therapeutic products derived from autologous human iPS cells or iPS cell-like cells, taking into consideration scientific and technological advances, ethics, regulatory rationale, and international trends in human stem cell-derived products. This led to the development of the Japanese official Notification No. 0907-4, "Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Autologous Human Induced Pluripotent Stem(-Like) Cells," issued by Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan, on September 7, 2012. The present paper addresses various aspects of products derived from autologous human iPS cells (or iPS cell-like cells), in addition to similar points to consider that are described previously for autologous human stem cell-based products. Major additional points include (1) possible existence of autologous human iPS cell-like cells that are different from iPS cells in terms of specific biological features; (2) the use of autologous human iPS(-like) cells as appropriate starting materials for regenerative medicine, where necessary and significant; (3) establishment of autologous human iPS(-like) cell lines and their characterization; (4) cell banking and/or possible establishment of intermediate cell lines derived from autologous human iPS(-like) cells at appropriate stage(s) of a manufacturing process, if necessary; and (5) concerns about the presence of undifferentiated cells in the final product; such cells may cause ectopic tissue formation and/or tumorigenesis. The ultimate goal of this guidance is to provide suitable medical opportunities as soon as possible to the patients with severe diseases that are difficult to treat with conventional modalities.

Keywords: Guideline, Autologous Human Induced Pluripotent Stem(-Like) Cells

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Hayakawa T*1, Aoi T*2, Umezawa A*3, Ozawa K*4, Sato Y, Sawa Y*5, Matsuyama A*6, Yamanaka S*7, Yamato M*8: A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from processing of allogeneic human induced pluripotent stem(-Like) cells.
Regenerative Therapy 2015;2:95-108.

As a series of endeavors to establish suitable measures for the sound development of regenerative medicine using human stem cell-based products, we studied scientific principles, concepts and basic technical elements to ensure the quality and safety of therapeutic products derived from allogeneic human induced pluripotent stem cells (iPS cells) or iPS cell-like cells, taking into consideration scientific and technological advances, ethics, regulatory rationale, and international trends in human stem cell-derived products. This led to the development of the Japanese official Notification No. 0907-5, "Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Allogeneic Human Induced Pluripotent Stem(-Like) Cells," issued by Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan, on September 7, 2012. The present paper addresses various aspects of products derived from allogeneic human iPS cells (or iPS cell-like cells), in addition to similar points to

consider that are described previously for allogeneic human stem cell-based products. Major additional points include 1) possible existence of allogeneic human iPS cell-like cells that are different from iPS cells in specific biological features; 2) the use of allogeneic human iPS(-like) cells as appropriate starting materials for regenerative medicine, where necessary and significant; 3) establishment of an allogeneic human iPS(-like) cell line and its characterization; 4) establishment of well-characterized stable cell banks and relevant intermediate cell products, if necessary; 5) concerns about the presence of undifferentiated cells in final products; such cells may cause ectopic tissue formation and/or tumorigenesis; and 6) concerns about undesirable immunological reactions that may be caused by the final products. The ultimate goal of this guidance is to provide suitable medical opportunities as soon as possible to the patients with severe diseases that are difficult to treat with conventional modalities.

Keywords: Guideline, Allogeneic Human Induced Pluripotent Stem(-Like) Cells

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Hayakawa T*1, Aoi T*2, Umezawa A*3, Ozawa K*4, Sato Y, Sawa Y*5, Matsuyama A*6, Yamanaka S*7, Yamato M*8: A study on ensuring the quality and safety of pharmaceuticals and medical devices derived from the processing of human embryonic stem cells.
Regenerative Therapy 2015;2:109-22.

As a series of endeavors to establish suitable measures for the sound development of regenerative medicine using human stem cell-based products, we studied scientific principles, concepts, and basic technical elements to ensure the quality and safety of therapeutic products derived from the processing of human embryonic stem cells (hESCs), taking into consideration scientific and technological advances, ethics, regulatory rationale, and international trends in human stem cell-derived products. This led to the development of the Japanese official Notification No. 0907-6, "Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices Derived from the Processing of Human Embryonic Stem Cells," issued by Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan, on September 7, 2012. The present paper addresses various aspects of products derived from hESCs, in addition to similar points to consider that are described previously for allogeneic human stem cell-based products. Major additional points include 1) establishment of hESCs; 2) establishment of stable and well-characterized cell banks of hESCs and relevant intermediate cell products; 3) concerns about the presence of undifferentiated cells in final products, which may result in ectopic tissue formation and/or tumorigenesis; and 4) concerns about undesirable immunological reactions caused by the final products. The ultimate goal of this series of guidelines on regenerative medicine is to provide suitable medical opportunities as soon as possible to the patients with severe diseases that are difficult to treat with conventional modalities. If these guidelines are interpreted and employed in a flexible and meaningful way in this context, they should serve as a useful means to achieve their goals.

Keywords: Guideline, human embryonic stem cells, human stem cell-derived products

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Yoshida T, Yoshioka Y*1, Morishita Y*1, Aoyama M*1, Tochigi S*1, Hirai T*1, Tanaka K*1, Nagano K*1, Kanada H*2, Tsunoda S*2, Nabeshi H, Yoshikawa T*1, Higashisaka K*1, Tsutsumi Y*1: Protein corona changes mediated by surface modification of amorphous silica nanoparticles suppress acute toxicity and activation of intrinsic coagulation cascade in mice.

Nanotechnology 2015;26:245101.

Recently, nanomaterial-mediated biological effects have been shown to be governed by the interaction of nanomaterials with some kinds of proteins in biological fluids, and the physical characteristics of the nanomaterials determine the extent and type of their interactions with proteins. Here, we examined the relationships between the surface properties of amorphous silica nanoparticles with diameters of 70 nm (nSP70), their interactions with some proteins in biological fluids, and their toxicity in mice after intravenous administration. The surface modification of nSP70 with amino groups (nSP70-N) prevented acute lethality and abnormal activation of the coagulation cascade found in the nSP70-treated group of mice. Since our previous study showed that coagulation factor XII played a role in the nSP70-mediated abnormal activation of the coagulation cascade, we examined the interaction of nSP70 and nSP70-N with coagulation factor XII. Coagulation factor XII bonded to the surface of nSP70 to a greater extent than that observed for nSP70-N, and consequently more activation of coagulation factor XII was observed for nSP70 than for nSP70-N. Collectively, our results suggest that controlling the interaction of nSP70 with blood coagulation factor XII by modifying the surface properties would help to inhibit the nSP70-mediated abnormal activation of the blood coagulation cascade.

Keywords: Coagulation, Nanoparticles, Protein adsorption

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Hattori T, Watanabe-Takahashi M[*1], Ohoka N, Hamabata T[*2], Furukawa K[*3], Nishikawa K[*1], Naito M: Proteasome inhibitors prevent cell death and prolong survival of mice challenged by Shiga toxin.
FEBS Open Bio. 2015;5:605-14.

Shiga toxin (Stx) causes fatal systemic complications. Stx induces apoptosis, but the mechanism of which is unclear. We report that Stx induced rapid reduction of short-lived anti-apoptotic proteins followed by activation of caspase 9 and the progression of apoptosis. Proteasome inhibitors prevented the reduction of anti-apoptotic proteins, and inhibited caspase activation and apoptosis, suggesting that the reduction of anti-apoptotic proteins is a prerequisite for Stx-induced apoptosis. A clinically approved proteasome inhibitor, bortezomib, prolonged the survival of mice challenged by Stx. These results imply that proteasome inhibition may be a novel approach to prevent the fatal effects of Stx.

Keywords: Shiga toxin, Apoptosis, Proteasome

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Tomoshige S[*], Naito M, Hashimoto Y[*], Ishikawa M[*]: Degradation of HaloTag-fused nuclear proteins using bestatin-HaloTag ligand hybrid molecules.
Org Biomol Chem. 2015;16:9746-50.

We have developed a protein knockdown technology using hybrid small molecules designed as conjugates of a ligand for the target protein and a ligand for ubiquitin ligase cellular inhibitor of apoptosis protein 1 (cIAP1). However, this technology has several limitations. Here, we report the development of a novel protein knockdown system to address these limitations. In this system, target proteins are fused with HaloTag to provide a common binding site for a degradation inducer. We designed and synthesized small molecules consisting of alkyl chloride as the HaloTag-binding degradation inducer, which binds to HaloTag, linked to BE04 (2), which binds to cIAP1. Using this system, we successfully knocked down HaloTag-fused cAMP responsive element binding protein 1 (HaloTag-CREB1) and HaloTag-fused c-jun (HaloTag-c-jun), which are ligand-unknown nuclear proteins, in living cells. HaloTag-binding degradation inducers can be synthesized easily, and are expected to be useful as biological tools for pan-degradation of HaloTag-fused proteins.

Keywords: Ubiquitin-proteasome system, cIAP1, HaloTag

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Shibata N, Ohoka N, Sugaki Y[*1], Onodera C[*1], Inoue M[*2], Sakuraba Y[*3], Takakura D, Hashii N, Kawasaki N, Gondo Y[*3], Naito M: Degradation of Stop Codon Read-through Mutant Proteins via the Ubiquitin-Proteasome System Causes Hereditary Disorders.

J Biol Chem. 2015;290:28428-37.

During translation, stop codon read-through occasionally happens when the stop codon is misread, skipped, or mutated, resulting in the production of aberrant proteins with C-terminal extension. These extended proteins are potentially deleterious, but their regulation is poorly understood. Here we show in vitro and in vivo evidence that mouse cFLIP-L with a 46-amino acid extension encoded by a read-through mutant gene is rapidly degraded by the ubiquitin-proteasome system, causing hepatocyte apoptosis during embryogenesis. The extended peptide interacts with an E3 ubiquitin ligase, TRIM21, to induce ubiquitylation of the mutant protein. In humans, 20 read-through mutations are related to hereditary disorders, and extended peptides found in human PNPO and HSD3B2 similarly destabilize these proteins, involving TRIM21 for PNPO degradation.

Our findings indicate that degradation of aberrant proteins with C-terminal extension encoded by read-through mutant genes is a mechanism for loss of function resulting in hereditary disorders.

Keywords: Read-through mutation, Ubiquitin-proteasome system, cFLIP-L

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Nishikawa K*1, Iwaya K*2, Kinoshita M*2, Fujiwara Y*3, Akao M*3, Sonoda M*3, Thiruppathi S, Suzuki T, Hiroi S*2, Seki S*2, Sakamoto T*2: Resveratrol increases CD68(+) Kupffer cells colocalized with adipose differentiation-related protein and ameliorates high-fat-diet-induced fatty liver in mice.

Mol Nutr Food Res. 2015;59:1155-70.

This study purposed to elucidate the effect of resveratrol on fatty liver in mice fed a high-fat (HF) diet, and to investigate the role of liver macrophages (Kupffer cells).

C57BL/6 mice were fed with either a control diet, HF diet (50% fat), or HF supplemented with 0.2% resveratrol (HF + res) diet, for 8 weeks. Compared with the HF group, the HF + res group exhibited markedly attenuated fatty liver, and reduced lipid droplets (LDs) in hepatocytes. Proteomic analysis demonstrated that the most downregulated protein in the livers of the HF + res group was adipose differentiation-related protein (ADFP), which is a major constituent of LDs and reflects lipid accumulation in cells. The HF + res group exhibited greatly increased numbers of CD68(+) Kupffer cells with phagocytic activity. situ hybridization analysis, compared with the HF group.

Keywords: Resveratrol, Fatty liver

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Kondo J*1, Nomura Y, Kitahara Y*1, Obika S*3, Torigoe H*2: Crystal structure of 2',4'-BNANC[N-Me]-modified antisense gapmer in complex with the target RNA.

Chemical Communications 2016;52:2354-7.

It has been confirmed by our previous studies that a 20,40-BNANC[N–Me]-modified antisense gapmer displays high affinity and selectivity to the target RNA strand, promising mRNA inhibitory activity and excellent nuclease resistance. Herein, we have obtained a crystal structure that provides insights into these excellent antisense properties.

Keywords: Antisense, BNA, Crystal structure

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Haishima Y, Kawakami T, Fukui C, Tanoue A*1, Yuba T*2, Ozono S, Kumada H*3, Inoue K, Morikawa T, Takahashi M, Fujisawa A*4, Yamasaki K*5, Nomura Y, Isama K, Chung U*4, Ogawa K, Niimi S, Yoshida M: Characterization of alternative plasticizers in polyvinyl chloride sheets for blood containers.

J Vinyl Add Technol. 2015;doi:10.1002/vnl.21472.

This study aimed to optimize the ratio of dioctyl 4-cyclohexene-1,2-dicarboxylate (DOTH) and di-isononyl-cyclohexane-1,2-dicarboxylate (DINCH®) for use as plasticizers in poly(vinyl chloride) (PVC) sheets. We also evaluated the biological safety of DOTH for its potential to be part of a safe PVC-based blood container. The suppression of hemolysis in mannitol-adenine-phosphate / red cell concentrates (MAP/

RCC) with DOTH/(DINCH®-PVC) sheets and the elution of plasticizers from the sheets increased with higher DOTH compositions. The properties of the PVC sheet containing DOTH and DINCH® in the ratio of 25:33 parts against PVC 100 parts as a weight were almost identical to the PVC sheet made of di(2-ethylhexyl) phthalate. From a subchronic toxicity test, DOTH did not show any adverse effects on all organs, including the testes, epididymis, liver, and kidneys. The no-observed-adverse-effect level was 300 mg/kg body weight/day in a rat. These results suggest that DOTH/DINCH® (25:33) is a promising candidate for the replacement of di(2-ethylhexyl) phthalate in blood containers.

Keywords: PVC medical device, blood containers, hemolysis

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迫田秀行，新見伸吾：疲労き裂進展特性による人工関節用高度架橋超高分子量ポリエチレンの耐久性評価．*臨床バイオメカニクス* 2015;36:197-200.

Ultra-high molecular weight polyethylene (UHMWPE) has been widely used as an articulating surface of artificial joints. Recent advancements of materials such as improved wear resistance by cross-linking and improved stability by the addition of an anti-oxidant are expected to reduce aseptic loosening, leading to the longer life of artificial joints. On the other hand, there is still a possibility of fatigue failure at sites under a large load, such as a post of artificial knee joints and a rim of artificial hip joints. In this study, we investigated the fatigue characteristics of highly cross-linked UHMWPE, which is now commonly used clinically, using fatigue crack propagation tests, and compared them with the results of our previous study using highly cross-linked UHMWPE by delamination tests to evaluate the clinical relevance of the tests.

It was found that the fatigue crack propagation characteristics of remelted highly cross-linked UHMWPE were significantly lower compared to virgin UHMWPE. These results were consistent with reports of rim cracking found in retrieved acetabular liners made of remelted highly cross-linked UHMWPE. On the other hand, the results were not consistent with our previous study showing the improved fatigue characteristics of highly cross-linked UHMWPE by delamination tests. Therefore, it was indicated that the mechanisms of failure are different between delamination and cracking. It was considered necessary to assess both delamination resistance and fatigue crack propagation properties in order to evaluate the durability of UHMWPE components.

Keywords: artificial joint, cross-linking, fatigue crack propagation

Sakoda H, Niimi S: Impact of lipid-induced degradation on the mechanical properties of ultra-high molecular weight polyethylene for joint replacements.
Journal of the Mechanical Behavior of Biomedical Materials 2016;53:218-25.

Gamma or electron beam irradiation of ultra-high molecular weight polyethylene (UHMWPE) used in artificial joints for sterilization and/or crosslinking purposes generates free radicals in the material, which causes long-term oxidative degradation of UHMWPE. Recently, another mechanism for the degradation of UHMWPE by the absorption of lipids during in vivo clinical use was proposed. However, knowledge on lipid-induced degradation is quite limited, compared with that on radical-induced degradation. In this study, lipid-induced degradation was simulated using squalene absorption and subsequent accelerated aging, and its impact on the mechanical properties of UHMWPE was evaluated. The simulated lipid-induced degradation caused an increased elastic modulus and decreased elongation with maximum degradation at the surfaces. These results imply that degradation of UHMWPE may occur during in vivo long-term use, even if free radicals are completely eliminated. Therefore, further investigation is required to clarify the impact of lipid-induced degradation on clinical outcomes, such as the wear and fatigue characteristics of UHMWPE components.

Keywords: UHMWPE, lipids, degradation

Abdi R*, Moore R*, Sakai S, Donnelly CB*,

Mounayar M*, Sackstein R*: HCELL expression on murine MSC licenses pancreatotropism and confers durable reversal of autoimmune diabetes in NOD mice.

Stem Cells 2015;33:1523-31.

Type 1 diabetes (T1D) is an immune-mediated disease resulting in destruction of insulin-producing pancreatic beta cells. Mesenchymal stem cells (MSCs) possess potent immunomodulatory properties, garnering increasing attention as cellular therapy for T1D and other immunologic diseases. However, MSCs generally lack homing molecules, hindering their colonization at inflammatory sites following intravenous (IV) administration. Here, we analyzed whether enforced E-selectin ligand expression on murine MSCs could impact their effect in reversing hyperglycemia in nonobese diabetic (NOD) mice. Although murine MSCs natively do not express the E-selectin-binding determinant sialyl Lewis^x (sLe^x), we found that fucosyltransferase-mediated α(1,3)-exofucosylation of murine MSCs resulted in sLe^x display uniquely on cell surface CD44 thereby creating hematopoietic cell E-/L-selectin ligand (HCELL), the E-selectin-binding glycoform of CD44. Following IV infusion into diabetic NOD mice, allogeneic HCELL$^+$ MSCs showed 3-fold greater peri-islet infiltrates compared to buffer-treated (i.e., HCELL$^-$) MSCs, with distribution in proximity to E-selectin-expressing microvessels. Exofucosylation had no effect on MSC immunosuppressive capacity in *in vitro* assays; however, although engraftment was temporary for both HCELL$^+$ and HCELL$^-$ MSCs, administration of HCELL$^+$ MSCs resulted in durable reversal of hyperglycemia, whereas only transient reversal was observed following administration of HCELL$^-$ MSCs. Notably, exofucosylation of MSCs generated from CD44$^{-/-}$ mice induced prominent membrane expression of sLe^x, but IV administration of these MSCs into hyperglycemic NOD mice showed no enhanced pancreatotropism or reversal of hyperglycemia. These findings provide evidence that glycan engineering to enforce HCELL expression boosts trafficking of infused MSCs to pancreatic islets of NOD mice and substantially improves their efficacy in reversing autoimmune diabetes.

Keywords: HCELL, glycan engineering, mesenchymal stem cell

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Kikuchihara Y*[1], Abe H*[1,2], Tanaka T*[1,2], Kato M*[1], Wang L*[1], Ikarashi Y, Yoshida T*[1], Shibutani M*[1]: Relationship between brain accumulation of manganese and aberration of hippocampal adult neurogenesis after oral exposure to manganese chloride in mice.

Toxicology 2015;331:24-34.

We previously found persistent aberration of hippocampal adult neurogenesis, along with brain manganese (Mn) accumulation, in mouse offspring after developmental exposure to 800-ppm dietary Mn. Reduction of parvalbumin (Pvalb)$^+$γ-aminobutyric acid (GABA)-ergic interneurons in the hilus of the dentate gyrus along with promoter region hypermethylation are thought to be responsible for this aberrant neurogenesis. The present study was conducted to examine the relationship between the induction of aberrant neurogenesis and brain Mn accumulation after oral Mn exposure as well as the responsible mechanism in young adult animals. We used two groups of mice with 28- or 56-day exposure periods to oral $MnCl_2{\cdot}xH_2O$ at 800 ppm as Mn, a dose sufficient to lead to aberrant neurogenesis after developmental exposure. A third group of mice received intravenous injections of Mn at 5-mg/kg body weight once weekly for 28 days. The 28-day oral Mn exposure did not cause aberrations in neurogenesis. In contrast, 56-day oral exposure caused aberrations in neurogenesis suggestive of reductions in type 2b and type 3 progenitor cells and immature granule cells in the dentate subgranular zone. Brain Mn accumulation in 56-day exposed cases, as well as in directly Mn-injected cases occurred in parallel with reduction of Pvalb$^+$ GABAergic interneurons in the dentate hilus, suggesting that this may be responsible for aberrant neurogenesis. For reduction of Pvalb$^+$ interneurons, suppression of brain-derived neurotrophic factor-mediated signaling of mature granule cells may occur via suppression of c-Fos-mediated neuronal plasticity due to direct Mn-toxicity rather than promoter region hypermethylation of *Pvalb*.

Keywords: Manganese, Hippocampal adult neurogenesis, GABAergic interneuron

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Simazaki D*1, Kubota R, Suzuki T*2, Akiba M*1, Nishimura T*3, Kunikane S*1: Occurrence of selected pharmaceuticals at drinking water purification plants in Japan and implications for human health.
Water Res. 2015;76:187-200.

The present study was performed to determine the occurrence of 64 pharmaceuticals and metabolites in source water and finished water at 6 drinking water purification plants and 2 industrial water purification plants across Japan. The analytical methods employed were sample concentration using solid-phase extraction cartridges and instrumental analysis by liquid chromatography with tandem mass spectrometry (LCeMS/MS), liquid chromatography with mass spectrometry (LC/MS), or trimethylsilyl derivatization followed by gas chromatography with mass spectrometry (GC/MS). Thirty-seven of the 64 target substances were detected in the source water samples. The maximum concentrations in the source water were mostly below 50 ng/L except for 13 substances. In particular, residual concentrations of iopamidol (contrast agent) exceeded 1000 ng/L at most facilities. Most of the residual pharmaceuticals and metabolites in the source water samples were removed in the course of conventional and/or advanced drinking water treatments, except for 7 pharmaceuticals and 1 metabolite, i.e., amantadine, carbamazepine, diclofenac, epinastine, fenofibrate, ibuprofen, iopamidol, and oseltamivir acid. The removal ratios of the advanced water treatment processes including ozonation and granular activated carbon filtration were typically much higher than those of the conventional treatment processes. The margins of exposure estimated by the ratio of daily minimum therapeutic dose to daily intake via drinking water were substantial, and therefore the pharmacological and physiological impacts of ingesting those residual substances via drinking water would be negligible.

Keywords: Drinking water, Pharmaceutical, Water treatment

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久保田領志，小林憲弘，齋藤信裕*1，鈴木俊也*2，小杉有希*2，田中美奈子*3，塚本多矩*4，平林達也*5，五十嵐良明：固相抽出－液体クロマトグラフ－質量分析計による水道水中フェノール類の検査法の開発とその妥当性評価.
水道協会雑誌 2015;84(7):2-15.

水道水中フェノール類の標準検査法を迅速・簡便化するため，固相抽出－液体クロマトグラフ－質量分析法を検討した．その結果，全15化合物のピーク分離が良好なLC条件を確立し，また，固相カラムの乾燥時間の短縮化や溶出溶媒量の少量化等により，前処理時間を現行法の1/2以下に大幅に短縮できる方法を開発した．開発した検査法について，多施設共同試験による妥当性評価試験を実施した．水道水を用いて添加回収試験を行い，妥当性評価ガイドラインの性能パラメータが目標を満たすか評価した結果，真度（回収率），併行精度及び室間精度から外挿して評価した室内精度も目標を満たしたことから，本検査法は妥当であると判断した．

Keywords: Drinking water, Phenols, SPE-LC-MS

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Nishi I*1, Kawakami T, Onodera S*2: Monitoring the concentrations of nonsteroidal anti-inflammatory drugs and cyclooxygenase-inhibiting activities in the surface waters of the Tone Canal and Edo River Basin.
J Environ Sci Health Part A. 2015;50:1108-15.

Environmental pollution by pharmaceuticals has become a major problem in many countries worldwide. However, little is known about the concentrations of pharmaceuticals in water sources in Japan. The objective of this study was to clarify variations in the concentrations of seven nonsteroidal anti-inflammatory drugs (NSAIDs) and in cyclooxygenase(COX)-inhibiting activities in river water and domestic wastewater collected from the Tone Canal and the Edo River Basin in Japan. Total NSAID concentrations were higher in the Tone Canal than in the Edo River, and the highest concentration was observed at the domestic wastewater inflow point located in the Tone Canal (concentration averages of salicylic acid, ibuprofen, felbinac, naproxen, mefenamic acid, diclofenac, and

ketoprofen in wastewater samples were 55.3, 162.9, 39.7, 11.8, 30.8, 259.7, and 48.3 ng/L, respectively). Gas chromatography-tandem mass spectrometry showed that wastewater samples collected during cooler seasons contained higher levels of COX-inhibiting activity. COX-inhibiting activities were highly correlated with NSAID concentrations (particularly for ketoprofen and diclofenac); however, other COX inhibitors, such as NSAIDs that were not examined in this study and/or other chemicals with COX-inhibiting activity, could exist in the water samples because the concentrations of NSAIDs obtained from the water samples did not account for the total COX-inhibiting activities observed. Therefore, COX inhibition assays may be helpful for evaluating the aquatic toxicity of COX inhibitors. In this study, we demonstrated that COX inhibitors in surface water may influence aquatic organisms more than was expected based on NSAID concentrations. Thus, further studies examining other COX inhibitors in the aquatic environment are necessary.

Keywords: cyclooxygenase, nonsteroidal anti-inflammatory drugs, river water

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Nakanishi J[*1], Morimoto Y[*2], Ogura I[*1], Kobayashi N, Naya M[*3], Ema M[*1], Endoh S[*1], Shimada M[*4], Ogami A[*2], Myojyo T[*2], Oyabu T[*2], Gamo M[*1], Kishimoto A[*5], Igarashi T[*1], Hanai S[*1]: Risk Assessment of the Carbon Nanotube Group.
Risk Analysis 2015;35:1940–56.

This study assessed the health risks via inhalation and derived the occupational exposure limit (OEL) for the carbon nanotube (CNT) group rather than individual CNT material. We devised two methods: the integration of the intratracheal instillation (IT) data with the inhalation (IH) data, and the "biaxial approach."

Keywords: CNT toxicity, OEL, risk assessment

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Matsunaga K[*1], Kuroda Y[*2], Sakai S, Adachi R, Teshima R, Yagami A[*1], Itagaki H[*2]: Anaphylactic augmentation by epicutaneous sensitization to acid-hydrolyzed wheat protein in a guinea pig model.
J Toxicol Sci. 2015;40:745-52.

Recent reports suggest that hydrolyzed wheat protein (HWP) variants such as Glupearl® 19S (GP19S) induce immediate-type hypersensitivity via epicutaneous (EC) sensitization. The identification of strong allergens is a key step in product assessment before commercial launch. However, few reports have described the estimation of actual and potential anaphylactic sensitizing capacity. In this study we assessed the strength of both the actual and potential anaphylactic sensitizing capacity by investigating the immediate-type hypersensitivity inducing potential of HWP compared with gluten. We assessed these strengths via the EC route using an EC or intradermal (ID) sensitization method. We quantified the strength of immediate-type hypersensitivity by evaluating the titer of serum antibodies isolated from sensitized subjects using passive cutaneous anaphylaxis (PCA) reactions. We also evaluated the cross-reactivity between GP19S and gluten. GP19S and gluten applied by both the sensitization methods induced obvious IgG1-mediated PCA reactions. GP19S had stronger sensitizing potential than gluten, according to the serum titers and dye spot diameters. The difference in antibody titers between GP19S and gluten was 16-fold for the EC method versus 2-fold for the ID method. GP19S cross-reacted with gluten. Acid hydrolysis of gluten increased anaphylactic sensitizing capacity in the EC method. To our knowledge, our study is the first to quantitatively confirm that HWP and gluten can induce immediate-type hypersensitivity through an intact skin. These findings suggest that acid-HWP imposes a higher risk of EC sensitization than gluten because of the ease with which the former confers a sensitizing effect through the intact skin.

Keywords: acid-hydrolyzed wheat protein, cross-reactivity, epicutaneous sensitization

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久保田領志，小林憲弘，五十嵐良明：水道水質検査精度管理のための統一試料調査に関する経年分析（平成17 ～ 22年度）：無機物.
水道協会雑誌 2015;84(12):15-22.

厚生労働省が水道水質検査機関を対象に実施している水道水質検査精度管理のための統一試料調査において，平成17 ～ 22年度の6か年分の無機物の調査結果について経年分析を行った．検査法の使用機関割合の推移は，多くの年度でICP-MSが主であり，ICP-AES，FL-AASの順であった．棄却機関の割合については，FL-AAS，ICP-MS及びICP-AESの3群間で有意差が認められ，ICP-MSが他の2検査法に比べて低割合を示した．複数年度で対象となったアルミニウムについては，|Z|≧3の機関の割合は減少し，|Z|≦2や2＜|Z|＜3では増加傾向となった．また，棄却機関数は増加となったが増加機関数は数機関であり，検査回数を重ねることで分析精度が向上していると評価できた.

Keywords: External quality assessment, Water quality standard, Inorganic substances

Kawakami T, Isama K, Ikarashi Y: Survey of isothiazolinones and other preservatives in household wet tissue products in Japan.
J Environ Chem. 2015;25:207-14.

Recently, many cases of contact dermatitis due to isothiazolinone preservatives in several types of household products used for cooling the body have been reported. As a result, the concentrations of isothiazolinone preservatives in these products were investigated. However, concentrations of isothiazolinone preservatives in other types of household products have not been studied adequately. In this study, 19 preservatives (including isothiazolinones) in 32 wet tissue products were investigated because these products come in direct contact with the skin. 2-Methyl-4-isothiazolin-3-one (MI), 5-chloro-2-methyl-4-isothiazolin-3-one (CMI), and benzisothiazolin-3-one (BIT) were detected in 19 samples (0.46-48 μg/g-wet), 17 samples (trace amount [tr.]-52 μg/g-wet), and one sample (67 μg/g-wet), respectively. Five types of parabens were detected in 21 samples (tr.-834 μg/g-wet). 2-Bromo-2-nitropropane-1,3-diol (Bronopol), 3-iodo-2-propynyl N-butylcarbamate (IPBC), and phenoxyethanol were detected in 12 samples (4.7-254 μg/g-wet), 11 samples (tr.-62 μg/g-wet), and 4 samples (65-1159 μg/g-wet), respectively. The concentration levels of isothiazolinone preservatives detected in this study perhaps induce allergic contact dermatitis in patients who are already sensitive to these preservatives. However, only 3 products described the use of isothiazolinone preservatives and a cautionary note about the possibility of contact dermatitis due to isothiazolinone preservatives was not provided. We also found that preservatives detected in the samples were different from those indicated on the product (in some cases, name of preservatives were not indicated at all). The use of such products may expose consumers to the risk of contact dermatitis; moreover, when contact dermatitis occurs, the identification of the substance that causes it may be delayed. Therefore, it is desirable that manufacturers provide information about the components of wet tissue products on the product labels.

Keywords: contact dermatitis, isothiazolinone preservatives, wet tissue

小林憲弘，久保田領志，齋藤信裕*1，木村謙治*2，宮﨑悦子*2，平林達也*3，水田裕進*4，木村慎一*4，宮本紫織*5，大倉敏裕*5，中村弘揮*6，粕谷智浩*7，古川浩司*8，塚本多矩*9，市川千種*9，髙原玲華*10，林田寛司*10，京野完*11，佐久井徳広*11，山本五秋*12，齊藤香織*12，五十嵐良明：水道水中のイミノクタジン・ジクワット・パラコートLC/MS/MS一斉分析法の妥当性評価.
環境科学会誌 2016;29:3–16.

著者らが開発した水道水中のイミノクタジン，ジクワットおよびパラコートLC/MS/MS一斉分析法を水道水質検査に適用できるかどうかを評価するため，12機関（水道事業体4機関，衛生研究所1機関，登録検査機関3機関および分析機器メーカー4機関）において，分析法の妥当性を評価した.

Keywords: validation, drinking water, agricultural chemicals

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久保田領志，小林憲弘，五十嵐良明：水道水質検査精度管理のための統一試料調査に関する経年分析（平成17～22年度）：有機物.
水道協会雑誌 2016;85(2):9-15.

厚生労働省が実施している水道水質検査精度管理のための統一試料調査において，平成17～22年度の6か年分の有機物の調査結果の経年分析を行った．Zスコア3以上（|Z|≧3）となった機関の参加総数に対する割合は，平成19年度のフェノール類で高く，唯一10%を超えた．検査法別に見ると，棄却された機関の割合は，SPE-GC/MSを使った場合がPT-GC/MSやHS-GC/MSに比べて有意に高割合であった．複数回調査対象項目として選定されたジクロロフェノール類について，Zスコア区分ごとの機関の割合を比較すると，|Z|≧3の機関の割合の減少とともに|Z|≦2及び2<|Z|<3の機関の割合が増加した．同一項目の調査回数を重ねることで，分析精度が改善していると評価できた.

Keywords: External quality assessment, Water quality standard, Organic substances

植草義徳，鍋師裕美，中村里香，堤智昭，蜂須賀暁子，松田りえ子，手島玲子：市販流通食品中の放射性セシウム調査（平成24年度および平成25年度）.
食品衛生学雑誌 2015;56:49-56.

We surveyed the concentration of radioactive cesium in foods purchased at markets in areas where possible contamination has been a concern after the Fukushima accident. In fiscal years 2012 and 2013, we surveyed 1,735 and 1,674 foods, respectively, using a NaI (Tl) scintillation spectrometer for the screening test and a γ-ray spectrometer with a germanium semiconductor detector for the final test. Only 3 and 4 samples (0.2% of our total samples) exceeded the regulatory limit (100 Bq/kg) for radioactive cesium in fiscal years 2012 and 2013, respectively. Our surveillance indicates that the pre-shipment monitoring of foods by local governments has been working effectively.

Keywords: radioactive cesium, scintillation spectrometer, germanium semiconductor detector

Uekusa Y, Takatsuki S, Tsutsumi T, Matsuda R, Akiyama H, Hachisuka A, Teshima R, Watanabe T: Follow-up investigation of polychlorinated biphenyl concentrations in fish from tsunami-stricken areas of Japan.
Organohalogen Compounds 2015;77:432-5.

We quantified PCBs and the concentrations of 209 congeners as percentages of the total PCB concentration in 80 follow-up fish samples obtained not only from tsunami-stricken areas but also from an area unlikely to have been affected by the tsunami after the Great East Japan Earthquake in 2011. Total PCB concentrations in the samples from the tsunami-stricken areas ranged from 0.32 to 223 ng/g, whereas those in samples from the negative control area ranged from 0.75 to 128 ng/g. The maximum PCB concentration found was lower than the provisional regulatory limit (oceans, 500 ng/g) in Japan. The percentage of each chlorinated congener appeared similar among the samples; tetra- to hepta-chlorinated congeners dominated. Our results support our previous findings that fish samples from markets in tsunami-stricken areas were unlikely to have been contaminated with PCBs at high concentrations.

Keywords: PCBs, fish, HRGC-HRMS

植草義徳，鍋師裕美，片岡洋平，渡邉敬浩，蜂須賀暁子，穐山浩，堤智昭，松田りえ子，手島玲子：福島第一原子力発電所事故に由来した放射性セシウムが検出された食品のウラン濃度の調査.
日本食品化学学会誌 2016;23:43-8.

The concentration of uranium (U-238) in various foods containing radioactive cesium (Cs-134 and Cs-137) derived from the Fukushima Daiichi nuclear power plant accident was determined using inductively coupled plasma mass spectroscopy. U-238 concentration in the foods that Cs-134 concentration was below the limits of detection and that was obtained before the accident, were also investigated. U-238 was detected in all 87 samples investigated and the concentration ranged from 0.038 to 130 mBq/kg. In addition, no correlation was observed between the concentration of radioactive cesium and U-238. The range of U-238 concentration observed in the post-accident food samples was similar to that in the food samples that Cs-134 concentration was below the limits of detection and that in the pre-accident food samples, and to the literature values in foods previously reported. These results suggest that the U-238 concentration was not

significantly different in the foods between before and after the accident.
Keywords: uranium, ICP-MS

片岡洋平，渡邉敬浩，林智子，手島玲子，松田りえ子：清涼飲料水中の鉛，総ヒ素，カドミウムの一斉定量を目的としたICP-OES法，ICP-MS法，電気加熱式原子吸光法の開発.
食品衛生学雑誌 2015;56:88-95.

In this study, we developed methods to quantify lead, total arsenic and cadmium contained in various kinds of soft drinks, and we evaluated their performance. The samples were digested by common methods to prepare solutions for measurement by ICP-OES, ICP-MS and graphite furnace atomic absorption spectrometry (GF-AAS). After digestion, internal standard was added to the digestion solutions for measurements by ICP-OES and ICP-MS. For measurement by GF-AAS, additional purification of the digestion solution was conducted by back-extraction of the three metals into nitric acid solution after extraction into an organic solvent with ammonium pyrrolidine dithiocarbamate. Performance of the developed methods were evaluated for eight kinds of soft drinks.
Keywords: lead, total arsenic, cadmium

Watanabe T, Kikuchi H, Matsuda R, Hayashi T, Akaki K*, Teshima R: Performance evaluation of an improved GC-MS method to quantify methylmercury in fish.
Food Hyg Saf Sci. 2015;56:69-76.

Here, we set out to improve our previously developed methylmercury analytical method, involving phenyl derivatization and gas chromatography-mass spectrometry (GC-MS). In the improved method, phenylation of methylmercury with sodium tetraphenylborate was carried out in a toluene/water two-phase system, instead of in water alone. The modification enabled derivatization at optimum pH, and the formation of by-products was dramatically reduced. In addition, adsorption of methyl phenyl mercury in the GC system was suppressed by co-injection of PEG200, enabling continuous analysis without loss of sensitivity. The performance of the improved analytical method was independently evaluated by three analysts using certified reference materials and methylmercury-spiked fresh fish samples. The present analytical method was validated as suitable for determination of compliance with the provisional regulation value for methylmercury in fish, set in the Food Sanitation law.
Keywords: methylmercury, GC-MS, fresh fish

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Ito A*1, Taguchi T*1, Mogi T*1, Wake H*1, Tanaami T*1, Hada S*1, Akiyama H, Teshima R, Sasaki N*2, Yamada A*2, Ozeki Y*2: Identification of genetically modified organisms by detection of target gene pattern using DNA microarrays.
Jpn J Food Chem Safety. 2015;22:133-8.

For the identification and quantification of genetically modified organisms (GMOs), one possible simple alternative method relies on the detection of DNA fragments synthesized using random primers without the need for nucleic acid amplification (for example, PCR) on DNA microarrays. Here, we tested simple detection protocols with a DNA microarray, and consequently, we were able to identify five selected GM maize lines by the pattern of spots detected on the DNA microarray. Our protocol requires no specific primers in the target DNA synthesis steps; all target DNA is synthesized by random 9-mer primers in one tube. This study suggests the possibility of detecting transgenes in GMOs and identifying GMO lines by the pattern of independently detected spots, irrespective of the position of the target gene sequences in the genomic DNA of each GMO line.
Keywords: DNA microarray, genetically modified organism (GMO), screening detection

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Nabeshi H, Tsutsumi T, Uekusa Y, Hachisuka A, Matsuda R, Teshima R: Surveillance of Strontium-90 in Foods after the Fukushima Daiichi Nuclear Power Plant Accident.
Food Hyg Saf Sci. 2015;56:133-43.

As a result of the Fukushima Daiichi nuclear power plant (NPP) accident, various radionuclides were released into the environment. In this study, we surveyed strontium-90 (^{90}Sr) concentrations in several foodstuffs. Strontium-90 is thought to be the third most

important residual radionuclide in food collected after the Fukushima Daiichi, NPP accident after following cesium-137 (^{137}Cs) and cesium-134 (^{134}Cs). Results of ^{90}Sr analyses indicated that ^{90}Sr was detect in 25 of the 40 radioactive cesium (r-Cs) positive samples collected in areas around the Fukushima Daiichi NPP, ranging in distance from 50 to 250 km. R-Cs positive samples were defined as containing both ^{134}Cs and ^{137}Cs which are considered to be indicators of the after-effects of the Fukushima Daiichi NPP accident. We also detected ^{90}Sr in 8 of 13 r-Cs negative samples, in which ^{134}Cs was not detected. Strontium-90 concentrations in the r-Cs positive samples did not significantly exceed the ^{90}Sr concentrations in r-Cs negative samples or the ^{90}Sr concentration ranges in comparable food groups found in previous surveys before the Fukushima Daiichi NPP accident. Thus, ^{90}Sr concentrations in r-Cs positive samples were indistinguishable from the background ^{90}Sr concentrations arising from global fallout prior to the Fukushima accident, suggesting that no marked increase of ^{90}Sr concentrations has occurred in r-Cs positive samples as a result of the Fukushima Daiichi NPP accident.

Keywords: the Fukushima Daiichi nuclear power plant (NPP) accident, strontium-90 (^{90}Sr), surveillance

Brennan JC[*1], He G[*1], Tsutsumi T, Zhao J[*1], Wirth E[*2], Fulton MH[*2], Denison MS[*1]: Development of species-specific Ah receptor-responsive third generation CALUX cell lines with enhanced responsiveness and improved detection limits.

Environ Sci Technol. 2015;49:11903-12.

The Ah receptor (AhR)-responsive CALUX (chemically activated luciferase expression) cell bioassay is commonly used for rapid screening of samples for the presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), dioxin-like compounds, and AhR agonists/antagonists. By increasing the number of AhR DNA recognition sites (dioxin responsive elements), we previously generated a novel third generation (G3) recombinant AhR-responsive mouse CALUX cell line (H1L7.5c3) with a significantly enhanced response to DLCs compared to existing AhR-CALUX cell bioassays. However, the elevated background luciferase activity of these cells and the absence of comparable G3 cell lines derived from other species have limited their utility for screening purposes. Here, we describe the development and characterization of species-specific G3 recombinant AhR-responsive CALUX cell lines (rat, human, and guinea pig) that exhibit significantly improved limit of detection and dramatically increased TCDD induction response. The low background luciferase activity, low minimal detection limit (0.1 pM TCDD) and enhanced induction response of the rat G3 cell line (H4L7.5c2) over the H1L7.5c3 mouse G3 cells, identifies them as a more optimal cell line for screening purposes. The utility of the new G3 CALUX cell lines were demonstrated by screening sediment extracts and a small chemical compound library for the presence of AhR agonists. The improved limit of detection and increased response of these new G3 CALUX cell lines will facilitate species-specific analysis of DLCs and AhR agonists in samples with low levels of contamination and/or in small sample volumes.

Keywords: Ah receptor, CALUX, AhR agonists

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Hirai T[*1], Yoshioka Y[*1,2,3], Takahashi H[*1,2], Ichihashi K[*1], Udaka A[*1], Mori T[*4], Nishijima N[*1], Yoshida T[*1], Nagano K[*5], Kamada H[*5,6], Tsunoda S[*5,6], Takagi T[*7,8], Ishii K[*9,10], Nabeshi H, Yoshikawa T[*1], Higashisaka K[*1,5], Tsutsumi Y[*1,4,6]: Cutaneous exposure to agglomerates of silica nanoparticles and allergen results in IgE-biased immune response and increased sensitivity to anaphylaxis in mice.

Part Fibre Toxicol. 2015;12:16.

BACKGROUND:

The skin is a key route of human exposure to nanomaterials, which typically occurs simultaneously with exposure to other chemical and environmental allergen. However, little is known about the hazards of nanomaterial exposure via the skin, particularly when accompanied by exposure to other substances.

RESULTS:

Repeated topical treatment of both ears and the shaved upper back of NC/Nga mice, which are models for human atopic dermatitis (AD), with a mixture of mite extract and silica nanoparticles induced AD-like skin lesions. Measurements of ear thickness and histologic analyses revealed that cutaneous exposure to silica nanoparticles did not aggravate AD-like skin

lesions. Instead, concurrent cutaneous exposure to mite allergens and silica nanoparticles resulted in the low-level production of allergen-specific IgGs, including both the Th2-related IgG1 and Th1-related IgG2a subtypes, with few changes in allergen-specific IgE concentrations and in Th1 and Th2 immune responses. In addition, these changes in immune responses increased the sensitivity to anaphylaxis. Low-level IgG production was induced when the mice were exposed to allergen-silica nanoparticle agglomerates but not when the mice exposed to nanoparticles applied separately from the allergen or to well-dispersed nanoparticles.

CONCLUSIONS:

Our data suggest that silica nanoparticles themselves do not directly affect the allergen-specific immune response after concurrent topical application of nanoparticles and allergen. However, when present in allergen-adsorbed agglomerates, silica nanoparticles led to a low IgG/IgE ratio, a key risk factor of human atopic allergies. We suggest that minimizing interactions between nanomaterials and allergens will increase the safety of nanomaterials applied to skin.

Keywords: atopic dermatitis, agglomerate, nanomaterials

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Saito-Shida S, Nemoto S, Teshima R: Multiresidue determination of pesticides in tea by gas chromatography-tandem mass spectrometry.
J Environ Sci Health B. 2015;50:760-76.

An efficient and reliable GC-MS/MS method for the multiresidue determination of pesticides in tea was developed by modifying the Japanese official multiresidue method. Sample preparation was carefully optimized for the efficient removal of coextracted matrix components. The optimal sample preparation procedure involved swelling of the sample in water; extraction with acetonitrile; removal of water by salting-out; and sequential cleanup by ODS, graphitized carbon black/primary secondary amine (GCB/PSA) and silica gel cartridges prior to GC-MS/MS analysis. The recoveries of 162 pesticides from fortified (at 0.01 mg kg-1) green tea, oolong tea, black tea and matcha (powdered green tea) were mostly (95–98% of the tested pesticides) within the range of 70–120%, with relative standard deviations of <20%. Poor recovery of triazole pesticides was considered to be due to low recovery from the silica gel cartridges. The test solutions obtained by the modified method contained relatively small amounts of pigments, caffeine and other matrix components and were cleaner than those obtained by the original Japanese official multiresidue method. No interfering peaks were observed in the blank chromatograms, indicating the high selectivity of the modified method. The overall results suggest that the developed method is suitable for the quantitative analysis of GC-amenable pesticide residues in tea.

Keywords: GC-MS/MS, pesticides, tea

今井浩一*，尾上恵子*，石井里枝*，髙野真理子*，根本了，手島玲子：LC-MS/MSによる農産物および畜水産物中のイプフェンカルバゾン分析法の開発.
食品衛生学雑誌 2015;56:205-10.

A method for the determination of ipfencarbazone in agricultural products, livestock products and seafood by LC-MS/MS was developed. Agricultural samples were extracted with acetone. An aliquot of crude

extract was partitioned with n-hexane and sat. sodium chloride solution. Cleanup was performed using GC/PSA and C18 cartridges. In the case of livestock products and seafood, samples were extracted with a mixture of acetone and n-hexane, and the organic layer was collected. After acetonitrile-hexane partitioning, the extract was cleaned up using PAS and C18 cartridges. The gradient LC separation was performed on a C18 column with acetonitrile-water containing acetic acid as a mobile phase, and MS with positive ion electrospray ionization was used for detection. The average recoveries (n＝5) of ipfencarbazone from 16 kinds of agricultural products, livestock products and seafood spiked at the MRLs or at the uniform limits (0.01 ppm) were 73–101%, and the relative standard deviations were 1.3–5.1%. The limit of quantitation of the developed method was 0.01 mg/kg for ipfencarbazone.

Keywords: ipfencarbazone, agricultural product, LC-MS/MS

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福田優作*，片岡洋平，佐野勇気*，滝澤和宏*，渡邉敬浩，手島玲子：ミネラルウォーター類中のシアンおよび臭素酸を対象とした分析法の開発と適用性の検証.

食品衛生学雑誌 2015;56:256-62.

We developed and evaluated methods of quantifying cyanide (cyanide ion and cyanogen chloride) and bromic acid in mineral waters (MW). After performance evaluation, recovery studies were performed on 110 kinds of MW products to examine the applicability of the methods. The approximate proportion of the MW samples, in which the recovery rate of these anionic compounds was within 90 to 110%, was 95% in the cyanide ion and bromic acid analysis and 45% in the cyanogen chloride analysis. We observed low rates of recovery of cyanogen chloride from some MW products with pH values around neutral. To increase the recovery rate, we propose adding phosphoric acid buffer to adjust the pH of these MW samples. The retention times for bromic acid in some MW products differed from that in standard solution. We concluded that carbonic acid influences the retention times. It may be necessary to exclude carbon dioxide from the MW samples by degassing to synchronize the retention times of bromic acid in the MW samples and the standard solution.

Keywords: cyanide ion, cyanogen chloride, bromic acid

* （一財）日本冷凍食品検査協会

南谷臣昭*，永井宏幸*，多田裕之*，後藤黄太郎*，根本了：LC-MSによる農産物中のブトロキシジムの分析.

食品衛生学雑誌 2015;56:233-9.

An analytical method for the determination of butroxydim in agricultural products by LC-MS was developed. Butroxydim was extracted with acetonitrile and an aliquot of the crude extract was cleaned up on an octadecyl silanized silica gel (C18) cartridge column (1,000 mg), followed by a salting-out step to remove water. Before purification on a silica gel (SI) cartridge column (690 mg), polar matrices were precipitated by adding ethyl acetate, n-hexane and anhydrous sodium sulfate successively. This process effectively removed caffeine and catechins and improved recovery when analyzing residual butroxydim in tea leaves. Recovery and repeatability were good; the relative standard deviations were less than 5% for all 12 tested agricultural products (brown rice, soybean, potato, spinach, cabbage, apple, orange, grapefruit, lemon, tomato, peas with pods, and tea). Average recoveries for 11 agricultural products, except for lemon, were 74–92%.

Keywords: butroxydim, agricultural product, LC-MS

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Saito-Shida S, Nemoto S, Teshima R, Akiyama H: Quantitative analysis of pesticide residues in vegetables and fruits by liquid chromatography quadrupole time-of-flight mass spectrometry.

Food Addit Contam Part A. 2016;33:119-27.

The applicability of liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) for the quantitative analysis of pesticide residues in vegetables and fruits was demonstrated. The LC-QTOF-MS parameters, such as cone voltage, capillary voltage, collision energy and mass extraction window, were carefully optimized for the analysis of pesticide residues. The LC-QTOF-MS method was validated for 149 pesticides in four

vegetables and fruits, i.e. apple, potato, cabbage and spinach, at a spiking level of 0.01 mg kg-1. The samples were prepared according to the Japanese official multi-residue method with a modification to the column clean-up procedure. Of the 149 pesticides, recoveries in the range of 70–120% were achieved for 147 pesticides in apple, 145 in potato, 141 in cabbage and 131 in spinach, with intra-day precisions (RSDs) of < 25% and inter-day precisions (RSDs) of < 30%, which are within the acceptable range given in the Japanese method validation guideline. Matrix effects were negligible for the majority of the target pesticides. Except for spiroxamine in spinach, no interfering peaks were observed in the blank samples. The target pesticides, except those with low sensitivity, achieved calibration curves with satisfactory linearity, with correlation coefficients (r) greater than 0.995 in the concentration range of 0.002–0.1 μg ml-1. Furthermore, the majority of the target pesticides provided more than one fragment ion or isotope ion that could be used for confirmation. The overall results suggest that LC-QTOF-MS is a powerful tool for the quantification of pesticide residues in vegetables and fruits at the level of 0.01 mg kg-1.

Keywords: pesticide, multi-residue method, liquid chromatography quadrupole time-of-flight mass spectrometry

鍋師裕美，堤智昭，植草義徳，松田りえ子，穐山浩，手島玲子，蜂須賀暁子：調理による牛肉・山菜類・果実類の放射性セシウム濃度及び総量の変化.

RADIOISOTOPES 2016;65:45-58.

牛肉，山菜類，果実類，キノコを用いて調理前後の放射性セシウム濃度を測定し，除去効果を検討した結果，調味液へ浸漬してから牛肉を乾燥させた場合や山菜類をあく抜きした場合では，調理前の80％以上の放射性セシウムが除去され，調味液への浸漬やゆで調理，及びこれらの工程後の水さらしが放射性セシウム除去に有効であることが示された．しかし，牛肉及び果実の単純な乾燥，果実類のジャム，焼きシイタケ，山菜類のてんぷらでは，放射性セシウムはほとんど食品から除去されなかった．調理法によっては，放射性セシウムの総量に変化はないものの，水分除去等により濃度が上昇することがあるため注意が必要である．

Keywords: radioactive cesium, removal effect, cooking

Amakura Y[*1], Tsutsumi T, Yoshimura M[*1], Nakamura M[*2], Handa H[*2], Matsuda R, Watanabe T:

Detection of Aryl Hydrocarbon Receptor Activation by Some Chemicals Related in Food Hygiene by Using a Reporter Gene Assay.

Foods 2016;5:doi:10.3390/foods5010015.

The purpose of this study was to examine whether a simple bioassay used for the detection of dioxins (DXNs) could be applied to detect trace amounts of harmful DXN-like substances in food products. To identify substances with possible DXN-like activity, we assessed the ability of various compounds in the environment to bind the aryl hydrocarbon receptor (AhR) that binds specifically to DXNs. The compounds tested included 19 polycyclic aromatic hydrocarbons (PAHs), 20 PAH derivatives (nitrated, halogenated, and aminated derivatives), 23 pesticides, six amino acids, and eight amino acid metabolites. The AhR binding activities (AhR activity) of these compounds were measured using the chemical activated luciferase gene expression (CALUX) reporter gene assay system. The majority of the PAHs exhibited marked AhR activity that increased in a concentration-dependent manner. Furthermore, there was a positive link between AhR activity and the number of aromatic rings in the PAH derivatives. Conversely, there appeared to be a negative correlation between AhR activity and the number of chlorine residues present on halogenated PAH derivatives. However, there was no correlation between AhR activity and the number and position of substituents among nitrated and aminated derivatives. Among the pesticides tested, the indole-type compounds carbendazim and thiabendazole showed high levels of activity. Similarly, the indole compound tryptamine was the only amino acid metabolite to induce AhR activity. The results are useful in understanding the identification and characterization of AhR ligands in the CALUX assay.

Keywords: aryl hydrocarbon receptor, reporter gene assay, food hygiene

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Sato K, Ohtsuki T, Tatebe C, Takiguchi H, Nakamura R[*], Otabe A[*], Oobayashi Y[*], Akiyama H:

Determination method of 2-(5-benzyl-3,6-

dioxopiperazin-2-yl)acetic acid in aspartame using high performance liquid chromatography.
Jpn J Food Chem Safety. 2015;22:170-4.

2-(5-Benzyl-3,6-dioxopiperazin-2-yl)acetic acid (DKP) is one of the degradation products of aspartame (APM). The Joint FAO/WHO Expert Committee on Food Additives (JECFA), USA, EU, and Japan has set 1.5 wt% as the maximum level of DKP in APM. In this study, we developed and validated a high-performance liquid chromatography (HPLC) method to determine the content of DKP in APM, based on the approach recommended in the Food Chemical Codex (FCC) from USA. For the separation of analyte, the column was investigated with 5 and 10 μm particle size. In the recovery test, the developed method gave satisfactory recoveries (99.1% and 99.5%) and repeatabilities (0.4%) from APM spiked with two concentrations (viz. 0.15 and 1.5 wt%). The limits of detection and quantification for DKP are estimated to be 0.0005 and 0.002 wt%, respectively. In the analyses of ten commercial APM samples, the DKP contents determined using the developed method are all in good agreement with those obtained using the conventional FCC method. This developed method is therefore applicable for the determination of DKP contents in commercial-grade APM.
Keywords: 5-benzyl-3,6-dioxo-2-piperazineacetic acid, aspartame, HPLC

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多田敦子，杉本直樹，小林義和*1，濱田ひかり*1，石附京子，秋山卓美，伊藤裕才*2，川原信夫*3，山﨑壮*4，穐山浩：味認識装置による既存添加物苦味料及び関連苦味化合物の品質評価.
日本食品化学学会誌 2015;22:25-31.

天然由来の食品添加物苦味料9種，苦味試薬22種及び渋味試薬3種を用いて，味認識装置の適用性について調べた．味認識装置の6種の脂質膜センサから得られるデータを元に，試料毎に味要素10項目の味強度値を算出した．各試料につき，味要素10項目の内絶対値が最も高かった値を100とし，同じ試料の他の味要素項目の味強度値を相対的に変換した%換算値を算出した．%換算値を試料毎にレーダーグラフにプロットし，各試料の味質をレーダーグラフのパターンを元に比較した．その結果，試料の味質は大きく５つのタイプに分類された．含有苦味成分が類似する試料は味質のパターンも類似しているため，味質のタイプは，苦味物質の化学構造の種類により特徴的であることが分かった．さらに，各試料の味要素10項目の%換算値を用いて,主成分分析（多変量解析の1つ）を行ったところ，苦味物質の味質を明確に5つのタイプに分類できた．本研究により，味認識装置が天然由来の食品添加物苦味料及び苦味試薬の味質の評価に適用可能であることが明らかとなった．本研究の結果から，苦味物質中の苦味成分が未解明であっても，味認識装置による味質の評価を行うことで，苦味成分の化学構造タイプを推定できるものと示唆された．本研究により得られた結果は，他の市販苦味製品の味質の評価にも役に立つものと期待される．
Keywords：食品添加物，苦味料，味認識装置

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多田敦子，石附京子，杉本直樹，吉松嘉代*1，川原信夫*1，末松孝子*2，有福和紀*3，深井俊夫*4，田村幸吉*5，大槻崇，田原麻衣子，山﨑壮*6，穐山浩：既存添加物カンゾウ油性抽出物の成分組成の多変量解析に基づく基原植物種の検討.
食品衛生学雑誌 2015;56:217-27.

既存添加物製品の基原植物の確認は品質や安全性確保の上から重要である．既存添加物カンゾウ油性抽出物（酸化防止剤）の基原として，既存添加物名簿収載品目リストにはカンゾウ属植物の*Glycyrrhiza uralensis*，*G. inflata*又は*G. glabra*の根又は根茎と記されているが，カンゾウ油性抽出物流通製品がどのカンゾウ属植物種の油性抽出物の成分組成に類似するかは確認されていない．本研究で，カンゾウ油性抽出物流通製品8検体をLC/MSにより分析した結果，7製品では*G. glabra*に特徴的なglabridinが，1製品では*G. inflata*に特徴的なlicochalcone Aが検出された．さらにこれら流通製品に加え，各カンゾウ属植物種の乾燥試料や市販のカンゾウ由来製品のエタノール抽出物等，計31検体をLC/MS（SIR）およびNMRで測定し，主成分分析（多変量解析）を行った結果，カンゾウ油性抽出物製品の基原種は主に*G. glabra*であると確認された．またNMR測定結果を用いる多変量解析が，既存添加物製品の基原種推定に有用かつ簡便な方法であることが示唆された．
Keywords：カンゾウ油性抽出物，NMR，主成分分析

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Ohtsuki T, Nakamura R*1, Kubo S*1, Otabe A*1, Oobayashi Y*1, Suzuki S*1, Yoshida Mika*2, Yoshida Mitsuya*2, Tatebe C, Sato K, Akiyama H: Development of an HPLC Method with an ODS Column to Determine Low Levels of Aspartame Diastereomers in Aspartame.
PLOS One 2016;11:e0152174.

α-L-Aspartyl-D-phenylalanine methyl ester (L, D-APM) and α-D-aspartyl-L-phenylalanine methyl ester (D, L-APM) are diastereomers of aspartame (*N*-L-α-Aspartyl-L-phenylalanine-1-methyl ester, L, L-APM). The Joint FAO/WHO Expert Committee on Food Additives has set 0.04 wt% as the maximum permitted level of the sum of L, D-APM and D, L-APM in commercially available L, L-APM. In this study, we developed and validated a simple high-performance liquid chromatography (HPLC) method using an ODS column to determine L, D-APM and D, L-APM in L, L-APM. The limits of detection and quantification, respectively, of L, D-APM and D, L-APM were found to be 0.0012 wt% and 0.004 wt%. This method gave excellent accuracy, repeatability, and reproducibility in a recovery test performed on five different days. Moreover, the method was successfully applied to the determination of these diastereomers in commercial L, L-APM samples. Thus, the developed method is a simple, useful, and practical tool for determining L, D-APM and D, L-APM levels in L, L-APM.
Keywords: Aspartame, diastereomers, HPLC

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熊井康人，河﨑裕美，酒井昌昭*1，浦嶋幸雄*1，山田信之*2，関根百合子*2，工藤礼佳*2，中里光男*3，早藤知恵子*3，宮川弘之*3，山嶋裕季子*3，西岡千鶴*4，酒井國嘉*5，玉城宏幸*6，古謝あゆ子*6，建部千絵，大槻崇，久保田浩樹，佐藤恭子，穐山浩，河村葉子：マーケットバスケット方式による小児の食品添加物一日摂取量の推定（2009年度）．
日本食品化学学会誌 2015;22:181-7.

マーケットバスケット方式による小児（1 ～ 6歳）の食品添加物（色素及び保存料，甘味料，製造用剤）の一日摂取量の推定を行った．対象とする加工食品の喫食量リストは，国民健康・栄養調査（2001-2002年）及び国民健康・栄養研究所の調査結果（2003年）に基づき作成した．

最も高い一日摂取量を示したのはオルトリン酸（9.4 mg/kg体重/日，リンとして）あり，次いで，縮合リン酸（0.76 mg/kg 体重/日，リンとして），プロピレングリコール（0.47 mg/kg 体重/日）であった．小児の食品添加物推定一日摂取量をJECFAの一日摂取許容量（ADI）及び最大耐用一日摂取量（MTDI）と比較した．ADIに対する推定一日摂取量の割合は，色素及び保存料，甘味料，プロピレングリコールでは0 ～ 1.9%であり，MTDIに対するリン化合物の一日摂取量割合は15%であった．
Keywords：マーケットバスケット方式，食品添加物，一日摂取量

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日本食品化学学会誌 2015;22:188-94.

2014年度におけるマーケットバスケット方式による日本の小児（1 ～ 6歳）の食品添加物（色素及び保存料，甘味料，製造用剤）の一日摂取量の推定を行った．対象とする加工食品の喫食量リストは，国民健康・栄養研究所の調査結果（2003年）に基づき作成した．対象とする加工食品の喫食量リストは，国民健康・栄養研究所が2003年に実施した特別集計業務の調査結果に基づき作成した．最も高い一日摂取量を示したのはオルトリン酸（11 mg/kg 体重/日，リンとして）であり，次いで，縮合リン酸（1.0 mg/kg 体重/日，リンとして），プロピレングリコール（0.73 mg/kg 体重/日）であった．

小児の食品添加物推定一日摂取量をJECFAまたは食品安全委員会が設定する一日摂取許容量（ADI）及び最大耐用一日摂取量（MTDI）と比較した．ADIに対する推定一日摂取量の割合は，色素及び保存料，甘味料，プ

ロピレングリコールでは0 ～ 2.9%であり，MTDIに対するリン化合物の一日摂取量割合は18%であった．
Keywords：マーケットバスケット方式，食品添加物，一日摂取量

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Amakura Y*1, Yoshimura M*1, Yoshida T*1, Tada A, Ito Y*2, Yamazaki T*3, Sugimoto N, Akiyama H: Chromatographic evaluation of the components of grape skin extract used as food additives.
Jpn J Food Chem Safety. 2015;22:108-14.

Grape skin extract, a food manufacturing agent registered in the List of Existing Food Additives in Japan, was evaluated by high performance liquid chromatographic (HPLC) method. Chemical constituents of these extracts were separated by repeated column chromatography, and 12 compounds were isolated and characterized as tryptamine, syringic acid, vanillic acid, ethyl gallate, (+)-catechin, (-)-epicatechin, luteoliflavan, quercetin, quercetin 3-*O*-glucuronide, myricetin 3-*O*-glucoside, procyanidin B-1, and procyanidin B-2 by spectroscopic methods. The presence of malvidin 3-*O*-glucoside as the major anthocyanin was confirmed by HPLC. A broad peak forming a swollen base line was attributed to a B type of proanthocyanidins, i.e., a condensed tannin oligomer, the number and weight averaged molecular weights of which were estimated, by gel permeation chromatography (GPC), to be 5999.6 and 21287.7, respectively. The proanthocyanidin content in commercial grape skin extract products was determined by colorimetric analysis with vanillin-sulfuric acid to be around 60% (catechin equivalent value).
Keywords: grape skin extract, anthocyanin, proanthocyanidin

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Amakura Y*1, Yoshimura M*1, Morimoto S*1, Yoshida T*1, Tada A, Ito Y*2, Yamazaki T*3, Sugimoto N, Akiyama H: Chromatographic evaluation and characterization of components of gentiana root extract used as food additives.
Chem Pharm Bull. 2016;64:78-82.

Gentian root extract is used as a bitter food additive in Japan. We investigated the constituents of this extract to acquire the chemical data needed for standardized specifications. Fourteen known compounds were isolated in addition to a mixture of gentisin and isogentisin: anofinic acid, 2-methoxyanofinic acid, furan-2-carboxylic acid, 5-hydroxymethyl-2-furfural, 2,3-dihydroxybenzoic acid, isovitexin, gentiopicroside, loganic acid, sweroside, vanillic acid, gentisin 7-*O*-primeveroside, isogentisin 3-*O*-primeveroside, 6'-*O*-glucosylgentiopicroside, and swertiajaposide D. Moreover, a new compound, loganic acid 7-(2'-hydroxy-3'-*O*-β-D-glucopyranosyl)benzoate (1), was also isolated. HPLC was used to analyze gentiopicroside and amarogentin, defined as the main constituents of gentian root extract in the List of Existing Food Additives in Japan.
Keywords: gentian root extract, Gentiana lutea, bittering agent

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西﨑雄三，多田敦子，石附京子，伊藤裕才*1，小野田絢*2，杉本直樹，穐山浩：モル吸光係数比を利用したジャマイカカッシア抽出物中のクアシン及びネオクアシンの新規定量法の開発．
食品衛生学雑誌 2015;56:185-93.

既存添加物ジャマイカカッシア抽出物中のクアシンおよびネオクアシンを，4-ヒドロキシ安息香酸（4HBA）とのモル吸光係数比（S/M）を利用し，HPLCを用いる新規定量法を開発した．4HBAとクアシンおよびネオクアシンの混合液を，定量NMR（qNMR）を用いてモル比（M）を，HPLCを用いて吸光度比（S）を算出し，4HBAに対するクアシンおよびネオクアシンのS/M, 0.84および0.85を求めた．添加物製品中のクアシンおよびネオクアシンを直接qNMRを用いて定量した値と，純度既知の4HBAを内標準物質として，S/MとHPLCを用いた定量値との差は，1.2%以下で有意な差異はなかった．本分析法は，添加物や加工食品中のクアシンおよびネオク

アシンの定量に適用可能であることが示された.
Keywords：モル吸光係数比，定量NMR，4-ヒドロキシ安息香酸

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Kawasaki H, Akiyama T, Tada A, Sekiguchi W, Nishizaki Y, Ito Y*, Sugimoto N, Akiyama H: Development of HILIC-LC/MS method for direct quantitation of 2-acetyl-4-tetrahydroxybutylimidazole in caramel III with the qNMR certified standard.
Jpn J Food Chem Safety. 2015;22:115-22.

A method LC/MS with HILIC column (HILIC-LC/MS) was developed for direct quantitation of 2-acetyl-4-tetrahydroxybutylimidazole (THI), an undesired polar byproduct in caramel III colorants. To verify the reliability of the proposed analytical method for the quantification of THI in caramel III commercial products, we determined the absolute purity of a THI analytical standard using quantitative NMR (qNMR) and then performed absolute calibration and standard addition procedures using the analytical standard. The correlation coefficients were >0.99 and >0.97 for the absolute calibration and standard addition procedures, indicating satisfactory linearity of the respective calibration curves. The procedures also returned identical quantitation values in a sample. The THI content in six samples of caramel III commercial products in Japan was determined using the HILIC-LC/MS method. The THI content in each of these samples was lower than officially stipulated limits. The current JECFA standard method for determination of THI in caramel III by HPLC/UV using a 10-μm particle size C8 column with derivatization of THI-2,4-dinitrophenylhydrazone gave lower THI values than the proposed HILIC-LC/MS method due to sub-optimal peak separation by the column recommended in the JECFA standard method. Our data suggest that the analytical conditions of the current JECFA standard method should be improved.
Keywords: qNMR, HILIC, caramel

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Ito Y*, Ishizuki K, Sugimoto N, Tada A, Akiyama T, Sato K, Akiyama H, Goda Y: Confirmation of the configuration of two glucuronic acid units in glycyrrhizic acid.
Jpn J Food Chem Safety. 2015;22:32-7.

Glycyrrhizic acid (GA), a triterpenoid saponin containing two glucuronic acid (GlcA1 and GlcA2) units, is found in the roots of Glycyrrhiza plants, and has been widely used as a natural sweetener for foods as well as a natural medicine. Purified GA is commercially available from various manufacturers as an analytical standard or a biochemical reagent. While producers describe the configurations of GlcA1 and GlcA2 as α and β-forms, respectively, reports of the structural elucidation of GA have proposed that both GlcA units are β-form. To clarify this point, commercial GA from various sources was analyzed by 1D and 2D NMR studies. Results confirmed that the actual configuration of both GlcA units in GA is β-form.
Keywords: glycyrrhizic acid, glucuronic acid, natural sweetener

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食品衛生学雑誌 2015;56:57-67.

ポリエチレンテレフタレート製器具・容器包装のアンチモン（Sb）およびゲルマニウム（Ge）溶出試験における各測定法の性能を評価するため，試験室間共同試験を行った．当試験には18機関が参加し，濃度非明示の3検体（各2測定）について電気加熱方式原子吸光光度法（GF-AAS），誘導結合プラズマ発光強度測定法（ICP-OES）および誘導結合プラズマ質量分析法（ICP-MS）によりSbおよびGeの定量を行った．その結果，GF-AASおよびICP-OESでは，真度が98 ～ 107%，併行精度（RSDr）が1.7 ～ 7.5%，室間再現精度（RSDR）が2.0 ～ 18.8%であり，これらの性能は規格試験法として十分であった．また，ICP-MSでは，真度が99 ～ 106%，RSDrが0.7 ～ 2.2%，RSDRが2.2 ～ 10.5%であり，代替法として適用可能であった．しかし，一部の試験機関ではSbの定量値

が添加量よりも高かった．その一因として，検量線溶液中のSbがガラス器具に吸着したためと考えられた．そのため，Sbの試験を行う場合には，検量線溶液の濃度について細心の注意を払う必要があると考えられた．
Keywords：アンチモン，ゲルマニウム，試験室間共同試験

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柴田博*1，六鹿元雄，阿部裕，伊藤禎啓*2，大坂郁恵*3，大野春香*4，大野浩之*5，大野雄一郎*6，尾崎麻子*7，柿原芳輝*8，小林尚*9，城野克広*10，関戸晴子*11，薗部博則*12，高坂典子*13，但馬吉保*14，田中葵*15，田中秀幸*10，中西徹*16，野村千枝*17，羽石奈穂子*18，疋田晃典*19，三浦俊彦*20，山口未来，渡辺一成*21，穐山浩：ゴム製器具・容器包装における亜鉛溶出試験の試験室間共同試験．
食品衛生学雑誌 2015;56:123-31.

食品衛生法におけるゴム製器具･容器包装の亜鉛（Zn）試験法の性能を評価するため，水または4%酢酸による亜鉛溶液6種を検体として用いた試験室間共同試験を行った．当試験には18機関が参加し，濃度非明示の6検体（各2測定）についてフレーム方式原子吸光光度法，誘導結合プラズマ発光強度測定法および誘導結合プラズマ質量分析法によりZnの定量を行った．その結果，いずれの測定法においても真度が97 ～ 103%，併行精度（RSDr）が0.7 ～ 4.9%，室間再現精度（RSDR）が1.7 ～ 8.9%であり，性能パラメーターの値は目標値（真度：80 ～ 110%，RSDr：10%以下，RSDR：25%以下）を満たしており，規格試験法として十分な性能を有していることが判明した．
Keywords：亜鉛，溶出試験，試験室間共同試験

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Syaka A*, Kusumoto A*, Asakura H, Kawamoto K*: Whole genome sequences of eight *Campylobacter jejuni* isolates from wild birds.
Genome Announc. 2015;3:e00315-15.

We present here the draft genome sequences of 8 *Campylobacter jejuni* strains isolated from wild birds. The strains were initially isolated from swabs taken from resident wild birds in the Tokachi area of Japan. The genome sizes range from 1.65 to 1.77 Mbp.
Keywords: *Campylobacter jejuni*, wild birds, draft genome sequence

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Pascoe B*1, Meric G, Murray S*1, Yahara K*1, Mageiros L*1, Bowen R*1, Jones NH*1, Jeeves RE*1, Lappin-Scott HM*1, Asakura H, Sheppard SK*1,2: Enhanced biofilm formation and multi-host

transmission evolve from divergent genetic backgrounds in *Campylobacter jejuni*.
Environ Microbiol. 2015;17:4779-89.

In microaerophilic organisms such as Campylobacter, biofilms play a key role in transmission to humans as the bacteria are exposed to atmospheric oxygen concentrations when leaving the reservoir host gut. Our approach combines genome-wide association studies with traditional microbiology techniques to investigate the genetic basis of biofilm formation in 102 *Campylobacter jejuni* isolates. 30 genes exhibited statistically robust association with biofilm formation, which showed strain-to-strain diversity in host generalist ST-21 and ST-45 clonal complexes, suggesting the evolution of enhanced biofilm from different genetic backgrounds and a possible role in colonization of multiple hosts and transmission to humans.

Keywords: *Campylobacter jejuni*, biofilm formation, genome-wide association study (GWAS)

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Asakura H, Kawamoto K[*1], Murakami S[*2] Tachibana M, Kurazono H[*1], Makino S[*3], Yamamoto S[*4], Igimi S: Ex vivo proteomics of *Campylobacter jejuni* 81-176 reveal that FabG affects fatty acid composition to alter bacterial growth fitness in the chicken gut.
Res Microbiol. 2016;167:63-71.

Here we performed ex vivo proteomic analysis of *C. jejuni* 81-176 in chicken. At 0, 1 and 4 weeks p.i., inocula were recovered from chicken ceca by cell sorting using flow cytometry. iTRAQ-coupled 2D-LC-MS/MS analyses that detected 55 bacterial proteins, among which either 3 or 7 proteins exhibited >1.4-fold-increased expression at 1 or 4 weeks p.i. compared with those at 0 weeks p.i., respectively. Deletion of the fabG gene clearly decreased the proportion of bacterial unsaturated fatty acids (UFAs) and chicken colonization. These findings suggest a pivotal role of the fabG in UFA production, linked to bacterial adaptation in the poultry host.

Keywords: *Campylobacter jejuni*, chicken, proteomics

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Masuda K, Yamamoto S, Kubota K, Kurazono H[*1], Makino S[*2], Kasuga F, Igimi S, Asakura H: Evaluation of the dynamics of microbiological quality in lightly pickled napa cabbages during manufacture.
J Food Safety. 2015;35:458-65.

We examined indicator bacterial counts, prevalence of STEC and *Salmonella* spp., and bacterial community structure in intermediate products and the related facilities at a collaborative plant in which lightly pickled vegetables were manufactured. Plate counts showed a significant reduction in coliform during processing, whereas the reduction in total viable counts was relatively less than that of coliforms. No STEC and *Salmonella* spp. were recovered. 16S rRNA pyrosequencing analysis revealed process-by-process alteration of bacterial community composition in which the yields of Pseudomonas spp. were drastically affected by soaking in high concentration of NaCl. In summary, we demonstrate that the revised prerequisite program is indeed functional to reduce the microbial risks.

Keywords: Light pickles, microbiome, hygienic practice

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朝倉宏，山本詩織，橘理人，吉村昌徳[*1]，山本茂貴[*2]，五十君靜信：冷凍処理による鶏肉中でのカンピロバクター汚染低減効果に関する検討.
日本食品微生物学会雑誌 2015;32:159-62.

鶏肉におけるカンピロバクター汚染を流通段階で制御するための一手法として，冷凍処理の有効性を評価した．添加回収試験を通じ，-20℃での2週間の冷凍処理により，最大で約1.9-2.3対数個の減少を認めた．40%の自然汚染率を示す鶏挽肉を用いた検討では，1週間の冷凍処理により汚染率は約1／4にまで低減した．急速冷凍処理を行った食鳥部分肉の本菌汚染菌数は，チルド検体に比べ総じて低値を示した．以上より，冷凍処理は鶏肉におけるカンピロバクター汚染を低減する一手法であることが示された．

Keywords：カンピロバクター，冷凍，鶏肉汚染

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杉山広*1，荒川京子*1，柴田勝優*1，川上泰*2，森嶋康之*1，山崎浩*1，荒木潤*1,3，生野博*4，朝倉宏：わが国における土壌媒介寄生虫症，特に回虫症の発生とその汚染源の文献的および検査期間データに基づく調査.
食品衛生研究 2015;65:37-41.

土壌媒介性の回虫症に関する発生動向を調査した．2004年以降の発生数は大きく減少していたが，現在でも少数ながらも発生が継続していることが明らかとなった．また，症例の発生原因については不明ながら，輸入野菜の回虫卵汚染については一部明らかにされており，今回の調査でも1検体からの検出事例を認めた．一方で，国内で生産される野菜の汚染実態については検査数も少ないこと等から，今後感染源の調査にあたっては検討を継続する必要性が示唆された．

Keywords：回虫症，野菜，発生動向

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堀内朗子*1，荒川京子*2，秋葉達也*1，吉田建介*1，平田史子*1，松本奈保子*1，丸山弓美*1，奥津敬右*1，朝倉宏，杉山広*2：ストマッカーを利用した野菜等の回虫卵検査法の検討.
食品衛生研究 2015;65:45-50.

野菜からの回虫卵検査法として，細菌検査に汎用されるストマッカーを用いた方法について，従来法（沈殿報・浮遊法）との比較を行い，その有用性について評価した．鶏肉におけるカンピロバクター汚染を流通段階で制御するための一手法として，冷凍処理の有効性を評価した．検出感度については，ストマッカー法と従来法の間では有意な差異を認めなかった一方で，前者の方法では，虫卵分離に要する時間を大きく短縮することができ，その有効性が示された．

Keywords：回虫卵検査法，野菜，ストマッカー処理法

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Suzuki H, Okada Y: Enterohemorrhagic *Escherichia coli* (EHEC) infection and beef consumption in Japan.
Proceeding of 2nd AFSSA. 2015;79-82.

In 2004 and 2005, importing U.S. beef was almost stopped. And then, the amounts of U.S. beef were gradually increased but were not recovered to those before the ban. The amounts of the total imported beef and domestic beef were almost constant during this period. On the other hand, the number of food-borne EHEC patients and cases, and the total number of EHEC patients (not only food-borne cases) were fluctuated but relatively constant from 2000 to 2012. It is thought that the U.S. beef might not have a great effect on the situation of EHEC infection in Japan, and it is too short to estimate whether the new standard for raw beef preparation or the ban of raw beef liver is effective or not.

Keywords: EHEC, beef, Japan

Okada Y, Monden S, Suzuki H, Nakama A*, Ida M*, Igimi S: Antimicrobial susceptibilities of *Listeria monocytogenes* isolated from the imported and the domestic foods in Japan.
J Food Nutr Sci. 2015;3:70-3.

In vitro antimicrobial susceptibility of *Listeria monocytogenes* isolated from the imported and the domestic foods in Japan was determined by plate dilution method. Eleven isolates from domestic meat, meat products, liver, seafood and environment, and 16 isolates from imported meat and meat products were examined their susceptibilities against ampicillin, chloramphenicol, enrofloxacin, erythromycin, gentamicin, kanamycin, penicillin and tetracycline. All of the isolates except the one isolate from domestic scallop were susceptible to all the antibiotics tested. Only 1 isolate showed resistance to kanamycin and gentamicin. The minimum inhibitory concentration (MIC) for 50% of the strains and the MIC for 90% of the strains were comparable between the imported and the domestic food origins. These results suggest there were less differences of antimicrobial susceptibility between the two origins of Listeria isolates.

Keywords: *Listeria monocytogenes*, Antibiotic Susceptibility

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Ogawa T*, Okada Y, Kuse H*, Kemi M*, Suzuki H: Swelling of eyeball accompanied with lens luxation in four mongolian gerbils.

比較眼科研究 2015;34:21-7.

The Mongolian gerbils (Meriones unguiculatus) are one of the experimental animal models which were established in Japan1). Abnormally larger eyes were found unilaterally, either right or left, in five out of 686 post-weaning Mongolian gerbils, in our breeding colony, which was started from four pairs of MGW inbred albino strain gerbils and maintained for around one year. Ophthalmological and histopathological examinations were conducted on the eyeballs from four out of these five animals. The affected eyeballs were swollen and showed anterior synechia in all four cases. The lens with opacity of one animal moved freely in the intraocular space depending on the direction of animal's head and those of the other three animals were unevenly located near the Ciliary body or posterior position, and these ocular lesions were diagnosed as lens luxation. Histopathological examination revealed imperfect formation of the ciliary body and zonula ciliaris (Zinn's zonule), suggesting lens luxation due to abnormal formation of the zonula ciliaris that plays a role as a supporting system of the lens. However, these ocular lesions were accompanied by phenomena indicating elevated intraocular pressure, such as enlarged eyeball, edematous cornea, thinning of the cornea and retina, atrophy of the optic nerve, and cupping of the optic disk, and glaucoma was suggested. Thus, such abnormal zonula ciliaris (and its consequent lens luxation) might have occurred following glaucoma. It is suggested that genetic background might be involved in these ocular lesions because all four cases were occurred in the colony, although the prevalence was low.

Keywords：スナネズミ，眼球腫大，水晶体脱臼

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Kumagai Y[*1], Gilmour S[*1], Ota E[*2], Momose Y, Onishi T[*3], Bilano VL[*1], Kasuga F, Sekizaki T[*1], Shibuya K[*1]: Estimating the burden of foodborne diseases in Japan.

Bull World Health Organ. 2015;93:540-9C.

When assessing the burden posed by foodborne diseases using methods developed by the WHO's Foodborne Disease Burden Epidemiology Reference Group (FERG), foodborne disease caused by Campylobacter, Salmonella and EHEC led to an estimated loss of 6099, 3145 and 463 DALYs in Japan, respectively, in 2011. These estimated burdens are based on the pyramid reconstruction method, and are much higher than those indicated by routine surveillance data. Most of the burden posed by foodborne disease in Japan comes from secondary complications. The tools developed by FERG appear useful in estimating disease burdens and setting priorities in the field of food safety.

Keywords: DALYs, FERG, foodborne diseases

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Saito H[*1], Toho M[*2], Tanaka T[*3], Noda M: Development of a practical method to detect noroviruses contamination in composite meals.

Food Environ Virol. 2015;7:239-48

Various methods to detect foodborne viruses including norovirus (NoV) in contaminated food have been developed. However, a practical method suitable for routine examination that can be applied for the detection of NoVs in oily, fatty, or emulsive food has not been established. In this study, we developed a new extraction and concentration method for detecting NoVs in contaminated composite meals. We spiked NoV-GI.4 or -GII.4 stool suspension into potato salad and stir-fried noodles. The food samples were suspended in homogenizing buffer and centrifuged to obtain a food emulsion. Then, anti-NoV-GI.4 or anti-NoV-GII.4 rabbit serum raised against recombinant virus-like particles or commercially available human gamma globulin and Staphylococcus aureus fixed with formalin as a source of protein A were added to the food emulsion. NoV-IgG-protein A-containing bacterial complexes were collected by centrifugation, and viral RNA was extracted. The detection limits of NoV RNA were 10-35 copies/g food for spiked NoVs in potato salad and stir-fried noodles. Human gamma globulin could also concentrate other NoV genotypes as well as other foodborne viruses, including sapovirus, hepatitis A virus, and adenovirus. This newly developed method can be used as to identify NoV contamination in composite foods and is also possibly applicable to other foodborne viruses.

Keywords: norovirus, food, detection

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入谷展弘*1，山元誠司*1,2，改田厚*1，阿部仁一郎*1，久保英幸*1，平井有紀*1，上林大起*1,2，野田衛，西尾孝之*1：2014－2015シーズンに大阪市で認められたノロウイルス流行.
大阪市立環境科学研究所報告　調査・研究年報 2015;77:13-6

2014-2015シーズンのノロウイルス（NoV）検出状況は2014年9月～12月にGII.3，2015年1月～3月にGII.17が主に検出され，時期によって流行するNoVの遺伝子型が異なっていた．NoV GII.3は主に低年齢層におけるヒトからヒトへの感染拡大であった．NoV GII.17の流行は大阪市において初めて認められ，主に成人層で流行していた.

Keywords: norovirus, GII.17, 2014-2015 season

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楠原一*1，赤地重宏*1，小林隆司*1，西中隆道*1，小林真美*2，山口江里*2，岩出義人*2，田沼正路*2，野田衛：ノロウイルスGII.17型の流行とその特徴について－三重県.
病原微生物検出情報 2015;36:91-2

2014/15シーズンのノロウイルスを原因とする食中毒などの健康被害事例と小児の感染性胃腸炎では，検出される遺伝子型に明らかな違いがみられた．健康被害事例からは，これまで検出例の少ない遺伝子型（GII.17）のノロウイルスが相次いで検出され，GII.17陽性検体には，市販のノロウイルス簡易検査キットでは陽性を示さないものもあった.

Keywords: norovirus, GII.17, commercial kits

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Yahata Y*1, Misaki T*1, Ishida Y*2, Nagira M*1, Watahiki M*3, Isobe J*3, Terajima J, Iyoda S*1, Mitobe J*1, Ohnishi M*1, Sata T*3, Taniguchi K*1, Tada Y*1, Okabe N*1, E. coli O111 Outbreak Investigation Team: Epidemiological analysis of a large enterohaemorrhagic Escherichia coli O111 outbreak in Japan associated with haemolytic uraemic syndrome and acute encephalopathy.
Epidemiol Infect. 2015;143:2721-32.

A large outbreak of enterohaemorrhagic Escherichia coli (EHEC) O111 and O157 occurred in Japan in April 2011. We conducted an unmatched case-control study and trace-back investigation to determine the source of EHEC O111 infection and risk factors for severe complications. Pulsed-field gel electrophoresis was performed to help define cases. A total of 86 individuals met the case definition. Of these, 40% experienced haemolytic uraemic syndrome (HUS), 24% acute encephalopathy, and 6% died. Illness was significantly associated with eating the raw beef dish yukhoe (odds ratio 19·64, 95% confidence interval 7·03-54·83), the likely food vehicle. EHEC O111 and its closely related stx-negative variants were found in the beef. HUS occurred most frequently in individuals aged 5-9 years, and this age group was significantly associated with acute encephalopathy. The prevalence of HUS and acute encephalopathy was higher than in previous non-O157-related outbreaks, indicating a high risk of severe complications.

Keywords: EHEC, HUS, O111

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Ban E*1, Yoshida Y*1, Wakushima M*1, Wajima T*2, Hamabata T*3, Ichikawa N*1, Abe H*4, Horiguchi Y*4, Hara-Kudo Y, Kage-Nakadai E*1, Yamamoto T*5, Wada T*5, Nishikawa Y*1: Characterization of unstable pEntYN10 from enterotoxigenic *Escherichia coli* (ETEC) O169:H41.
Virulence. 2015;6:735-44.

Enterotoxigenic *Escherichia coli* (ETEC) serotype O169:H41 has been an extremely destructive epidemic ETEC type worldwide. The strain harbors a large unstable plasmid that is regarded as responsible for its virulence, although its etiology has remained unknown. To examine its genetic background specifically on the unstable retention and responsibility in the unique adherence to epithelial cells and enterotoxin production, the complete sequence of a plasmid, pEntYN10, purified from the serotype strain was

determined. The length is 145,082 bp; its GC content is 46.15%. It contains 182 CDSs, which include three colonization factors (CFs), an enterotoxin, and large number of insertion sequences. The repertory of plasmid stability genes was extraordinarily scant. Uniquely, results showed that three CFs, CS6, CS8 (CFA/III)-like, and K88 (F4)-like were encoded redundantly in the plasmid with unique variations among previously known subtypes. These three CFs preserved their respective gene structures similarly to those of other ETEC strains reported previously with unique sequence variations respectively. It is particularly interesting that the K88-like gene cluster of pEntYN10 had two paralogous copies of faeG, which encodes the major component of fimbrial structure. It remains to be verified how the unique variations found in the CFs respectively affect the affinity to infected cells, host range, and virulence of the ETEC strain.
Keywords: ETEC, plasmid, genetic diversity

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西尾智裕[*1]，大塚佳代子[*2]，小田みどり[*1]，杉山寛治[*1]，工藤由起子：魚介類からの腸炎ビブリオ検出における遺伝子検出法の検討．
感染症学雑誌 2015;89:445-51.

魚介類からの腸炎ビブリオの効率的な検出を目的として，2種類のDNA抽出法（熱抽出法およびアルカリ熱抽出法）を腸炎ビブリオ種特異的遺伝子（易熱性溶血毒遺伝子tlhまたはrpoD遺伝子）および病原因子遺伝子（耐熱性溶血毒素TDH遺伝子tdh またはTDH類似性溶血毒素遺伝子trh）を対象とした3種類の遺伝子増幅法（PCR法，リアルタイムPCR法およびloop-mediated isothermal amplification（LAMP）法）と組み合わせて検出感度を検討した．腸炎ビブリオ種特異的遺伝子検出においては，アルカリ熱抽出法でDNAを抽出しtlh-リアルタイムPCR法およびrpoD—LAMP法にて検出することによって，供試した2菌株（tdh・trh1陽性株およびtrh2陽性株）および食品検体（カキおよびアカガイ）のいずれの組み合わせにおいて85-145 cfu/testの低菌数レベルで対象遺伝子が検出された．また，tdh検出においては，熱抽出法ではリアルタイムPCR法にて検出することによって85 cfu/testの菌数レベルでも両食品種で検出された．アルカリ熱抽出法では，LAMP法およびリアルタイムPCR法で85 cfu/testの菌数レベルでも検出された．さらに，trh検出においては，trh2の検出に限定すればアルカリ熱抽出法でのLAMP法で145 cfu/testの菌数レベルでも検出されたが，trh1およびtrh2の両方ではアルカリ熱抽出法でのPCR法が比較的高感度であった．しかし，今後さらにtrh検出法の検討が必要と考えられた．本研究では，アルカリ熱抽出法によってDNAを抽出し，tlh-リアルタイムPCR法またはrpoD-LAMP法，tdh-リアルタイムPCR法またはtdh-LAMP法，trh-PCR法およびtrh -LAMP法を行うことによって魚介類から腸炎ビブリオを比較的高感度に検出できることが示された．
Keywords：腸炎ビブリオ，リアルタイムPCR法，LAMP法

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森哲也[*1]，吉田信一郎[*2]，池本尚人[*3]，加藤一郎[*4]，林伸之[*5]，齋藤明美[*2]，市川希美[*1]，伊藤武[*1]，工藤由起子：ゼリー飲料および固形化成分を含有する粉末清涼飲料の細菌試験法の問題点とその改善法の検討．
日本食品微生物学会雑誌 2016;33:19-25.

ゼリー飲料における大腸菌群試験法，およびゲル状となる粉末清涼飲料における大腸菌群試験法および細菌数（生菌数）試験法について検討した．現行の大腸菌群試験法に従い2倍濃度のLB 10 mlにゼリー飲料検体10 mlを加えて培養する場合，ガス産生が正しく判定されない場合があることが判明した．そこで，LB 100 mlに検体10 mlを加えて培養する方法を検討した結果，ガス産生が確認された．また，コンニャクグルコマンナン等が含まれる粉末清涼飲料の試験では，10倍乳剤ではゲル強度が高いために，試験の操作が困難となるため，100倍乳剤を用いて検討した.大腸菌群の検査では，2倍濃度のLB 100 mlに100倍乳剤を加えて培養することによってガスの産生が確認され，現行の試験法と同等の検出感度での大腸菌群の検査が実施できた．一方，細菌数（生菌数）測定に関しては，混釈培養法でのコロニー数測定が困難であったため，今後さらに検討が必要である．
Keywords：ゼリー飲料，大腸菌群試験法，細菌数（生菌数）試験法

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Kobayashi N, Maeda E*1, Saito S*2, Furukawa I*3, Ohnishi T, Watanabe M, Terajima J, Hara-Kudo Y: Association of cell-adhesion activities with virulence in Shiga toxin-producing *Escherichia coli* O103:H2.
Biocontrol Science 2016;21:57-61.

The characteristics of 11 strains of Stx1-producing and Stx2-non-producing STEC O103:H2 were analyzed to investigate the differences in virulence in a single serotype of Shiga toxin (Stx)-producing *Escherichia coli* (STEC). Differences in the cell-adhesion activity to Caco-2 cells were observed among the strains. The activity of the one strain, isolated from a patient with hemolytic uremic syndrome was 4-20-fold higher than those of the other strains. Although the strains with high cell-adhesion activity showed high expressions of eae, espB, espD, and tir in the locus of enterocyte effacement related with cell-adhesion, those were not specific for this strain. In addition, the Stx1 production level of the strain was not particularly high. It was indicated that the high adhesion activity might be a potential factor to associate serious symptom.
Keywords: Shiga toxin-producing Escherichia coli, Virulence, Adhesion activity

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渡辺麻衣子，菊池裕：核酸増幅検査（NAT）を利用した真菌否定試験および迅速同定法の開発に関する研究.
医薬品医療機器レギュラトリーサイエンス 2016;47:150-7.

第十六改正日本薬局方（日局16）一般試験法の無菌試験は，無菌であることが求められている原薬又は製剤の出荷判定試験に適用される．培養開始から14日間後に細菌又は真菌の増殖を肉眼的に判定し，増殖が観察された場合には，培養法で微生物を検出する．真菌の場合，特徴を見出すための特別な熟練技術や，結果の判定のための専門知識が求められることに加え，培養が必要で時間がかかる．また，時に判定に対して客観性に乏しくなる場合がある．真菌についてもNATを利用した微生物迅速法による無菌試験法を開発する必要性が高い．本研究では，再生医療等製品にも適用可能な医薬品の出荷判定試験に用いる微生物迅速法による無菌試験法の導入を見据え，日局16の無菌試験法で規定された試験用菌株 *Aspergillus brasiliensis* および*Candida albicans*を含む合計10菌種を供試して，NATによる真菌の検出および検出された真菌の同定に用いるプライマーセットの比較検討を行った．日局16収載のプライマーセットを中心に，各プライマーセット間で検出力や同定精度を比較した．その結果，菌種を絞らず網羅的に真菌汚染の有無を検出できるプライマーセットおよび塩基配列相同率を指標として明瞭に菌種を同定できる可能性が最も高いプライマーセットを明らかにした．さらに，今後NATを真菌検査の迅速法として導入するにあたっての問題点を明らかにした.
Keywords：真菌，核酸増幅検査，無菌試験法

Ohnishi T, Furusawa H, Oyama R, Koike S*1, Yoshinari T, Kamata Y*2, Sugita-Konishi Y*3: Molecular epidemiological analysis of *Kudoa septempunctata* by random amplified polymorphic DNA analysis.
JJID. 2015;68:235-8.

Molecular epidemiological analysis of *Kudoa septempunctata* isolates from 34 olive flounders associated with foodborne disease outbreaks and from 6 reference samples was performed using random amplified polymorphic DNA (RAPD) analysis. The K. septempunctata isolates analyzed in this study were divided into 8 groups. Eight isolates obtained from the large Ehime Prefecture outbreak in Japan that had occurred on October 8, 2010, were further divided into 4 groups. Eight isolates obtained from Korean samples were divided into 3 groups. These groups included isolates that had been identified from the large Ehime Prefecture outbreak. These results indicated that the Korean isolates had similar genetic backgrounds to those involved in the Ehime Prefecture outbreak. Isolates associated with outbreaks with similar dates of onset tended to be classified in the same group, suggesting that the strains involved in these incidents were genetically related. These results demonstrated that RAPD analysis is a useful molecular epidemiological analysis method for *K. septempunctata*.
Keywords: *Kudoa*, Parasite, Food-borne disease

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Takeuchi F*1, Ogasawara Y*1, Kato K*1, Sekizuka T*1, Nozaki T*1.2, Sugita-Konishi Y*3, Ohnishi T, Kuroda M*1: Genetic variants of Kudoa septempunctata (Myxozoa: Multivalvulida), a flounder parasite causing foodborne disease.
J Fish Dis. 2016;39:667-72.

Foodborne disease outbreaks caused by raw olive flounders (Paralichthys olivaceus) parasitized with Kudoa septempunctata have been reported in Japan. Origins of olive flounders consumed in Japan vary, being either domestic or imported, and aquaculture-raised or natural. Although it is unknown whether different sources are associated with different outcomes, it is desirable to identify whether this is the case by determining whether unique K. septempunctata strains occur and if so, whether some are associated with foodborne illness. We here developed an intraspecific genotyping method, using the sequence variation of mitochondrial genes. We collected olive flounder samples from foodborne disease outbreaks, domestic fish farms or quarantine offices and investigated whether *K. septempunctata* genotype is associated with pathogenicity or geographic origin. The 104 samples were classified into three genotypes, ST1, ST2 and ST3. Frequency of symptomatic cases differed by genotypes, but the association was not statistically significant. Whereas *K. septempunctata* detected from aquaculture-raised and natural fish from Japan were either ST1 or ST2, those from fish inspected at quarantine from Korea to Japan were ST3. Our method can be applied to phylogeographic analysis of *K. septempunctata* and contribute to containing the foodborne disease.
Keywords: *Kudoa*, Parasite, Food-borne disease

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Takeuchi F*1, Ogasawara Y*1, Sekizuka T*1, Yokoyama H*2, Kamikawa R*3, Inagaki Y*4, Nozaki T*1.4, Sugita-Konishi Y*5, Ohnishi T, Kuroda M*1: The Mitochondrial Genomes of a Myxozoan Genus *Kudoa* Are Extremely Divergent in Metazoa.
PLOS One 2015;10:e0132030.

The Myxozoa are oligo-cellular parasites with alternate hosts--fish and annelid worms--and some myxozoan species harm farmed fish. The phylum Myxozoa, comprising 2,100 species, was difficult to position in the tree of life, due to its fast evolutionary rate. Recent phylogenomic studies utilizing an extensive number of nuclear-encoded genes have confirmed that Myxozoans belong to Cnidaria. Nevertheless, the evolution of parasitism and extreme body simplification in Myxozoa is not well understood, and no myxozoan mitochondrial DNA sequence has been reported to date. To further elucidate the evolution of Myxozoa, we sequenced the mitochondrial genomes of the myxozoan species *Kudoa septempunctata*, *K. hexapunctata* and K. iwatai and compared them with those of other metazoans. The Kudoa mitochondrial genomes code for ribosomal RNAs, transfer RNAs, eight proteins for oxidative phosphorylation and three proteins of unknown function, and they are among the metazoan mitochondrial genomes coding the fewest proteins. The mitochondrial-encoded proteins were extremely divergent, exhibiting the fastest evolutionary rate in Metazoa. Nevertheless, the dN/dS ratios of the protein genes in genus *Kudoa* were approximately 0.1 and similar to other cnidarians, indicating that the genes are under negative selection. Despite the divergent genetic content, active oxidative phosphorylation was indicated by the transcriptome, metabolism and structure of mitochondria in *K. septempunctata*. As possible causes, we attributed the divergence to the population genetic characteristics shared between the two most divergent clades, Ctenophora and Myxozoa, and to the parasitic lifestyle of Myxozoa. The fast-evolving, functional mitochondria of the genus Kudoa expanded our understanding of metazoan mitochondrial evolution.
Keywords: *Kudoa*, Parasite, Food-borne disease

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Lim CW*1, Yoshinari T, Layne J*2, Chan SH*1: Multi-mycotoxin screening reveals separate occurrence of aflatoxins and ochratoxin a in asian rice.

Journal of Agricultural Food Chemistry 2015;63:3104-13.

The determination of important regulated mycotoxins in rice has been reported previously but not in the individual matrix of white, brown, red, and basmati rice with respect to the matrix effect, recovery, and stability. A total of 190 Asian rices were examined for regulated mycotoxin contamination by the LC-ESI-MS/MS method. Mean recovery ranged from 70 to 120%. RSD values were lower than 15% for all analytes.

Keywords: mycotoxin, LC-ESI-MS/MS, rice

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Yoshinari T, Ohashi H*1, Abe R*1, Kaigome R*1, Ohkawa H*2, Sugita-Konishi Y*3: Development of a rapid method for the quantitative determination of deoxynivalenol using Quenchbody.

Analytica chimica acta 2015;888:126-30.

Quenchbody (Q-body) is a novel fluorescent biosensor based on the antigen-dependent removal of a quenching effect on a fluorophore attached to antibody domains. In order to develop a method using Q-body for the quantitative determination of deoxynivalenol (DON), a trichothecene mycotoxin produced by some *Fusarium* species, anti-DON Q-body was synthesized from the sequence information of a monoclonal antibody specific to DON. To validate the analytical method using Q-body, a spike-and-recovery experiment was performed using four spiked wheat samples. The recoveries were in the range of 94.9-100.2%. This data indicate that the Q-body system for the determination of DON in wheat samples was successfully developed and Q-body is expected to have a range of applications in the field of food safety.

Keywords: Q-body, wheat, deoxynivalenol

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Sakuda S*, Yoshinari T, Furukawa T*, Jermnak U*, Takagi K.*, Iimura K*, Yamamoto T*, Suzuki M*, Nagasawa H*: Search for aflatoxin and trichothecene production inhibitors and analysis of their modes of action.

Biosci Biotechnol Biochem. 2015;17:1-12.

Mycotoxin contamination of crops is a serious problem throughout the world because of its impact on human and animal health as well as economy. Inhibitors of mycotoxin production are useful not only for developing effective methods to prevent mycotoxin contamination, but also for investigating the molecular mechanisms of secondary metabolite production by fungi. We have been searching for mycotoxin production inhibitors among natural products and investigating their modes of action. In this article, we review aflatoxin and trichothecene production inhibitors, including our works on blasticidin S, methyl syringate, cyclo(l-Ala-l-Pro), respiration inhibitors, and precocene II.

Keywords: aflatoxin, trichothecene, inhibitor

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Tanda K*1, Eto R*2, Kato K*2, Oba M*2, Ueda A*2, Suemune H*1, Doi M*3, Demizu Y, Kurihara M, Tanaka M*2: Peptide foldamers composed of six-membered ring α,α-disubstituted α-amino acid with two changeable chiral acetal moieties.

Tetrahedron 2015;71:3909-14.

Chiral cyclic α,α-disubstituted α-amino acids with four chiral centers at their acetal moieties were synthesized. An X-ray crystallographic analysis of homo-chiral tripeptide with (2R,3R)-butane-2,3-diol acetal moieties revealed that the tripeptide formed both (P) and (M) helical structures, and all peptide main-chain N(i)-H were intramolecularly hydrogen-bonded with the side-chain acetal -O- of the same amino acid residues (i). The effect of the four chiral centers in the amino acid residue on the peptide backbone helical-screw control was very weak.

Keywords: conformation, peptide, α,α-disubstituted α-amino acid

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Gautam S*1, Kim T*1, Shoda T, Sen S*1, Deep D*1, Luthra R*1, Ferreira MT*2, Pinho MG*2, Spiegel DA*1: An activity-based probe for studying

crosslinking in live bacteria.

Angew Chem Int Ed. 2015;54:10492-6.

Penicillin-binding proteins (PBPs) catalyze the crosslinking of peptidoglycan (PG), an essential process for bacterial growth and survival, and a common antibiotic target. Yet, despite its importance, little is known about the spatiotemporal aspects of crosslinking—largely because of a lack of experimental tools for studying the reaction in live bacteria. Here we introduce such a tool: an activity-based probe that enables visualization and relative quantitation of crosslinking in vivo. In *Staphylococcus aureus*, we show that fluorescent mimics of the natural substrate of PBPs (PG stem peptide) are covalently incorporated into the cell wall, installing fluorophores in place of natural crosslinks. These fluorescent stem peptide mimics (FSPMs) are selectively recognized by a single PBP in S. aureus: PBP4. Thus, we were able to use FSPM pulse-labeling to localize PBP4 activity in live cells, showing that it is recruited to the septum in a manner dependent on wall teichoic acid.

Keywords: bacteria, biosensors, crosslinking

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Demizu Y, Oba M*1, Okitsu K, Yamashita H, Misawa T, Tanaka M*1, Kurihara M, Gellman SH*2: A preorganized β-amino acid bearing a guanidinium side chain and its use in cell-penetrating peptides.

Org Biomol Chem. 2015;13:5617-20.

A cyclic β-amino acid (APCGu) bearing a side-chain guanidinium group has been developed. The APCGu residue was incorporated into an α/β-peptide based on the Tat(47–57) fragment, leading to an oligomer with substantial helicity in methanol that enters HeLa cells much more readily than does the corresponding Tat α-peptide.

Keywords: foldamer, peptide, cell-penetrating peptide

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Shoda T, Kato M, Fujisato T, Okuhira K, Demizu Y, Inoue H*, Naito M, Kurihara M: Synthesis and evaluation of tamoxifen derivatives with a long alkyl side chain as selective estrogen receptor down-regulators.

Bioorg Med Chem. 2015;23:3091-6.

Estrogen receptors (ERs) play a major role in the growth of human breast cancer cells. An antagonist that acts as not only an inhibitor of ligand binding but also an inducer of the down-regulation of ER would be useful for the treatment for ER-positive breast cancer. We previously reported the design and synthesis of a selective estrogen receptor down-regulator (SERD), (E/Z)-4-(1-{4-[2-(dodecylamino)ethoxy] phenyl}-2- phenylbut-1-en-1-yl)phenol (C12), which is a tamoxifen derivative having a long alkyl chain on the amine moiety. This compound induced degradation of ERα via a proteasome-dependent pathway and showed an antagonistic effect in MCF-7 cells. With the aim of increasing the potency of SERDs, we designed and synthesized various tamoxifen derivatives that have various lengths and terminal groups of the long alkyl side chain. During the course of our investigation, C10F having a 10-fluorodecyl group on the amine moiety of 4-OHT was shown to be the most potent compound among the tamoxifen deriva- tives. Moreover, computational docking analysis suggested that the long alkyl chain interacted with the hydrophobic region on the surface of the ER, which is a binding site of helix 12 and coactivator. These results provide useful information to develop promising candidates as SERDs.

Keywords: breast cancer, estrogen receptor, SERD

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Usui K*, Yamamoto K*, Shimizu T*, Biao M*, Okazumi M*, Demizu Y, Kurihara M, Suemune H*: Synthesis and resolution of substituted [5] carbohelicenes.

J Org Chem. 2015;80:6502-8.

Three types of racemic [5]helicenyl acetates

(1a, 2, and 3a) were synthesized. The synthesis of 2 was achieved by regioselective oxidation using o-iodoxybenzoic acid. The enzymatic kinetic resolution of 1a－3a was studied.The conversion with the highest rate and ee was obtained using 1a as the substrate and lipase Amano PS-IM as the enzyme. The two enantiomers of 1-[5]helicenol 3b were separated using (1S)-10-camphorsulfonyl chloride as the chiral resolving agent.

Keywords: helicene, kinetic resolution, X-ray diffraction

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Demizu Y, Misawa T, Nagakubo T, Kanda Y, Okuhira K, Sekino Y, Naito M, Kurihara M: Structural development of stabilized helical peptides as inhibitors of estrogen receptor (ER)-mediated transcription.

Bioorg Med Chem. 2015;23:4132-8.

Three types of stabilized helical peptides containing disulfide bonds, C–C cross-linked side chains, or α,α-disubstituted amino acids (2-aminoisobutyric acid (Aib)) were designed and synthesized as inhibitors of estrogen receptor (ER)-coactivator interactions. Furthermore, heptaarginine (R7)-conjugated versions of the peptides were prepared, and their effects on ER-mediated transcription were evaluated at the cellular level (in ER-positive T47D cells). Among them, the R7-conjugated peptides 11 and 12 downregulated the mRNA expression of pS2 (an ER-mediated gene whose expression is upregulated by 17β-estradiol) by 95% (at a dose of 10 μM) and 87% (at a dose of 3 μM), respectively.

Keywords: estrogen receptor, protein-protein interaction, transcriptional inhibitor

Oba M*, Demizu Y, Yamashita H, Kurihara M, Tanaka M*: Plasmid DNA delivery using fluorescein-labeled arginine-rich peptides.

Bioorg Med Chem. 2015;23:4911-8.

Arginine (Arg)-rich peptides exhibit an effective cell-penetrating ability and deliver membrane-impermeable compounds into cells. In the present study, three types of Arg-rich peptides, R9 containing nine Arg residues, (RRG)3 containing six Arg and three glycine (Gly) residues, and (RRU)3 containing six Arg and three a-aminoisobutyric acid (Aib) residues, were evaluated for their plasmid DNA (pDNA) delivery and cell-penetrating abilities. The transfection efficiency of R9/pDNA complexes was much higher than those of (RRG)3 and (RRU)3/pDNA complexes, and was derived from the enhanced cellular uptake of R9/pDNA complexes. The replacement of three Arg residues with the neutral amino acid Gly and hydrophobic amino acid Aib drastically changed the cell-penetrating ability and physicochemical properties of peptide/pDNA complexes, resulting in markedly reduced transfection efficiency. A comparison of the R9 peptide administration forms between a peptide alone and peptide/pDNA complex revealed that the uptake of R9 peptides was more efficient for the complex than the peptide alone, but occurred through the same internalization mechanism. The results of the present study will contribute to the design of novel Arg-rich cell-penetrating peptides for pDNA delivery.

Keywords: cell-penetrating peptide, gene transfer, drug delivery system

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Sakakibara N*[1], Balboni G*[2], Congiu C*[2], Onnis V*[2], Demizu Y, Misawa T, Kurihara M, Kato Y*[1], Maruyama T*[1], Toyama M*[3], Okamoto M*[3], Baba M*[3]: Design, synthesis, and anti-HIV-1 activity of 1-substituted 3-(3,5-dimethylbenzyl)triazine derivatives.

Antiviral Chem Chemother. 2015;24:62-71.

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) is an attractive target for the development of drugs used in the treatment of HIV-1 infection and acquired immune deficiency syndrome (AIDS). We have continued the search for novel anti-HIV-1 agents using the structure–activity relationships of the successful 1,3-disubstituted and 1,3,6-trisubstituted uracil-type HIV-1 RT inhibitors. A series of new triazine analogs were synthesized using an established method. The anti-HIV-1 activities of these compounds were determined based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. The cytotoxicity of the compounds was evaluated by assessing the viability of mock-infected cells. Some of the compounds showed good-to-moderate activities against HIV-1, with half-maximal effective concentrations (EC50) in the submicromolar range. In particular, a dihydro-1-(4-aminobenzyl)triazine analog showed satisfactory anti-HIV-1 activity with an EC50 of 0.110 μM and a selectivity index (SI) of 909. Furthermore, molecular modeling analyses were performed to explore the major interactions between HIV-1 RT and potent inhibitors. These results may be important for further development of this class of compounds as anti-HIV-1 agents. The satisfactory anti-HIV-1 activity of triazine analogs may serve as the basis for further investigations of the behavior of this

class of compounds against drug-resistant mutants.
Keywords: AIDS, HIV, non-nucleoside reverse transcriptase inhibitors

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Sakakibara N*1, Igarashi J*2, Takata M*2, Demizu Y, Misawa T, Kurihara M, Konishi R*2, Kato Y*1, Maruyama T*1, Tsukamoto I*2: Synthesis, evaluation, and molecular docking studies of novel carbocyclic oxetanocin A (COA-Cl) derivatives as potential tube formation agents.
Chem Pharm Bull. 2015;63:701-9.

Six novel carbocyclic oxetanocin A analogs (2-chloro-C.OXT-A; COA-Cl) with various hydroxymethylated or spiro-conjugated cyclobutane rings at the N9-position of the 2-chloropurine moiety were synthesized and evaluated using human umbilical vein endothelial cells. All prepared compounds (2a–f) showed good to moderate activity with angiogenic potency. Among these compounds, 100 μM cis– trans-2′,3′-bis(hydroxymethyl)cyclobutyl derivative (2b), trans-3′-hydroxymethylcyclobutyl analog (2d), and 3′,3′-bis(hydroxymethyl)cyclobutyl derivative (2e) had greater angiogenic activity, with relative tube areas of 3.43 ± 0.44, 3.32 ± 0.53, and 3.59 ± 0.83 (mean ± standard deviation (S.D.)), respectively, which was comparable to COA-Cl (3.91 ± 0.78). These data may be important for further development of this class of compounds as potential tube formation agents.
Keywords: 2-chloro-carbocyclic oxetanocin A, 2-chloro-C.OXT-A, nucleoside derivative

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Demizu Y, Yamashita H, Doi M*1, Misawa T, Oba M*2, Tanaka M*2, Kurihara M: Topological study of the structures of heterochiral peptides with equal amounts of L-Leu and D-Leu.
J Org Chem. 2015;80:8597-603.

We designed and synthesized two dodecapeptides, Boc-(L-Leu-L-Leu-Aib-D-Leu-D-Leu-Aib)2-OMe (5) and Boc-L-Leu-L-Leu-Aib-(D-Leu-D-Leu-Aib)2-L-Leu-L-Leu-Aib-OMe (6), that contain equal amounts of L-Leu, D-Leu, and achiral Aib residues. The conformations of peptides 5 and 6 in the crystalline state were studied using X-ray crystallographic analysis. Peptide 5 formed a left-handed (M) α-helical structure, whereas peptide 6 was composed of a combination of fused (M) α-helical and right-handed (P) 310-helical structures. In solution, roughly equivalent amounts of (P) and (M) helices were present in 5, whereas the (M) α-helix was present in 6 as its dominant conformation.
Keywords: amino acid, peptide, helix

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Misawa T, Yorioka M, Demizu Y, Noguchi-Yachide T*1, Ohoka N, Kurashima-Kinoshita M, Motoyoshi H, Nojiri H*2, Kittaka A*2, Makishima M*3, Naito M, Kurihara M: Effects of alkyl side chains and terminal hydrophilicity on vitamin D receptor (VDR) agonistic activity based on the diphenylpentane skeleton.
Bioorg Med Chem Lett. 2015;25:5362-6.

Vitamin D receptor (VDR) is a family of nuclear receptors (NR) that regulates physiological effects such as the immune system, calcium homeostasis, and cell proliferation. We synthesized non-secosteroidal VDR ligands bearing a long alkyl chain based on the diphenylpentane skeleton. The VDR-mediated transcriptional activities of the synthesized compounds were evaluated using a reporter gene assay and HL-60 cell differentiation-inducing assay. We herein described the structure–activity relationship and effects of alkyl-chain length on VDR-mediated transcriptional activity.
Keywords: vitamin D receptor, non-secosteroidal VDR ligands, long alkyl chain

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Yamashita H, Oba M*, Misawa T, Tanaka M*, Hattori T, Naito M, Kurihara M, Demizu Y: A helix-stabilized cell-penetrating peptide as an intracellular-delivery tool.
ChemBioChem. 2016;17:137-40.

Two types of cationic cyclic α,α-disubstituted α-amino acids: ApiC2Gu (which possesses a lysine mimic side chain) and ApiC2Gu (which possesses an

arginine mimic side chain), were developed. These amino acids were incorporated into an arginine-based peptide sequence [(L-Arg-L-Arg-dAA)3 : dAA = ApiC2NH2 or ApiC2Gu], and the relationship between the secondary structures of the resulting peptides and their ability to pass through cell membranes was investigated. The peptide containing ApiC2Gu formed a stable α-helical structure and was more effective at penetrating cells than the nonhelical Arg nonapeptide (R9). Furthermore, the peptide was able to deliver plasmid DNA into various types of cells in a highly efficient manner.

Keywords: cell-penetrating peptides, non-proteinogenic amino acids, plasmid DNA delivery

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Demizu Y, Yamashita H, Misawa T, Doi M*1, Oba M*2, Tanaka M*2, Kurihara M: Handedness preferences of heterochiral helical peptides containing homochiral peptide segments.

Eur J Org Chem. 2016;840-6.

A homochiral L-Leu-L-Leu-Aib segment was incorpo- rated into the N- or C-termini of left-handed (M) helical peptides (D-Leu-L-Leu-Aib)n. We then investigated the preferred conformations of two sets of three peptides; i.e., Boc-L-Leu-L-Leu-Aib- (D-Leu-L-Leu-Aib)n -OMe (n = 1: 1; 2: 2; 3: 3) and Boc-(D-Leu-L-Leu-Aib)n -L-Leu-L-Leu-Aib-OMe (n = 1: 4; 2: 5; 3: 6), in solution and in the crystalline state. Nonapeptide 2 and dodecapeptide 3, each containing an N-terminal L-Leu-L-Leu-Aib segment, formed left-handed (M) helices as the preferred secondary structures in solution. In the crystalline state, nonapeptide 2 folded into an (M) α-helical structure. Peptides 4–6, each containing a C-terminal L-Leu-L-Leu-Aib segment, formed roughly equivalent amounts of right-handed (P) and (M) helices.

Keywords: amino acid, peptide, helix

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Demizu Y, Tsutsui K, Misawa T, Kurihara M: 1,4-Bis[(N-acetyl-L-phenylalanyl-glycyl-L-alanyl) aminomethyl]benzene.

Molbank 2016;doi:10.3390/M893.

The title compound was prepared by inducing amide bond formation between 1,4-bis(aminomethyl)benzene and tripeptide Ac-Phe-Gly-Ala-OH. The structure of the synthesized compound was determined on the basis of its 1H-nuclear magnetic resonance (NMR), 13C-NMR, and mass spectral data. Furthermore, the compound's preferred structure in solution and calculated conformation are also reported.

Keywords: peptide, foldamer, NMR analysis

Soga K, Abo H*1, Qin SY*1, Kyoutou T*1, Hiemori K*1, Tateno H*2, Matsumoto N*1, Hirabayashi J*2, Yamamoto K*1: Mammalian cell surface display as a novel method for developing engineered lectins with novel characteristics.

Biomolecules 2015;5:1540-62.

Leguminous lectins have a conserved carbohydrate recognition site comprising four loops (A-D). Here, we randomly mutated the sequence and length of loops C and D of peanut agglutinin (PNA) and expressed the proteins on the surface of mouse green fluorescent protein (GFP)-reporter cells. Flow cytometry, limiting dilution, and cDNA cloning were used to screen for several mutated PNAs with distinct properties. The mutated PNA clones obtained using NeuAcα2-6(Galβ1-3)GalNAc as a ligand showed preference for NeuAcα2-6(Galβ1-3)GalNAc rather than non-sialylated Galβ1-3GlcNAc, whereas wild-type PNA binds to Galβ1-3GlcNAc but not sialylated Galβ1-3GalNAc. Sequence analyses revealed that for all of the glycan-reactive mutated PNA clones, (i) loop C was eight amino acids in length, (ii) loop D was identical to that of wild-type PNA, (iii) residue 127 was asparagine, (iv) residue 125 was tryptophan, and (v) residue 130 was hydrophobic tyrosine, phenylalanine, or histidine. The sugar-binding ability of wild-type PNA was increased nine-fold when Tyr125 was mutated to tryptophan, and that of mutated clone C was increased more than 30-fold after His130 was changed to tyrosine. These results provide an insight into the relationship between the amino acid sequences of the carbohydrate recognition site and sugar-binding abilities of leguminous lectins.

Keywords: Carbohydrate-binding specificity, Cell surface display, Leguminous lectin

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Abo H[*1], Soga K, Tanaka A[*1], Tateno H[*2], Hirabayashi J[*2], Yamamoto K[*1]: Mutated leguminous lectin containing a heparin-binding like motif in a carbohydrate-binding loop specifically binds to heparin.

PLOS One 2015;10:e0145834.

We previously introduced random mutations in the sugar-binding loops of a leguminous lectin and screened the resulting mutated lectins for novel specificities using cell surface display. Screening of a mutated peanut agglutinin (PNA), revealed a mutated PNA with a distinct preference for heparin. Glycan microarray analyses using the mutated lectin fused to the Fc region of human immunoglobulin, revealed that a particular sulfated glycosaminoglycan (GAG), heparin, had the highest binding affinity for mutated PNA among 97 glycans tested, although wild-type PNA showed affinity towards Galβ1-3GalNAc and similar galactosylated glycans. Further analyses of binding specificity using an enzyme-linked immunoadsorbent assay demonstrated that the mutated PNA specifically binds to heparin, and weakly to de-2-O-sulfated heparin, but not to other GAG chains including de-6-O-sulfated and de-N-sulfated heparins. The mutated PNA had six amino acid substitutions within the eight amino acid-long sugar-binding loop. In this loop, the heparin-binding like motif comprised three arginine residues at positions 124, 128, and 129, and a histidine at position 125 was present. Substitution of each arginine or histidine residue to alanine reduced heparin-binding ability, indicating that all of these basic amino acid residues contributed to heparin binding. Inhibition assay demonstrated that heparin and dextran sulfate strongly inhibited mutated PNA binding to heparin in dose-dependent manner. The mutated PNA could distinguish between CHO cells and proteoglycan-deficient mutant cells. This is the first report establishing a novel leguminous lectin that preferentially binds to highly sulfated heparin and may provide novel GAG-binding probes to distinguish between heterogeneous GAG repeating units.

Keywords: Leguminous lectin, Heparin-binding motif, Specificity

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Nakamura K, Matsuoka H, Nakashima S[*], Kanda T[*], Nishimaki-Mogami T, Akiyama H: Oral administration of apple condensed tannins delays rheumatoid arthritis development in mice via down-regulation of T helper 17 (Th17) cell responses.

Mol Nutr Food Res. 2015;59:1406-10.

Apples are known to contain high concentrations of phenolic compounds such as condensed tannins. Consumption of condensed tannins has been reported to reduce the risk of many types of chronic diseases including allergies. However, their therapeutic effectiveness and potential in treating autoimmune disease remain controversial. Here, the effect of oral administration of apple condensed tannins (ACT) prepared from apples (Malus pumila cv. Fuji) on bovine type II collagen (CII)-induced arthritis in DBA1/J mice, a well-established murine model of human rheumatoid arthritis (RA), was evaluated. As compared to the control (without ACT administration) group, RA development was delayed and a significant reduction in the RA clinical score was observed in the ACT-administered group. Using cultured splenocytes isolated from CII-immunized mice, ACT-administration was shown to decrease the CII-induced increases in IL-17 expression and production in vitro. We propose that downregulation of T helper (Th) 17 cells is responsible for the ACT-induced RA suppression.

Keywords: Apple condensed tannins, Phytochemical, Rheumatoid arthritis

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Takabatake R[*1], Masubuchi T[*1], Futo S[*2], Minegishi Y[*3], Noguchi A, Kondo K, Teshima R, Kurashima T[*1], Mano J[*1], Kitta K[*1]: Selection of suitable DNA extraction methods for genetically modified maize 3272, and development and evaluation of an event-specific quantitative PCR method for 3272.

Shokuhin Eiseigaku Zasshi 2016;57:1-6.

A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) maize, 3272. We first attempted to obtain genome DNA from this maize using a DNeasy Plant Maxi kit and a DNeasy Plant Mini kit, which have been widely utilized in our previous studies, but DNA extraction yields from 3272 were markedly lower than those from non-GM maize

seeds. However, lowering of DNA extraction yields was not observed with GM quicker or Genomic-tip 20/G. We chose GM quicker for evaluation of the quantitative method. We prepared a standard plasmid for 3272 quantification. The conversion factor (Cf), which is required to calculate the amount of a genetically modified organism (GMO), was experimentally determined for two real-time PCR instruments, the Applied Biosystems 7900HT (the ABI 7900) and the Applied Biosystems 7500 (the ABI7500). The determined Cf values were 0.60 and 0.59 for the ABI 7900 and the ABI 7500, respectively. To evaluate the developed method, a blind test was conducted as part of an interlaboratory study. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSDr). The determined values were similar to those in our previous validation studies. The limit of quantitation for the method was estimated to be 0.5% or less, and we concluded that the developed method would be suitable and practical for detection and quantification of 3272.

Keywords: 3272, Genetically modified, Real-time PCR

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Obara T*1,2, Yamaguchi H*1, Satoh M*1, Iida Y*1, Sakai T*3, Aoki Y, Murai Y*4, Matsuura M*1, Sato M*1, Ohkubo T*5, Iseki K*6, Mano N*1: Prevalence, Determinants, and Reasons for the Non-Reporting of Adverse Drug Reactions by Pharmacists in the Miyagi and Hokkaido Regions of Japan.

Advances in Pharmacoepidemiology & Drug Safety 2015;4:191.

Little is known about the potential of adverse drug reaction (ADR) non-reporting by Japanese pharmacists. The aim of the present study was to clarify the prevalence, determinants, and reasons for ADR non-reporting by pharmacists in the Miyagi and Hokkaido regions of Japan. In this cross-sectional, self-administered questionnaire-based study, we contacted 3,164 pharmacists who belonged to the Miyagi Prefecture Hospital Pharmacists Association or the Hokkaido Society of Hospital Pharmacists during the 3-month period between January to March 2013. Of the 1,795 respondents 22.4% were <30 years of age, 25.6% were ≥ 50 years of age, and 42.1% were female. A total of 77.6% of the respondents did not have a personal history of ADR reporting. The multivariate logistic regression analysis showed that female sex (odds ratio, 1.52; 95% confidence interval, 1.17-1.97), having <10 years of practical experience (2.59, 1.39-4.82 for 5-9 years; 7.03, 2.94-16.83 for <5 years), working at a community pharmacy or drugstore (1.90, 1.16-3.12), having <5 pharmacists in the workplace (2.01, 1.48-2.75), and not understanding the ADR reporting system (5.93, 4.23-8.33) were significantly and independently associated with not having a personal history of ADR reporting. The most common reason for ADR non-reporting was “It was a well-known adverse drug reaction” (43.0%) followed by “Association between the drug and adverse reaction was not clear” (38.0%), “It was a minor adverse drug reaction” (29.0%), “Did not know how to make a report” (17.4%), and “Never been consulted about ADRs” (17.2%). As an understanding the ADR reporting system was strongly associated with ADR reporting, a more aggressive promotion of the ADR reporting system among pharmacists is warranted.

Keywords: Adverse drug reaction, Pharmacist, Questionnaire

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Obara T*1,2, Yamaguchi H*1, Iida Y*7, Satoh M*1, Sakai T*3, Aoki Y, Murai Y*4, Matsuura M*1, Sato M*1, Ohkubo T*5, Iseki K*6, Mano N*1: Knowledge of and Perspectives on Pharmacovigilance among Pharmacists in the Miyagi and Hokkaido Regions of Japan.

Journal of Pharmacovigilance 2016;4:192

The aim of the present study was to clarify the knowledge of and perspectives on pharmacovigilance among pharmacists in the Miyagi and Hokkaido regions of Japan. In this cross-sectional, self-administered questionnairebased study, we contacted

3,164 pharmacists who belonged to the Miyagi Prefecture Hospital Pharmacists Association or the Hokkaido Society of Hospital Pharmacists during the 3-month period between January and March 2013. Of the 1,851 respondents (<30 years, 22.2%; ≥ 50 years, 25.8%; women, 41.9%), 6.9%, 22.1%, and 71.0% answered "I understand what it is", "I have heard of it, but I do not understand what it is", and "I do not know what it is", respectively, to the question "Have you ever heard of the term 'pharmacovigilance'?". Multivariate logistic regression analysis revealed that being ≥ 50 years old (odds ratio [OR]: 6.10, 95% confidence interval [CI]: 1.99-18.72), having a doctoral degree (OR: 6.33; 95%CI: 3.19-12.57), and having ≥ 10 pharmacists in the workplace (OR: 2.08; 95%CI: 1.20-3.60) were significantly and independently associated with understanding "pharmacovigilance." Pharmacists who understood "pharmacovigilance" also tended to know more related terms and actions. Furthermore, 76.2% of the respondents thought that pharmacists should be responsible for pharmacovigilance in the clinical setting, and even though most of the pharmacists in Japan had insufficient knowledge of pharmacovigilance, 71.9% wished to acquire more.

Keywords: Pharmacovigilance, Pharmacist, Questionnaire

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Hanatani T, Sai K, Tohkin M*, Segawa K, Saito Y: Impact of Japanese regulatory action on metformin-associated lactic acidosis in type II diabetes patients. *Int J Clin Pharm.* 2015;37:537-45.

The impact of a regulatory action issued by the Japanese government in March 2012 regarding the risk of lactic acidosis in metformin treatment, including the high dose formulation (h-metformin), especially in the elderly, was assessed using a medical information database. The frequency of blood lactate measurements, and the rate of metformin prescriptions to the elderly were compared between the periods 1 year before and 1 year after the implementation of the regulatory action. The results showed that the regulatory action led to increased lactate measurement in the overall metformin users, but did not affect metformin prescription rate in the elderly patients. Our findings probably reflect the doctors' judgement that the benefits of metformin use outweigh the risk of lactic acidosis if lactate testing is performed regularly.

Keywords: Japanese regulatory action, Metformin, Lactic acidosis

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Takahashi H*1, Kaniwa N, Saito Y, Sai K, Hamaguchi T*2, Shirao K*2, Shimada Y*2, Matsumura Y*3, Ohtsu A*3, Yoshino T*3, Doi T*3, Takahashi A*4, Odaka Y*5, Okuyama M*5, Sawada J, Sakamoto H*5, Yoshida T*5: Construction of possible integrated predictive index based on EGFR and ANXA3 polymorphisms for chemotherapy response in fluoropyrimidine-treated Japanese gastric cancer patients using a bioinformatic method.
BMC Cancer. 2015;15:718.

We previously identified the SNP rs2293347 in the human epidermal growth factor receptor (EGFR) gene as a novel genetic factor related to fluoropyrimidine-chemotherapeutic response using a knowledge-based bioinformatic approach in which 119 fluoropyrimidine-treated gastric cancer patients were genotyped at 109,365 SNPs. In the present study, we reanalyzed the hypothesis-free genomic data using extended knowledge. We identified rs2867461 in annexin A3 (ANXA3) gene as another candidate. Logistic regression analysis showed that the performance of the rs2867461 + rs2293347 model was superior to those of the single factor models. The p value for a novel integrated predictive index (iEA) based on these two polymorphisms in EGFR and ANXA3 was 1.47 × 10(-8) by Fisher's exact test. These results suggest that the iEA index or a combination of polymorphisms in EGFR and ANXA3 may serve as predictive factors of drug response, and therefore could be useful for optimal selection of chemotherapy regimens.

Keywords: Single nucleotide polymorphisms, Genome-wide association study, Fluoropyrimidine

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Maekawa K, Nakamura R, Kaniwa N, Mizusawa S*1, Kitamoto A*1, Kitamoto T*1, Ukaji M, Matsuzawa Y, Sugiyama E, Uchida Y, Kurose K*2, Ueta M*3, Sotozono C*3, Ikeda H*4, Yagami A*5, Matsukura S*6, Kinoshita S*3, Muramatsu M*7, Ikezawa Z*6, Sekine A*1, Furuya H*8, Takahashi Y*4, Matsunaga K*5, Aihara M*6, Saito Y, JPDSC*9: Development of a simple genotyping method for the HLA-A*31:01-tagging SNP in Japanese.

Pharmacogenomics 2015;16:1689-99.

The aim of this study is to construct a simple, low-cost typing method for the surrogate marker of HLA-A*31:01, a risk factor for carbamazepine (CBZ)-related Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). DNAs from Japanese SJS/TEN patients was used for genotyping and developing the assay. HLA-A*31:01 was confirmed to be significantly associated with definite/probable cases of CBZ-related SJS/TEN (P = 0.0040). Three single nucleotide polymorphisms (SNPs), rs1150738, rs3869066 and rs259945, were in absolute linkage disequilibrium with HLA-A*31:01 in 210 Japanese SJS/TEN patients. Robust genotyping of rs3869066 in ZNRD1-AS1 was developed using polymerase chain reaction-restriction fragment length polymorphism assays. SNP genotyping is less time-consuming and cheaper than conventional HLA typing, and would be useful for identifying Japanese patients at risk of CBZ-related SJS/TEN.

Keywords: Carbamazepine, Stevens-Johnson syndrome/toxic epidermal necrolysis

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Ishikawa M, Saito K, Urata M, Kumagai Y*, Maekawa K, Saito Y: Comparison of circulating lipid profiles between fasting humans and three animal species used in preclinical studies: mice, rats and rabbits.

Lipids Health Dis. 2015;14:104.

Background: Circulating lipid metabolites are associated with many physiological and biological processes in the body, and therefore could be used as biomarkers for evaluating drug efficacy and safety in preclinical studies. However, differences in circulating lipid profiles among humans and animals often used in preclinical studies have not been fully investigated.

Methods: We performed lipidomic analysis to obtain circulating lipid profiles of fasted humans (Caucasian, n = 15) and three animal species used in preclinical studies (mice [BALB/c, n = 5], rats [Sprague-Dawley, n = 5], and rabbits [New Zealand White, n = 5]) by using liquid chromatography-mass spectrometry.

Results: Our data showed marked differences in lipid profiles among humans and these animal species. Furthermore, we observed that the levels of many lipid metabolites, such as poly-unsaturated fatty acid-containing cholesteryl esters, ether-type phosphoglycerolipids, and sulfatides, were significantly different ($p<0.05$) by more than 10-fold in these animals (depending on the animal species) from humans.

Conclusion: Our data could be useful while extrapolating the data on the biomarker candidates identified in preclinical studies into clinical studies.

Keywords: Preclinical studies, LC-MS, Circulating lipid metabolites

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Saito K, Uebanso T*1, Maekawa K, Ishikawa M, Taguchi R, Nammo T*1, Nishimaki-Mogami T, Udagawa H*1, Fujii M*2, Shibazaki Y*2, Yoneyama H*2, Yasuda K*1, Saito Y: Characterization of hepatic lipid profiles in a mouse model with nonalcoholic steatohepatitis and subsequent fibrosis.

Sci Rep. 2015;5:12466.

Nonalcoholic steatohepatitis (NASH) is a major health problem since it often leads to hepatocellular carcinoma. However, the underlying mechanisms of

NASH development and subsequent fibrosis have yet to be clarified. We compared comprehensive lipidomic profiles between mice with high fat diet (HFD)-induced steatosis and STAM mice with NASH and subsequent fibrosis. The STAM mouse is a model that demonstrates NASH progression resembling the disease in humans: STAM mice manifest NASH at 8 weeks, which progresses to fibrosis at 12 weeks, and finally develop hepatocellular carcinoma. Overall, 250 lipid molecules were detected in the liver using liquid chromatography-mass spectrometry. We found that STAM mice with NASH presented a significantly higher abundance of sphingolipids and lower levels of triacylglycerols than the HFD-fed control mice. The abundance of certain fatty acids in phospholipid side chains was also significantly different between STAM and control mice, although global levels of phosphatidylcholines and phosphatidylethanolamines were comparable. Finally, increase in levels of acylcarnitines and some diacylglycerols was observed in STAM mice toward the fibrosis stage, but not in age-matched control mice. Our study provides insights into the lipid status of the steatotic, NASH, and fibrotic liver that would help elucidate the molecular pathophysiology of NASH progression.

Keywords: Metabolomics, Lipid profile, Nonalcoholic fatty liver diseases

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Kaniwa N, Ueta M*1, Nakamura R, Okamoto-Uchida Y, Sugiyama E, Maekawa K, Takahashi Y*2, Furuya H*3, Yagami A*4, Matsukura S*5, Ikezawa Z*5, Matsunaga K*4, Sotozono C*1, Aihara M*5, Kinoshita S*1, Saito Y: Drugs causing severe ocular surface involvements in Japanese patients with Stevens-Johnson syndrome/toxic epidermal necrolysis.
Allergol Int. 2015;64:379-81.

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe cutaneous adverse drug reactions often affecting mucosal tissues like ocular surface. Drugs causing severe ocular surface involvements in Japanese patients with SJS/TEN were investigated. A total of 197 patients with SJS/TEN were enrolled. SJS/TEN patients who take acetaminophen show a significantly higher frequency of severe ocular surface disorders than patients taking other SJS/TEN frequently causative drugs such as carbamazepine, allopurinol, and quinolones. Patients taking antipyretic–analgesic medication, including acetaminophen and/or nonsteroidal anti-inflammatory drugs, for the treatment of common cold have a high frequency of SJS/TEN with severe ocular surface involvements compared with such medication taken for the treatment of other diseases. Our results suggest that not only cold medicines but also viral infections causing cold-like symptoms play some important roles in the development of severe ocular surface involvements.

Keywords: Drug-induced liver injury, Medical information database, Drug safety

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Saito Y, Stamp LK*1, Caudle KE*2, Hershfield MS*3, McDonagh EM*4, Callaghan JT*5, Tassaneeyakul W*6, Mushiroda T*7, Kamatani N*8, Goldspiel BR*9, Phillips EJ*10, Klein TE*4, Lee MT*7: Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for human leukocyte antigen B (HLA-B) genotype and allopurinol dosing: 2015 update.
Clin Pharmacol Ther. 2016;99:36-7

The Clinical Pharmacogenetics Implementation Consortium (CPIC) Guidelines for HLA-B*5801 Genotype and Allopurinol Dosing was originally published in February 2013. We reviewed the recent literature and concluded that none of the evidence would change the therapeutic recommendations in the original guideline; therefore, the original publication remains clinically current. However, we have updated the Supplemental Material and included additional resources for applying CPIC guidelines into the electronic health record. Up-to-date information can be found at PharmGKB (http://www.pharmgkb.org).

Keywords: Allopurinol, HLA, Severe cutaneous adverse reaction

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Juliandi B*1, Tanemura K*2, Igarashi K*3, Tominaga T*4, Furukawa Y, Otsuka M*2, Moriyama N, Ikegami D*3, Abematsu M*1, Sanosaka T*1, Tsujimura K*1, Narita M*3, Kanno J, Nakashima K*1: Reduced Adult Hippocampal Neurogenesis and Cognitive Impairments following Prenatal Treatment of the Antiepileptic Drug Valproic Acid.
Stem Cell Reports. 2015;5:996-1009.

Prenatal exposure to valproic acid (VPA), an established antiepileptic drug, has been reported to impair postnatal cognitive function in children born to VPA-treated epileptic mothers. However, how these defects arise and how they can be overcome remain unknown. Using mice, we found that comparable postnatal cognitive functional impairment is very likely correlated to the untimely enhancement of embryonic neurogenesis, which led to depletion of the neural precursor cell pool and consequently a decreased level of adult neurogenesis in the hippocampus. Moreover, hippocampal neurons in the offspring of VPA-treated mice showed abnormal morphology and activity. Surprisingly, these impairments could be ameliorated by voluntary running. Our study suggests that although prenatal exposure to antiepileptic drugs such as VPA may have detrimental effects that persist until adulthood, these effects may be offset by a simple physical activity such as running.

Keywords: valproic acid, hippocampal neurons, Embryonic Neurogenesis

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Ono R, Ishii M, Fujihara Y, Kitazawa M, Usami T, Kaneko-Ishino T, Kanno J, Ikawa M, Ishino F: Double strand break repair by capture of retrotransposon sequences and reverse-transcribed spliced mRNA sequences in mouse zygotes.
Sci Rep. 2015;5:12281.

The CRISPR/Cas system efficiently introduces double strand breaks (DSBs) at a genomic locus specified by a single guide RNA (sgRNA). The DSBs are subsequently repaired through non-homologous end joining (NHEJ) or homologous recombination (HR). Here, we demonstrate that DSBs introduced into mouse zygotes by the CRISPR/Cas system are repaired by the capture of DNA sequences deriving from retrotransposons, genomic DNA, mRNA and sgRNA. Among 93 mice analysed, 57 carried mutant alleles and 22 of them had long de novo insertion(s) at DSB-introduced sites; two were spliced mRNAs of Pcnt and Inadl without introns, indicating the involvement of reverse transcription (RT). Fifteen alleles included retrotransposons, mRNAs, and other sequences without evidence of RT. Two others were sgRNAs with one containing T7 promoter-derived sequence suggestive of a PCR product as its origin. In conclusion, RT-product-mediated DSB repair (RMDR) and non-RMDR repair were identified in the mouse zygote. We also confirmed that both RMDR and non-RMDR take place in CRISPR/Cas transfected NIH-3T3 cells. Finally, as two de novo MuERV-L insertions in C57BL/6 mice were shown to have characteristic features of RMDR in natural conditions, we hypothesize that RMDR contributes to the emergence of novel DNA sequences in the course of evolution.

Keywords: DSB, CRISPR/Cas, retrotransposon

Xu J*1,2, Alexander DB*1, Iigo M*1, Hamano H*3, Takahashi S*4, Yokoyama T*5, Kato M*5, Usami I*5, Tokuyama T*6, Tsutsumi M*7, Tamura M*8, Oguri T*9, Niimi A*9, Hayashi Y*10, Yokoyama Y*10, Tonegawa K*11, Fukamachi K*12, Futakuchi M*12, Sakai Y*12, Suzui M*12, Kamijima M*13, Hisanaga N*14, Omori T*15, Nakae D*16, Hirose A, Kanno J, Tsuda H*1: Chemokine (C-C motif) ligand 3 detection in the serum of persons exposed to asbestos.
A patient-based study. Cancer Sci. 2015;106:825-32.

Exposure to asbestos results in serious risk of developing lung and mesothelial diseases. Currently,

there are no biomarkers that can be used to diagnose asbestos exposure. The purpose of the present study was to determine whether the levels or detection rate of chemokine (C-C motif) ligand 3 (CCL3) in the serum are elevated in persons exposed to asbestos. The primary study group consisted of 76 healthy subjects not exposed to asbestos and 172 healthy subjects possibly exposed to asbestos. The secondary study group consisted of 535 subjects possibly exposed to asbestos and diagnosed with pleural plaque (412), benign hydrothorax (10), asbestosis (86), lung cancer (17), and malignant mesothelioma (10). All study subjects who were possibly exposed to asbestos had a certificate of asbestos exposure issued by the Japanese Ministry of Health, Labour and Welfare. For the primary study group, levels of serum CCL3 did not differ between the two groups. However, the detection rate of CCL3 in the serum of healthy subjects possibly exposed to asbestos (30.2%) was significantly higher ($P < 0.001$) than for the control group (6.6%). The pleural plaque, benign hydrothorax, asbestosis, and lung cancer groups had serum CCL3 levels and detection rates similar to that of healthy subjects possibly exposed to asbestos. The CCL3 chemokine was detected in the serum of 9 of the 10 patients diagnosed with malignant mesothelioma. Three of the patients with malignant mesothelioma had exceptionally high CCL3 levels. Malignant mesothelioma cells from four biopsy cases and an autopsy case were positive for CCL3, possibly identifying the source of the CCL3 in the three malignant mesothelioma patients with exceptionally high serum CCL3 levels. In conclusion, a significantly higher percentage of healthy persons possibly exposed to asbestos had detectable levels of serum CCL3 compared to healthy unexposed control subjects.

Keywords: asbestos, chemokine CCL3, mesothelioma

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Hirabayashi Y, Tsuboi I*1, Kuramoto K*2, Kusunoki Y*3, Inoue T: Cell cycle of primitive hematopoietic progenitors decelerated in senescent mice is reactively accelerated after 2-Gy whole-body irradiation.

Exp Biol Med (Maywood). 2016;241:485-92.

Aging is considered to be a functional retardation of continuous xenobiotic responses over a lifetime after the developmental period; thus, the effects of ionizing radiation over a lifetime may be somewhat accounted for by a modifier of aging effects. This study was conducted to evaluate the possible/synergic effects of radiation during aging by determining cell-cycle parameters of hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs), such as the percent of cells in cycling, the generation doubling time, and the cumulative cycling-cell fraction, by bromodeoxyuridine-ultraviolet assay, which enables the determination of their cycling capacity in vivo. Colony-forming progenitor cells, such as colony-forming unit (CFU)-granulocyte/macrophage (GM), CFU in the spleen on

day 9 (CFU-S9), and CFU-S on day 13 (CFU-S13) for mature, less mature, and immature HPCs, respectively, were evaluated in young and old mice (6 weeks and 21 months of age, respectively) with or without 2-Gy whole-body irradiation and a 4-week recovery period. Then, cell-cycle parameters were evaluated and compared. As a result, the generation doubling time of all types of HPC was prolonged by the irradiation in both young and old mouse groups, except that of CFU-S13 in old mice, which showed acceleration of the cell cycle following the irradiation. In addition, only CFU-S13 in irradiated old mice showed a significant increase in the cumulative cycling-cell-fraction ratio. Significant changes due to the effects of aging and irradiation on HPCs were observed only in the immature HPCs, i.e., the cell cycle of immature HPCs was suppressed by aging without irradiation and was, in contrast, accelerated as the cells recovered from radiation-induced damage. This suggests that the mechanisms of peripheral blood recovery after 2-Gy whole-body irradiation are markedly different between young and old mice, although 21-month-old mice showed almost the same level of recovery as the young mice.

Keywords: Whole-body irradiation, cell cycle, senescence

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Seed TM*1, Xiao S*2, Manley N*2, Nikolich-Zugich J*3, Pugh J*3, Van den Brink M*4, Hirabayashi Y, Yasutomo K*5, Iwama A*6, Koyasu S*7, Shterev I*8, Sempowski G*8, Macchiarini F*9, Nakachi K*10, Kunugi KC*11, Hammer CG*11, Dewerd LA*11: An interlaboratory comparison of dosimetry for a multi-institutional radiobiological research project: Observations, problems, solutions and lessons learned. *Int J Radiat Biol.* 2016;92:59-70

PURPOSE: An interlaboratory comparison of radiation dosimetry was conducted to determine the accuracy of doses being used experimentally for animal exposures within a large multi-institutional research project. The background and approach to this effort are described and discussed in terms of basic findings, problems and solutions. METHODS: Dosimetry tests were carried out utilizing optically stimulated luminescence (OSL) dosimeters embedded midline into mouse carcasses and thermal luminescence dosimeters (TLD) embedded midline into acrylic phantoms. RESULTS: The effort demonstrated that the majority (4/7) of the laboratories was able to deliver sufficiently accurate exposures having maximum dosing errors of </=5%. Comparable rates of 'dosimetric compliance' were noted between OSL- and TLD-based tests. Data analysis showed a highly linear relationship between 'measured' and 'target' doses, with errors falling largely between 0 and 20%. Outliers were most notable for OSL-based tests, while multiple tests by 'non-compliant' laboratories using orthovoltage X-rays contributed heavily to the wide variation in dosing errors. CONCLUSIONS: For the dosimetrically non-compliant laboratories, the relatively high rates of dosing errors were problematic, potentially compromising the quality of ongoing radiobiological research. This dosimetry effort proved to be instructive in establishing rigorous reviews of basic dosimetry protocols ensuring that dosing errors were minimized.

Keywords: Dosimetry, dose-response curve, ionizing radiation

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Fujimoto N*, Kanno J: Increase in prostate stem cell antigen expression in prostatic hyperplasia induced by testosterone and 17β-estradiol in C57BL mice. *J Steroid Biochem Mol Biol.* 2016;158:56-62.

Estradiol (E2) is known to act synergistically with testosterone (T) for the development of prostatic hyperplasia in rats and dogs, but murine prostate is less responsive to hormonal stimulation. However, a recent study revealed that the combined administration of E2 and T induced prostatic hyperplasia with bladder

outlet obstruction in C57BL mice. To understand the mechanisms underlying the hormonal induction of prostatic hyperplasia, the expression of growth factors and their receptors, androgen receptor, estrogen receptor (ER), and prostatic secretory proteins was investigated. Ten-week-old male C57BL mice were treated with T (30mg) or T+E2 (0.5mg) for 10 weeks, and prostatic lobes were dissected and subjected to quantitative RT-PCR and immunoblotting analysis. T administration appeared to induce glandular prostatic growth, while with T+E2 administration this growth was greater and accompanied by extreme bladder enlargement. The expression of prostate stem cell antigen (PSCA) mRNA and protein was increased in prostate tissue in the T group. The combined administration of E2 with T prominently enhanced PSCA expression, along with increased insulin growth factor 1 mRNA levels and decreased estrogen receptor β mRNA expression. The synergistic effect of E2 on the expression of PSCA suggests that this protein may play an important role in the hormone-induced development of prostatic hyperplasia.

Keywords: Estradiol, Prostate stem cell antigen, Testosterone

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Nakamura H*[1], Yamashita N*[1], Kanamaru Y*[2], Tachibana T*[1], Sekino Y, Chen S*[1], Gotoh T*[2], Tanaka F*[1], Goshima Y*[1]: Quantitative analysis of intraneuronal transport in human iPS neurons.
J Pharmacol Sci. 2015;128:170-8.

Induced pluripotent stem (iPS) cells are promising tools to investigate disease mechanism and develop new drugs. Intraneuronal transport, which is fundamental for neuronal survival and function, is vulnerable to various pharmacological and chemical agents and is disrupted in some neurodegenerative disorders. We applied a quantification method for axonal transport by counting CM-DiI-labeled particles traveling along the neurite, which allowed us to monitor and quantitate, for the first time, intraneuronal transport in human neurons differentiated from iPS cells (iCell neurons). We evaluated the acute effects of several anti-neoplastic agents that have been previously shown to affect intraneuronal transport. Vincristine, paclitaxel and oxaliplatin decreased the number of moving particle along neurites. Cisplatin, however, produced no effect on intraneuronal transport, which is in contrast to our previous report indicating that it inhibits transport in chick dorsal root ganglion neurons. Our system may be a useful method for assessing intraneuronal transport and neurotoxicity in human iPS neurons.

Keywords: Anti-neoplastic agents, Neurotoxicity, iPS cell

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Matsuo J*, Nakamura Y*, Izumi-Nakaseko H*, Ando K*, Sekino Y, Sugiyama A*: Possible effects of inhibition of IKr and IKs on field-potential waveforms in the human iPS cell-derived cardiomyocytes sheet.
J Pharmacol Sci. 2015;128:92-5.

In order to investigate how IKr and IKs inhibitions affect waveforms of the field potential in the human iPS cell-derived cardiomyocytes sheet, we analyzed the effects of E-4031 and chromanol 293B on the maximum upslope and peak amplitude of its second wave (n = 7 for each drug). E-4031 in 10-100 nM as well as chromanol 293B in 3-30 μM prolonged the field-potential duration, whereas E-4031 decreased the upslope in 10-100 nM and amplitude at 100 nM, which was not observed by chromanol 293B. Thus, the decrease of the upslope can be used as a supplemental marker of drug-induced IKr inhibition.

Keywords: Field potential, IKr, iPS-derived cardiomyocytes

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Irie T, Kikura-Hanajiri R, Usami M, Uchiyama N, Goda Y, Sekino Y: MAM-2201, a synthetic cannabinoid drug of abuse, suppresses the synaptic input to cerebellar Purkinje cells via activation of presynaptic CB1 receptors.
Neuropharmacology 2015;95:479-91.

Herbal products containing synthetic cannabinoids-initially sold as legal alternatives to marijuana-have become major drugs of abuse. Among the synthetic cannabinoids, [1-(5-fluoropentyl)-1H-indol-3-yl](4-methyl-1-naphthalenyl)-methanone (MAM-2201)

has been recently detected in herbal products and has psychoactive and intoxicating effects in humans, suggesting that MAM-2201 alters brain function. Nevertheless, the pharmacological actions of MAM-2201 on cannabinoid receptor type 1 (CB1R) and neuronal functions have not been elucidated. We found that MAM-2201 acted as an agonist of human CB1Rs expressed in AtT-20 cells. In whole-cell patch-clamp recordings made from Purkinje cells (PCs) in slice preparations of the mouse cerebellum, we also found that MAM-2201 inhibited glutamate release at parallel fiber-PC synapses via activation of presynaptic CB1Rs. MAM-2201 inhibited neurotransmitter release with an inhibitory concentration 50% of 0.36 μM. MAM-2201 caused greater inhibition of neurotransmitter release than Δ(9)-tetrahydrocannabinol within the range of 0.1-30 μM and JWH-018, one of the most popular and potent synthetic cannabinoids detected in the herbal products, within the range of 0.03-3 μM. MAM-2201 caused a concentration-dependent suppression of GABA release onto PCs. Furthermore, MAM-2201 induced suppression of glutamate release at climbing fiber-PC synapses, leading to reduced dendritic Ca(2+) transients in PCs. These results suggest that MAM-2201 is likely to suppress neurotransmitter release at CB1R-expressing synapses in humans. The reduction of neurotransmitter release from CB1R-containing synapses could contribute to some of the symptoms of synthetic cannabinoid intoxication including impairments in cerebellum-dependent motor coordination and motor learning.

Keywords: Cannabinoid receptor type 1, Cerebellum, Neurotransmitter release

OharaY, Koganezawa N*, Yamazaki H*, Roppongi RT*, Sato K, Sekino Y, Shirao T*: Early-stage development of human induced pluripotent stem cell (hiPSC)-derived neurons.

J Neurosci Res. 2015;93:1804-13.

Recent advances in human induced pluripotent stem cells (hiPSCs) offer new possibilities for biomedical research and clinical applications. Differentiated neurons from hiPSCs are expected to be useful for developing novel methods of treatment for various neurological diseases. However, the detailed process of functional maturation of hiPSC-derived neurons (hiPS neurons) remains poorly understood. This study analyzes development of hiPS neurons, focusing specifically on early developmental stages through 48 hr after cell seeding; development was compared with that of primary cultured neurons derived from the rat hippocampus. At 5 hr after cell seeding, neurite formation occurs in a similar manner in both neuronal populations. However, very few neurons with axonal polarization were observed in the hiPS neurons even after 48 hr, indicating that hiPS neurons differentiate more slowly than rat neurons. We further investigated the elongation speed of axons and found that hiPS neuronal axons were slower. In addition, we characterized the growth cones. The localization patterns of skeletal proteins F-actin, microtubule, and drebrin were similar to those of rat neurons, and actin depolymerization by cytochalasin D induced similar changes in cytoskeletal distribution in the growth cones between hiPS neurons and rat neurons. These results indicate that, during the very early developmental stage, hiPS neurons develop comparably to rat hippocampal neurons with regard to axonal differentiation, but the growth of axons is slower.

Keywords: axonal development, cytoskeletal proteins, growth cones

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Gyobu S*1, Miyata H*1, Ikawa M*1, Yamazaki D, Takeshima H*2, Suzuki J*1, Nagata S*1: A Role of TMEM16E Carrying a Scrambling Domain in Sperm Motility.

Mol Cell Biol. 2015;36:645-59.

Transmembrane protein 16E (TMEM16E) belongs to the TMEM16 family of proteins that have 10 transmembrane regions and appears to localize intracellularly. Although TMEM16E mutations cause bone fragility and muscular dystrophy in humans, its biochemical function is unknown. In the TMEM16 family, TMEM16A and -16B serve as Ca(2+)-dependent Cl(-) channels, while TMEM16C, -16D, -16F, -16G, and -16J support Ca(2+)-dependent phospholipid scrambling. Here, we show that TMEM16E carries a segment composed of 35 amino acids homologous to the scrambling domain in TMEM16F. When the corresponding segment of TMEM16A was replaced by this 35-amino-acid segment of TMEM16E, the chimeric molecule localized to the plasma membrane

and supported Ca(2+)-dependent scrambling. We next established TMEM16E-deficient mice, which appeared to have normal skeletal muscle. However, fertility was decreased in the males. We found that TMEM16E was expressed in germ cells in early spermatogenesis and thereafter and localized to sperm tail. TMEM16E(-/-) sperm showed no apparent defect in morphology, beating, mitochondrial function, capacitation, or binding to zona pellucida. However, they showed reduced motility and inefficient fertilization of cumulus-free but zona-intact eggs in vitro. Our results suggest that TMEM16E may function as a phospholipid scramblase at inner membranes and that its defect affects sperm motility.

Keywords: TMEM16E, Scramble, Sperm

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Hirata N, Yamada S, Asanagi M, Sekino Y, Kanda Y: Nicotine induces mitochondrial fission through mitofusin degradation in human multipotent embryonic carcinoma cells.

Biochem Biophys Res Commun. 2016;470:300-5.

Nicotine is considered to contribute to the health risks associated with cigarette smoking. Nicotine exerts its cellular functions by acting on nicotinic acetylcholine receptors (nAChRs), and adversely affects normal embryonic development. However, nicotine toxicity has not been elucidated inhuman embryonic stage. In the present study, we examined the cytotoxic effects of nicotine in human multipotent embryonal carcinoma cell line NT2/D1. We found that exposure to 10 μM nicotine decreased intracellular ATP levels and inhibited proliferation of NT2/D1 cells. Because nicotinesuppressed energy production, which is a critical mitochondrial function, we further assessed the effects of nicotine on mitochondrial dynamics. Staining with MitoTracker revealed that 10 μM nicotine induced mitochondrial fragmentation. The levels of the mitochondrial fusion proteins, mitofusins 1 and 2, were also reduced in cells exposed to nicotine. These nicotine effects were blocked by treatment with mecamylamine, a nonselective nAChR antagonist. These data suggest that nicotine degrades mitofusin in NT2/D1 cells and thus induces mitochondrial dysfunction and cell growth inhibition in a nAChR-dependent manner. Thus, mitochondrial function in embryonic cells could be used to assess the developmental toxicity of chemicals.

Keywords: Embryonic cells, Mitochondrial fission, Mitofusin

Asakura K*1, Hayashi S*2, Ojima A*3, Taniguchi T*4, Miyamoto N*4, Nakamori C*5, Nagasawa C*5, Kitamura T*6, Osada T*7, Honnda Y*8, Kasai C*9, Ando H*10, Kanda Y, Sekino Y, Sawada K*11:

Improvement of acquisition and analysis methods in multi-electrode array experiments with iPS cell-derived cardiomyocyte.

J Pharmacol Toxicol Methods. 2015;75:17-26.

INTRODUCTION: Multi-electrode array (MEA) systems and human induced pluripotent stem (iPS) cell-derived cardiomyocytes are frequently used to characterize the electrophysiological effects of drug candidates for the prediction of QT prolongation and proarrhythmic potential. However, the optimal experimental conditions for obtaining reliable experimental data, such as high-pass filter (HPF) frequency and cell plating density, remain to be determined.

METHODS: Extracellular field potentials (FPs) were recorded from iPS cell-derived cardiomyocyte sheets by using the MED64 and MEA2100 multi-electrode array systems. Effects of HPF frequency (0.1 or 1Hz) on FP duration (FPD) were assessed in the presence and absence of moxifloxacin, terfenadine, and aspirin. The influence of cell density on FP characteristics recorded through a 0.1-Hz HPF was examined. The relationship between FP and action potential (AP) was elucidated by simultaneous recording of FP and AP using a membrane potential dye.

RESULTS: Many of the FP waveforms recorded through a 1-Hz HPF were markedly deformed and appeared differentiated compared with those recorded through a 0.1-Hz HPF. The concentration-response curves for FPD in the presence of terfenadine reached a steady state at concentrations of 0.1 and 0.3 μM when a 0.1-Hz HPF was used. In contrast, FPD decreased at a concentration of 0.3 μM with a characteristic bell-shaped concentration-response curve when a 1-Hz HPF was used. The amplitude of the first and second peaks in the FP waveform increased with increasing cell plating density. The second peak of the FP waveform roughly coincided with AP signal at 50% repolarization,

and the negative deflection at the second peak of the FP waveform in the presence of E-4031 corresponded to early afterdepolarization and triggered activity.

DISCUSSION: FP can be used to assess the QT prolongation and proarrhythmic potential of drug candidates; however, experimental conditions such as HPF frequency are important for obtaining reliable data.Keyword : Field potential, Human induced pluripotent stem cell-derived cardiomyocytes, Membrane potential dye.

Keywords: Field potential, Human induced pluripotent stem cell-derived cardiomyocytes, Multi-electrode array

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Yamada S, Kotake Y, Nakano M, Sekino Y, Kanda Y: Tributyltin induces mitochondrial fission through NAD-IDH dependent mitofusin degradation in human embryonic carcinoma cells.
Metallomics 2015;7:1240-6.

Organotin compounds, such as tributyltin (TBT), are well-known endocrine disruptors. TBT acts at the nanomolar level through genomic pathways via the peroxisome proliferator activated receptor (PPAR)/retinoid X receptor (RXR). We recently reported that TBT inhibits cell growth and the ATP content in the human embryonic carcinoma cell line NT2/D1 via a non-genomic pathway involving NAD(+)-dependent isocitrate dehydrogenase (NAD-IDH), which metabolizes isocitrate to α-ketoglutarate. However, the molecular mechanisms by which NAD-IDH mediates TBT toxicity remain unclear. In the present study, we evaluated the effects of TBT on mitochondrial NAD-IDH and energy production. Staining with MitoTracker revealed that nanomolar TBT levels induced mitochondrial fragmentation. TBT also degraded the mitochondrial fusion proteins, mitofusins 1 and 2. Interestingly, apigenin, an inhibitor of NAD-IDH, mimicked the effects of TBT. Incubation with an α-ketoglutarate analogue partially recovered TBT-induced mitochondrial dysfunction, supporting the involvement of NAD-IDH. Our data suggest that nanomolar TBT levels impair mitochondrial quality control via NAD-IDH in NT2/D1 cells. Thus, mitochondrial function in embryonic cells could be used to assess cytotoxicity associated with metal exposure.

Keywords: TBT, Mitochondrial dynamics, Embryonic carcinoma

Kubo T, Kuroda Y, Horiuchi S, Kim SR, Sekino Y, Ishida S: Upregulations of metallothionein gene expressions and tolerance to heavy metal toxicity by three dimensional cultivation of HepG2 cells on VECELL 3-D inserts.
J Toxicol Sci. 2016;41:147-53.

The VECELL 3-D insert is a new culture scaffold consisting of collagen-coated ePTFE (expanded polytetrafluoroethylene) mesh. We analyzed the effects of VECELL 3-D inserts on the functionality of HepG2, a human hepatocellular carcinoma cell line. HepG2 cells cultured on VECELL 3-D inserts maintained a round shape, while those cultured on a standard culture plate or collagen-coated cell culture plate showed a flattened and cubic epithelial-like shape. HepG2 cells cultured on VECELL 3-D inserts had showed upregulated expression of metallothionein genes and in turn a higher tolerance to toxicity induced by heavy metals. These results suggest that HepG2 cell functions were changed by the cell morphology that is induced by culturing on a VECELL 3-D insert.

Keywords: VECELL 3-D insert, cell function change, metallothionein

Usami M, Mitsunaga K*1, Miyajima A, Takamatu M*2, Kazama S*2, Irie T, Doi O*3, Takizawa T*2: Effects of 13 developmentally toxic chemicals on the migration of rat cephalic neural crest cells in vitro.
Congenit Anom (Kyoto). 2016;56:52-9.

The inhibition of neural crest cell (NCC) migration has been considered as a possible pathogenic mechanism underlying chemical developmental toxicity. In this study, we examined the effects of 13 developmentally toxic chemicals on the migration of rat cephalic NCCs (cNCCs) by using a simple in vitro assay. cNCCs were cultured for 48 h as emigrants from rhombencephalic neural tubes explanted from rat embryos at day 10.5 of gestation. The chemicals were added to the culture medium at 24 h of culture. Migration of cNCCs was measured as the change in the radius (radius ratio) calculated from the circular spread of cNCCs between 24 and 48 h of culture. Of the chemicals examined, 13-cis-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate inhibited the migration of cNCCs at their embryotoxic concentrations; no effects were observed for acetaminophen, caffeine, indium, phenytoin, selenite, tributyltin, and valproic acid. In a cNCC proliferation assay, ethanol, ibuprofen, salicylic acid, selenate, and tributyltin inhibited cell proliferation, suggesting the contribution of the reduced cell number to the inhibited migration of cNCCs. It was determined that several developmentally toxic chemicals inhibited the migration of cNCCs, the effects of which were manifested as various craniofacial abnormalities.

Keywords: Developmental toxicity, Migration assay, Neural crest cell

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Kijima A, Ishii Y, Takasu S, Matsushita K, Kuroda K, Hibi D, Suzuki Y, Nohmi T, Umemura T: Chemical structure-related mechanisms underlying *in vivo* genotoxicity induced by nitrofurantoin and its constituent moieties in *gpt* delta rats.
Toxicology 2015;331:125-35.

Nitrofurans are antimicrobial compounds containing a nitro group at the 5-position of the furan ring and an amine or hydrazide side chain derivative. One member of the nitrofurans, nitrofurantoin (NFT), is a renal carcinogen in male rats despite its still controversial genotoxicity. We investigated chemical structure-related modes of action of NFT, and reporter gene mutation assays for NFT and its constituent moieties were performed. NFT, 5-nitro-2-furaldehyde (NFA), or 1-aminohydantoin (AHD) was administered to male F344 *gpt* delta rats by gavage for 4 or 13 weeks at a carcinogenic or the maximum tolerated dose. NFT caused a significant increase in *gpt* mutant frequency (MF) at 13 weeks with G-base substitution mutations. An increase in *gpt* MF was also observed in the NFA-treated group at 13 weeks, but not in the AHD-treated group. 8-Hydroxydeoxyguanosine (8-OHdG) levels in the kidney DNA of NFT-treated rats were significantly increased after 4 weeks. NFT caused accumulation of hyaline droplets indicated by positive immunostaining and western blot analysis for α2u-globulin in the proximal tubules. An additional study, in which female *gpt* delta rats were given NFT at the same dose used for males, was performed to mitigate the effect of α2u-globulin. NFT exerted the same effects on female rat kidneys to the same extent as males in terms of *gpt* MF and 8-OHdG level. Thus, it is highly probable that the structure of the nitro furan plays a key role in NFT-induced genotoxicity and genotoxic mechanisms including oxidative DNA damage are involved in NFT-induced renal carcinogenesis. α2u-globulin-mediated nephropathy may be a prerequisite for NFT-induced renal carcinogenesis in male rats, and additionally NFT could be a latent carcinogen in female rats and other animal species.

Keywords: *in vivo* mutagenicity, nitrofurantoin, *gpt* delta rat

Akagi J, Toyoda T, Cho YM, Mizuta Y, Nohmi T, Nishikawa A, Ogawa K: Validation study of the combined repeated-dose toxicity and genotoxicity assay using *gpt* delta rats.
Cancer Sci. 2015;106:529-41.

Transgenic rodents carrying reporter genes to detect organ-specific *in vivo* genetic alterations are useful for risk assessment of genotoxicity that causes cancer. Thus, the Organization for Economic Co-operation and Development (OECD) has established the guideline for genotoxicity tests using transgenic animals, which may be combined with repeated-dose toxicity studies. Here, we provide evidence to support equivalence of *gpt* delta and wild-type (WT) rats in terms of toxicological responses to a genotoxic hepatocarcinogen, diethylnitrosamine (DEN), and a nongenotoxic hepatocarcinogen, di(2-ethylhexyl)

phthalate (DEHP). DEHP-treated *gpt* delta rats showed similar increases in liver and kidney weights, serum albumin, albumin/globulin (A/G) ratios, and incidence of diffuse hepatocyte hypertrophy compared to WT F344 and SD rats. DEN-treated *gpt* delta rats showed equivalent increases in the number and area of precancerous GST-P-positive foci in the liver compared to WT rats. The livers of DEN-treated *gpt* delta rats also showed increased frequencies of *gpt* and Spi$^-$ mutations; such changes were not observed in DEHP-treated *gpt* delta rats. These results indicated that *gpt* delta rats (both F344 and SD backgrounds) displayed comparable DEHP-induced toxicity and DEN-induced genotoxicity as those observed in WT rats. With regard to the administration period, the general toxicity of 1.2% DEHP was evident throughout the experimental period, and the genotoxicity of 10 ppm DEN could be detected a 2-week of administration and further increased at 4-week. These results suggested that combined assays using *gpt* delta rats could detect both general toxicity and genotoxicity by canonical 4-week administration protocol; therefore it would be applicable for risk assessment and then ultimately serve to reduce cancer risks of human being from environmental chemicals.

Keywords: genotoxicity, *gpt* delta rat, reduction of animal use

Onami S, Cho YM, Toyoda T, Akagi J, Fujiwara S*, Ochiai R*, Tsujino K*, Nishikawa A, Ogawa K: Orally administered glycidol and its fatty acid esters as well as 3-MCPD fatty acid esters are metabolized to 3-MCPD in the F344 rat.

Regul Toxicol Pharmacol. 2015;73:726-31.

IARC has classified glycidol and 3-monochloropropane-1,2-diol (3-MCPD) as group 2A and 2B, respectively. Their esters are generated in foodstuffs during processing and there are concerns that they may be hydrolyzed to the carcinogenic forms *in vivo*. Thus, we conducted two studies. In the first, we administered glycidol and 3-MCPD and associated esters (glycidol oleate: GO, glycidol linoleate: GL, 3-MCPD dipalmitate: CDP, 3-MCPD monopalmitate: CMP, 3-MCPD dioleate: CDO) to male F344 rats by single oral gavage. After 30 minutes, 3-MCPD was detected in serum from all groups. Glycidol was detected in serum from the rats given glycidol or GL and CDP and CDO in serum from rats given these compounds. In the second, we examined if metabolism occurs on simple reaction with rat intestinal contents (gastric, duodenal and cecal contents) from male F344 *gpt* delta rats. Newly produced 3-MCPD was detected in all gut contents incubated with the three 3-MCPD fatty acid esters and in gastric and duodenal contents incubated with glycidol and in duodenal and cecal contents incubated with GO. Although our observation was performed at 1 time point, the results showed that not only 3-MCPD esters but also glycidol and glycidol esters are metabolized into 3-MCPD in the rat.

Keywords: glycidol, 3-MCPD, fatty acid ester

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Goto K*, Ogawa K: Lanthanum deposition is frequently observed in the gastric mucosa of dialysis patients with lanthanum carbonate therapy: a clinicopathologic study of 13 cases, including 1 case of lanthanum granuloma in the colon and 2 nongranulomatous gastric cases.

Int J Surg Pathol. 2016;24:89-92.

Lanthanum carbonate (LC) has been used as a phosphate binder agent for treating hyperphosphatemia in dialysis patients since 2005 in the United States and 2009 in Japan. Owing to its reported safety profile and tolerability, the use of LC is increasing. However, 4 articles regarding lanthanum deposition in the gastroduodenal mucosa have recently been published, 2-5 including 1 study wherein abdominal computed tomography revealed a high-density area corresponding to lanthanum deposition in the gastric mucosa in 42 (60%) of 70 patients treated with LC. To obtain a more precise incidence rate of lanthanum deposition based on pathologic investigation, we surveyed 153 pathological specimens of the digestive tract, which were biopsied or resected from 103 dialysis patients, including 19 patients treated with LC, from May 2009 to May 2015 in a single institute (Kainan Hospital, Japan). In this article, we present our clinicopathologic survey data in these patients, including 1 case with lanthanum deposition in the colon and 2 nongranulomatous cases.

Keywords: lanthanum, granuloma, dialysis

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Toyoda T, Cho YM, Akagi J, Mizuta Y, Hirata T, Nishikawa A, Ogawa K: Early detection of genotoxic urinary bladder carcinogens by immunohistochemistry for γ-H2AX.

Toxicol Sci. 2015;148:400-8.

DNA double-strand breaks (DSBs) induced by exposure to genotoxic agents are known to cause genome instability and cancer development. To evaluate the applicability of γ-H2AX, a sensitive marker of DSBs, in the early detection of genotoxicity and carcinogenicity of chemicals using animal models, we examined γ-H2AX expression in urinary bladders of rats. Six-week-old male F344 rats were orally treated for 4 weeks with a total of 12 chemicals divided into 4 categories based on genotoxicity and carcinogenicity in the urinary bladder. Animals were sacrificed at the end of administration or after 2 weeks of recovery, and immunohistochemistry for γ-H2AX was performed. At week 4, γ-H2AX expression in bladder epithelial cells was significantly increased by all 4 genotoxic bladder carcinogens as compared with the controls, while the three chemicals that were genotoxic but not carcinogenic in the bladders did not cause upregulation of γ-H2AX. After the recovery period, γ-H2AX expression was markedly reduced in all groups but remained significantly elevated in rats treated with 3 of the 4 genotoxic bladder carcinogens. Although slight increases in γ-H2AX expression were induced by a weak bladder carcinogen with equivocal genotoxicity (phenethyl isothiocyanate) and 2 nongenotoxic bladder carcinogens (melamine and uracil) at week 4, these differences were not significant and were thought to be associated with activated proliferation by urothelial hyperplasia, as demonstrated by increased Ki67-positive cells. These results suggested that γ-H2AX may be a potential biomarker for the early detection of genotoxic bladder carcinogens.

Keywords: urinary bladder, γ-H2AX, genotoxicity

Tsaalbi-Shtylik A[*1], Ferrás C[*1], Pauw B[*1], Hendriks G[*1], Temviriyanukul P[*1], Carlée L[*1], Calléja F[*1], van Hees S[*1], Akagi J, Iwai S[*2], Hanaoka F[*3], Jansen JG[*1], de Wind N[*1]: Excision of translesion synthesis errors orchestrates responses to helix-distorting DNA lesions.

J Cell Biol. 2015;209:33-46.

In addition to correcting mispaired nucleotides, DNA mismatch repair (MMR) proteins have been implicated in mutagenic, cell cycle, and apoptotic responses to agents that induce structurally aberrant nucleotide lesions. Here, we investigated the mechanistic basis for these responses by exposing cell lines with single or combined genetic defects in nucleotide excision repair (NER), postreplicative translesion synthesis (TLS), and MMR to low-dose ultraviolet light during S phase. Our data reveal that the MMR heterodimer Msh2/Msh6 mediates the excision of incorrect nucleotides that are incorporated by TLS opposite helix-distorting, noninstructive DNA photolesions. The resulting single-stranded DNA patches induce canonical Rpa-Atr-Chk1-mediated checkpoints and, in the next cell cycle, collapse to double-stranded DNA breaks that trigger apoptosis. In conclusion, a novel MMR-related DNA excision repair pathway controls TLS a posteriori, while initiating cellular responses to environmentally relevant densities of genotoxic lesions. These results may provide a rationale for the colorectal cancer tropism in Lynch syndrome, which is caused by inherited MMR gene defects.

Keywords: DNA repair, translesion synthesis, DNA mismatch repair

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Inoue K, Morikawa T, Takahashi M, Yoshida M, Ogawa K: Obstructive nephropathy induced with DL-potassium hydrogen tartrate in F344 rats.

J Toxicol Pathol. 2015;28:89-97.

We experienced obstructive nephropathy in F344 rats treated with DL-potassium hydrogen tartrate (PHT) in a 13-week oral repeated dose toxicity study. Six-week-old male and female F344/DuCrj rats were fed a diet containing up to 2.0% PHT for 13 weeks. Microscopical findings including irregular dilation of the distal tubule lumen, foreign body giant cells, inflammatory cell infiltration, and regeneration of renal tubules were observed focally or multifocally in the renal cortex and/or medulla in the 0.5% and higher dosage groups of both sexes. The severity of these lesions increased in a dose-dependent manner. In the urinalysis, an increase in protein and white blood cells or the concentration of tartaric acid was detected in

the 0.5% PHT and higher dosage groups of both sexes or males, respectively, though conventional blood biochemical analysis did not indicate failure of renal function. These results indicate that the PHT induces obstructive nephropathy in rats. There were no other treatment-related changes in other organs.

Keywords: obstructive nephropathy, kidney, DL-potassium hydrogen tartrate

Yoshida M, Inoue K, Takahashi M: Predictive modes of action of pesticides in uterine adenocarcinoma development in rats.

J Toxicol Pathol. 2015;28:207-16.

Endometrial adenocarcinoma in the uterine corpus is a malignant cancer that occurs in menopausal women and aged rodents. Because of the similarities in pathogenesis and morphology of endometrial adenocarcinoma in rodents and humans, prediction of the modes of action (MOA) in uterine carcinogenesis is important for extrapolation of rodent data to humans. Three MOAs have been accepted as major pathways for uterine carcinogenesis in rodents: 1) estrogenic activity, 2) increased serum 17beta-estradiiol (E2) to progesterone (P4) ratio and 3) modulation of estrogen metabolism to produce 4-hydroxyestradiol via P450 induction. Inhibition of estrogen excretion and increased aromatase in situ in the tumor are also a potential pathway. Here, chemicals showing uterine carcinogenicity were chosen from approximately 300 pesticides evaluated in Japan within the past decade, and their mechanisms were predicted using parameters from mechanistic and toxicity studies. Seven pesticides increased uterine tumor formation in rats, and the pathways of 4 pesticides could be predicted based on various mechanistic studies. The MOAs of cyenopyrafen and benthiavalicarb-isopropyl were predicted to be modulation of estrogen metabolism, while those of pyriminobac-methyl and spirodiclofen were predicted to be increased E2 to P4 ratio. The driven pathways of metazosulfuron and isopyrazam could not be predicted using several mechanistic studies. No mechanistic studies have been reported for sedaxane, which has a chemical structure and toxicological profile similar to isopyrazam. Our results indicated that appropriate mechanistic studies are useful for mechanism prediction in risk assessment. From this analysis, a flowchart showing a decision tree for predictive MOAs in uterine carcinogenesis was proposed.

Keywords: uterine carcinogenesis, prediction, pesticide

Shirota M*, Kawashima J*, Nakamura T*, Kamiie J*, Shirota K*, Yoshida M: Dose-dependent acceleration in the delayed effects of neonatal oral exposure to low-dose 17α-ethynylestradiol on reproductive functions in female Sprague-Dawley rats.

J Toxicol Sci. 2015;40:727-38.

Xenoestrogen exposure during the critical period of sexual differentiation of the brain causes delayed effects on female reproduction. We investigated the internal dose of orally administered ethynylestradiol (EE) during the critical period and its delayed effects by administering 0 (vehicle control), 0.4, or 2 μg/kg EE to female Sprague-Dawley rats for 5 days from postnatal day (PND) 1. Determination of serum EE level 24 hr after the initial dosing and 6 and 24 hr after the final dosing of 2 μg/kg indicated that the administered EE entered the circulation and cleared after every administration. Although the treatment did not affect physical development, including growth, eyelid opening, and vaginal opening, the estrous cycle was arrested from postnatal week (PNW) 12 even with 0.4 μg/kg EE, with an inverse correlation between doses and arresting ages. Although ovarian morphology at PNW 22-23 indicated that the treatment caused long-term anovulation and cystic follicle formation, the number of primordial follicles at PNW 22-23 was similar among the groups. Because this number was lower than that at PND 10 in all groups, primordial follicles may have been consumed under long-term anovulation. The treatment also caused other abnormalities, including mammary gland hyperplasia, increase in pituitary and liver weights, and decrease in the uterine weight. Because the highest circulating EE level in the 2 μg/kg-treated neonates is considered to be comparable to the physiological range of estradiol-17β, we concluded that a slight increase in the circulating estrogens during the neonatal period exerts irreversible delayed effects.

Keywords: 17α-ethynylestradiol, sexual differentiation, estrous cycle

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Sakamoto Y, Yoshida M, Tamura K, Takahashi M, Kodama Y, Inoue K: Dose-dependent difference of nuclear receptors involved in murine liver hypertrophy by piperonyl butoxide.
J Toxicol Sci. 2015;40:787-96.

Nuclear receptors play important roles in chemically induced liver hypertrophy in rodents. To clarify the involvement of constitutive androstane receptor (CAR) and other nuclear receptors in mouse liver hypertrophy induced by different doses of piperonyl butoxide (PBO), wild-type and CAR-knockout mice were administered PBO (200, 1,000, or 5,000 ppm) in the basal diet for 1 week. Increased liver weight and diffuse hepatocellular hypertrophy were observed at 5,000 ppm for both genotypes, accompanied by increased Cyp3a11 mRNA and CYP3A protein expression, suggesting that CAR-independent pathway, possibly pregnane X receptor (PXR), plays a major role in the induction of hypertrophy. Moreover, wild-type mice at 5,000 ppm showed enhanced hepatocellular hypertrophy and strong positive staining for CYP2B in the centrilobular area, suggesting the localized contribution of CAR. At 1,000 ppm, only wild-type mice showed liver weight increase and centrilobular hepatocellular hypertrophy concurrent with elevated Cyp2b10 mRNA expression and strong CYP2B staining, indicating that CAR was essential at 1,000 ppm. We concluded that high-dose PBO induced hypertrophy via CAR and another pathway, while lower dose of PBO induced a pathway mediated predominantly by CAR. The dose-responsiveness on liver hypertrophy is important for understanding the involvement of nuclear receptors.
Keywords: constitutive androstane receptor, liver hypertrophy, piperonyl butoxide

Maeda J, Inoue K, Ichimura R, Takahashi M, Kodama Y, Saito N*, Yoshida M: Essential role of constitutive androstane receptor in *Ginkgo biloba* extract induced liver hypertrophy and hepatocarcinogenesis.
Food Chem Toxicol. 2015;83:201-9.

Ginkgo biloba extract (GBE) is commonly used as a herbal supplement. The National Toxicology Program (NTP) study of GBE reported clear evidence of hepatocarcinogenicity in mice. To clarify the mode of action (MOA) for hepatocarcinogenesis by GBE, we investigated the involvement of the constitutive androstane receptor (CAR) in hepatocarcinogenesis induced by GBE using CAR-knockout (CARKO) and wild type (WT) mice. We used the same lot of GBE that was used for the NTP study. In 1-week GBE dietary treatment, hepatocellular DNA replication was increased in WT mice but not in CARKO mice. In 4- or 13-week treatment, greater hepatic Cyp2b10 induction and hepatocellular hypertrophy were observed in WT mice, whereas these effects of GBE were much smaller in CARKO mice. In a two-stage hepatocarcinogenesis model initiated by diethylnitrosamine, 27-week treatment with GBE resulted in an increase of eosinophilic altered foci and adenomas in WT mice. By contrast, foci and adenomas were clearly less evident in CARKO mice. These results indicate that GBE-induced hepatocarcinogenesis is mainly CAR-mediated. Since CAR-mediated MOA for hepatocarcinogenesis in rodents is considered to be qualitatively implausible for humans, our findings would be helpful to evaluate the carcinogenic characterization of GBE to humans.
Keywords: constitutive androstane receptor, *Ginkgo biloba* extract, hepatocarcinogenesis

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Kuwata K, Inoue K, Ichimura R, Takahashi M, Kodama Y, Yoshida M: Constitutive active/androstane receptor, peroxisome proliferator-activated receptor α, and cytotoxicity are involved in oxadiazon-induced liver tumor development in mice.
Food Chem Toxicol. 2016;88:75-86.

Oxadiazon (OX) is a protoporphyrinogen oxidase-inhibiting herbicide that induces porphyria and liver tumors in rodents. Although porphyria is generally considered to be a risk factor for liver tumor development, the mechanisms through which OX mediates tumor development are unclear. Therefore, in this study, we investigated the mechanisms of tumor development by focusing on constitutive active/androstane receptor (CAR), which is essential for the development of tumors in response to several chemicals. After 1, 4, or 13 weeks of dietary treatment with 1000 ppm OX, hepatic Cyp2b10 expression was induced in wild-type (WT) mice. However, this effect was blocked in CAR-knockout (CARKO) mice. Hepatic Cyp4a10 expression, indicative of peroxisome proliferator-activated receptor α (PPARα) activation, and cytotoxic changes in hepatocytes were also

observed in both groups of mice. After initiation by diethylnitrosamine, 26-week treatment with OX resulted in an increase in proliferative lesions, including foci and adenomas, in both genotypes, and the incidence and multiplicity of proliferative lesions in CARKO mice were higher than those in control mice but lower than those in WT mice. These results suggested that CAR, PPARα activation, and cytotoxicity were involved in the development of liver tumors. Moreover, porphyrin was not apparently involved in OX-induced tumor development.

Keywords: oxadiazon, constitutive androstane receptor, hepatocarcinogenesis

Ichimura R, Takahashi M, Morikawa T, Inoue K, Kuwata K, Usuda K[*1], Yokosuka M[*2], Watanabe G[*1], Yoshida M: The critical hormone-sensitive window for the development of delayed effects extends to 10 days after birth in female rats postnatally exposed to 17alpha-ethynylestradiol.

Biol Reprod. 2015;93:32.

Neonatal exposure to estrogens is known to cause delayed effects, a late-occurring adverse effect on adult female reproductive functions, such as early onset of age-matched abnormal estrous cycling. However, the critical period in which neonates are sensitive to delayed effects inducible exogenous estrogen exposure has not been clearly identified. To clarify this window, we examined the intensity and timing of delayed effects using rats exposed to ethynylestradiol (EE) at various postnatal ages. After subcutaneous administration of a single dose of EE (20 μg/kg, which induces delayed effects) on Postnatal Day (PND) 0, 5, 10 or 14 in Wistar rats, hypothalamic and hormonal alterations in young adults and long-term estrous cycling status were investigated as indicators of delayed effects. In young adults, peak luteinizing hormone concentrations at the time of the luteinizing hormone surge showed decreasing trend, and KiSS1 mRNA expression of the anterior hypothalamus and number of KiSS1-positive cells in the anteroventral periventricular nucleus were significantly decreased in the PND 0, 5, and 10 groups. The reduction in KiSS1 mRNA and KiSS1-postive cells was inversely correlated with age at time of exposure. These groups also exhibited early onset of abnormal estrous cycling, starting from 17 wk of age in the PND0 group and 19 wk of age in the PND5 and PND10 groups. These indicators were not apparent in the PND14 group. Our results suggest that PND0-PND10 is the critical window of susceptibility for delayed effects, and PND14 is presumed to be the provisional endpoint of the window.

Keywords: early development, estrous cycle, kisspeptin

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Yoshida M, Suzuki S[*], Takahashi M, Ichimura R, Inoue K, Taya K[*], Watanabe G[*]: Predominant role of the hypothalamic-pituitary axis, not the ovary, in different types of abnormal cycle induction by postnatal exposure to high dose p-tert-octylphenol in rats.

Reprod Toxicol. 2015;57:21-8.

To determine whether it is the hypothalamic-pituitary axis or the ovary that plays the predominant role in abnormal estrous cycling induction by postnatal exposure to estrogenic compounds, female rats were subcutaneously injected with 100 mg/kg p-tert-octylphenol or vehicle for 5 or 15 days after birth (OP-PND5, OP-PND15 or control). Ovaries were exchanged between control and treated groups on PND28. Controls receiving control or OP-PND5 ovaries showed normal cycles within 4 weeks after the exchange, and corpora lutea were detected in transplanted ovaries. Controls receiving OP-PND15 ovaries consistently increased persistent estrus (PE). OP-PND15 rats receiving control or OP-PND15 ovaries immediately descended into PE, and tranplanted ovaries were atrophic with cystic follicles, indicating anovulation. OP-PND5 rats receiving control or OP-PND5 ovaries showed early onset of PE after normal cycling. The hypothalamic-pituitary axis is predominant in abnormal cycling induction by postnatal exposure to OP. OP-PND15 ovaries were impaired compared to other groups.

Keywords: hypothalamic-pituitary axis, postnatal exposure, p-tert-octylphenol

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Abbasi A[*1], Khalaj M[*1], Akiyama K[*1], Mukai Y[*1], Matsumoto H[*1], Acosta TA[*1], Said N[*2], Yoshida M, Kunieda T[*1]: Lack of Rev7 function results in

development of tubulostromal adenomas in mouse ovary.

Mol Cell Endocrinol. 2015;412:19-25.

Rev7 is a subunit of Polζ, one of the translesion DNA synthesis (TLS) polymerases involved in DNA damage repair. We recently found that Rev7 is also essential for germ cell development in mouse. In the present study, we found the development of ovarian tumors in Rev7 mutant mouse, suggesting the involvement of TLS deficiency in the etiology of ovarian tumor. The Rev7 mutant mice showed complete lack of oocytes and follicles in the ovary. The lack of follicles causes a significant increase of gonadotropin level and an increase in the proliferation of ovarian cells. As a result, the weight of the ovaries of Rev7 mutant mice increased with age and they developed tubulostromal adenomas. However, the remarkable overgrowth of ovaries occurred after gonadotropin level decreases at older ages, suggesting gonadotropin-independent progression of the ovarian tumors. In addition, the Rev7 mutant fibroblasts and ovarian cells showed significant accumulation of DNA damage. These findings suggest that not only increased gonadotropin levels but also lack of DNA damage repair function could be responsible for the development of ovarian tumors in the Rev7 mutant mouse.

Keywords: ovarian tumor, Rev7, translesion DNA polymerase

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Shiga T[*1], Nakamura TJ[*1], Komine C[*1], Goto Y[*2], Mizoguchi Y[*1], Yoshida M, Kondo Y[*3], Kawaguchi M[*1]: A single neonatal injection of ethinyl estradiol impairs passive avoidance learning and reduces expression of estrogen receptor α in the hippocampus and cortex of adult female rats.

PLOS One 2016;11:e0146136.

Although perinatal exposure of female rats to estrogenic compounds produces irreversible changes in brain function, it is still unclear how the amount and timing of exposure to those substances affect learning function, or if exposure alters estrogen receptor α (ERα) expression in the hippocampus and cortex. In adult female rats, we investigated the effects of neonatal exposure to a model estrogenic compound, ethinyl estradiol (EE), on passive avoidance learning and ERα expression. Female Wistar-Imamichi rats were subcutaneously injected with oil, 0.02 mg/kg EE, 2 mg/kg EE, or 20 mg/kg 17β-estradiol within 24 h after birth. All females were tested for passive avoidance learning at the age of 6 weeks. Neonatal 0.02 mg/kg EE administration significantly disrupted passive avoidance compared with oil treatment in gonadally intact females. In a second experiment, another set of experimental females, treated as described above, was ovariectomized under pentobarbital anesthesia at 10 weeks of age. At 15-17 weeks of age, half of each group received a subcutaneous injection of 5 μg estradiol benzoate a day before the passive avoidance learning test. Passive avoidance learning behavior was impaired by the 0.02 mg/kg EE dose, but notably only in the estradiol benzoate-injected group. At 17-19 weeks of age, hippocampal and cortical samples were collected from rats with or without the 5 μg estradiol benzoate injection, and western blots used to determine ERα expression. A significant decrease in ERα expression was observed in the hippocampus of the estradiol-injected, neonatal EE-treated females. The results demonstrated that exposure to EE immediately after birth decreased learning ability in adult female rats, and that this may be at least partly mediated by the decreased expression of ERα in the hippocampus.

Keywords: ethinyl estradiol, estrogen receptor α, hippocampus

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Honma M: Evaluation of the *in vivo* genotoxicity of Allura Red AC (Food Red No. 40).

Food and Chemical Toxicology 2015;84:270-5.

Allura Red AC (Food Red No. 40) is a red azo dye that is used for food coloring in beverage and confectionary products. To clarify the *in vivo* genotoxicity, we treated mice with Allura Red AC and investigated the induction of DNA damage, clastogenicity, and mutagenicity using Comet assays, micronucleus tests, and transgenic gene mutation assays, respectively. No genotoxic effect was observed in any of the genotoxic endpoints. These data clearly

show no evidence of *in vivo* genotoxic potential of Allura Red AC administered up to the maximum doses in mice.

Keywords: Allura Red AC, red azo dye, *in vivo* genotoxicity

Barber C[*1], Amberg A[*2], Custer L[*3], Dobo KL[*4], Glowienke S[*5], Van Gompel J[*6], Gutsell S[*7], Harvey J[*8], Honma M, Kenyon MO[*4], Kruhlak N[*9], Muster W[*10], Stavitskaya L[*9], Teasdale A[*11], Vessey J[*1], Wichard J[*12]: Establishing best practise in the application of expert review of mutagenicity under ICH M7.

Regul Toxicol Pharmacol. 2015;73:367-77.

The ICH M7 guidelines for the assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals allows for the consideration of in silico predictions in place of *in vitro* studies. This represents a significant advance in the acceptance of (Q)SAR models and has resulted from positive interactions between modellers, regulatory agencies and industry with a shared purpose of developing effective processes to minimise risk. This paper discusses key scientific principles that should be applied when evaluating in silico predictions with a focus on accuracy and scientific rigour that will support a consistent and practical route to regulatory submission.

Keywords: ICH M7 guideline, impurities, (Q)SAR

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Canipa S[*1], Cayley A[*1], Drewe WC[*1], Williams RV[*1], Hamada S[*2], Hirose A, Honma M, Morita T: Using *in vitro* structural alerts for chromosome damage to predict *in vivo* activity and direct future testing.

Mutagenesis 2016;31:17-25.

While the *in vivo* genotoxicity of a compound may not always correlate well with its activity in in vitro test systems, for certain compound classes a good overlap may exist between the two endpoints. The difficulty, however, lies in establishing the cases where this relationship holds true and selecting the most appropriate protocol to highlight any potential *in vivo* hazard. With this in mind, a project was initiated in which existing structural alerts for *in vitro* chromosome damage in the expert system Derek Nexus were assessed for their relevance to *in vivo* activity by assessing their predictivity against an *in vivo* chromosome damage data set. An expert assessment was then made of selected alerts. Information regarding the findings from specific *in vivo* tests was added to the alert along with any significant correlations between activity and test protocol or mechanism. A total of 32 *in vitro* alerts were updated using this method resulting in a significant improvement in the coverage of *in vivo* chromosome damage in Derek Nexus against a data set compiled by the mammalian mutagenicity study group of Japan. The detailed information relating to *in vivo* activity and protocol added to the alerts in combination with the mechanistic information provided will prove useful in directing the further testing of compounds of interest.

Keywords: genotoxicity, hazard, predictivity

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Kanemaru Y[*1], Suzuki T, Niimi N, Grúz P, Matsumoto K[*2], Adachi N[*3], Honma M, Nohmi T: Catalytic and non-catalytic roles of DNA polymerase κ in the protection of human cells against genotoxic stresses.

Environ Mol Mutagen. 2015;56:650-62.

DNA polymerase κ (Pol κ) is a specialized DNA polymerase involved in translesion DNA synthesis. Although its bypass activities across lesions are well characterized in biochemistry, its cellular protective roles against genotoxic insults are still elusive. To better understand the *in vivo* protective roles, we have established a human cell line deficient in the expression of Pol κ (KO) and another expressing catalytically dead Pol κ (CD), to examine the cytotoxic

sensitivity to 11 genotoxins including ultraviolet C light (UV). These cell lines were established in a genetic background of Nalm-6-MSH+, a human lymphoblastic cell line that has high efficiency for gene targeting, and functional p53 and mismatch repair activities. We classified the genotoxins into four groups. Group 1 includes benzo[a]pyrene diolepoxide, mitomycin C, and bleomycin, where the sensitivity was equally higher in KO and CD than in the cell line expressing wild-type Pol κ (WT). Group 2 includes hydrogen peroxide and menadione, where hypersensitivity was observed only in KO. Group 3 includes methyl methanesulfonate and ethyl methanesulfonate, where hypersensitivity was observed only in CD. Group 4 includes UV and three chemicals, where the chemicals exhibited similar cytotoxicity to all three cell lines. The results suggest that Pol κ not only protects cells from genotoxic DNA lesions via DNA polymerase activities, but also contributes to genome integrity by acting as a non-catalytic protein against oxidative damage caused by hydrogen peroxide and menadione. The non-catalytic roles of Pol κ in protection against oxidative damage by hydrogen peroxide are discussed.

Keywords: translesion DNA synthesis, genotoxic stresses, DNA polymerase κ

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Keka IS*1, Mohiuddin*1, Maede Y*1, Rahman MM*1, Sakuma T*2, Honma M, Yamamoto T*2, Takeda S*1, Sasanuma H*1: Smarcal1 promotes double-strand-break repair by nonhomologous end-joining.
Nucleic Acids Res. 2015;43:6359-72.

Smarcal1 is a SWI/SNF-family protein with an ATPase domain involved in DNA-annealing activities and a binding site for the RPA single-strand-DNA-binding protein. Although the role played by Smarcal1 in the maintenance of replication forks has been established, it remains unknown whether Smarcal1 contributes to genomic DNA maintenance outside of the S phase. We disrupted the SMARCAL1 gene in both the chicken DT40 and the human TK6 B cell lines. The resulting SMARCAL1(-/-) clones exhibited sensitivity to chemotherapeutic topoisomerase 2 inhibitors, just as nonhomologous end-joining (NHEJ) null-deficient cells do. SMARCAL1(-/-) cells also exhibited an increase in radiosensitivity in the G1 phase. Moreover, the loss of Smarcal1 in NHEJ null-deficient cells does not further increase their radiosensitivity. These results demonstrate that Smarcal1 is required for efficient NHEJ-mediated DSB repair. Both inactivation of the ATPase domain and deletion of the RPA-binding site cause the same phenotype as does null-mutation of Smarcal1, suggesting that Smarcal1 enhances NHEJ, presumably by interacting with RPA at unwound single-strand sequences and then facilitating annealing at DSB ends. SMARCAL1(-/-)cells showed a poor accumulation of Ku70/DNA-PKcs and XRCC4 at DNA-damage sites. We propose that Smarcal1 maintains the duplex status of DSBs to ensure proper recruitment of NHEJ factors to DSB sites.

Keywords: SWI/SNF-family, nonhomologous end-joining, DSB repair

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Petkov PI*1, Patlewicz G*2, Schultz TW*3, Honma M, Todorov M*1, Kotov S*1, Dimitrov SD*1, Donner EM*2, Mekenyan OG*1: A feasibility study: Can information collected to classify for mutagenicity be informative in predicting carcinogenicity?.
Regul Tox Pharm. 2015;72:17-25.

Carcinogenicity is a complex endpoint of high concern yet the rodent bioassay still used is costly to run in terms of time, money and animals. Therefore carcinogenicity has been the subject of many different efforts to both develop short-term tests and non-testing approaches capable of predicting genotoxic carcinogenic potential. In our previous publication (Mekenyan et al., 2012) we presented an *in vitro-in vivo* extrapolation workflow to help investigate the differences between *in vitro* and *in vivo* genotoxicity tests. The outcomes facilitated the development of new (Q)SAR models and for directing testing. Here we have refined this workflow by grouping specific tests together on the basis of their ability to detect DNA and/or protein damage at different levels of biological organization. This revised workflow, akin to an Integrated Approach to Testing and Assessment (IATA) informed by mechanistic understanding

was helpful in rationalizing inconsistent study outcomes and categorizing a test set of carcinogens with mutagenicity data on the basis of regulatory mutagenicity classifications. Rodent genotoxic carcinogens were found to be correctly predicted with a high sensitivity (90-100%) and a low rate of false positives (3-10%). The insights derived are useful to consider when developing future (non-)testing approaches to address regulatory purposes.

Keywords: Carcinogenicity classification, Integrated Approaches to Testing and Assessment (IATA), (Q) SAR

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Masumura K, Sakamoto Y, Kumita W, Honma M, Nishikawa A, Nohmi T: Genomic integration of lambda EG10 transgene in *gpt* delta transgenic rodents.

Genes and Environment 2015;37:24.

Transgenic *gpt* delta mouse and rat models were developed to perform *gpt* and Spi$^-$ assays for *in vivo* mutagenicity tests. The animals were established by integration of lambda EG10 phage DNA as a transgene into the genome. To identify the site and pattern of genomic integration of the transgene copies, genomic DNAs extracted from C57BL/6J *gpt* delta mice and F344 *gpt* delta rats were applied to whole genome sequencing and mate-pair analysis. The result confirmed that multi-copy lambda EG10 transgenes are inserted at a single position in the mouse chromosome 17. The junction contains 70 bp of overlapped genomic sequences, and it has short homology at both ends. A copy number analysis suggested that the inserted transgenes may contain 41 head-to-tail junctions and 16 junctions of other types such as rearranged abnormal junctions. It suggested that the number of intact copies could be approximately 40 at maximum. In the F344 *gpt* delta rats, transgenes are inserted at a single position in the rat chromosome 4. The junction contains no overlapped sequence but 72-kb genomic sequence including one gene was deleted. The inserted transgenes may contain 15 head-to-tail junctions and two rearranged junctions. It suggested that the number of intact copies could be 14 at maximum. One germline base substitution in the *gpt* gene rescued from *gpt* delta rats was characterized. PCR primers for quick genotyping of *gpt* delta mice and rats have been designed.

Keywords: *gpt* delta transgenic rodents, transgene, whole genome sequencing

Aoki Y*1, Hashimoto A*1, Sugawara Y*1, Hiyoshi-Arai K*1, Goto S*2, Masumura K, Nohmi T: Alterations in the mutagenicity and mutation spectrum induced by benzo[*a*]pyrene instilled in the lungs of *gpt* delta mice of various ages.

Genes and Environment 2015;37:7.

To examine whether the mutagenic potential of lung exposure to air-borne environmental mutagens is age dependent, we administered 1 mg of benzo[*a*] pyrene intratracheally to 11- and 24-month old (middle-aged and old, respectively) *gpt* delta transgenic mice and then analyzed the benzo[*a*]pyrene-induced and spontaneous *in vivo* mutations and mutation spectrum in the lungs. The mutant frequencies in the lungs of the 11- and 24-month–old control (vehicle-treated) *gpt* delta mice were $1.14 \pm 0.22 \times 10^{-5}$ and $1.00 \pm 0.20 \times 10^{-5}$, respectively, which are significantly higher than that observed for the control 3-month–old (young) mice ($0.59 \pm 0.13 \times 10^{-5}$) in our previous studies, indicating that spontaneous mutation in the lung increases with age. The mutant frequencies in 11- and 24-month–old mice treated with benzo [*a*] pyrene were 1.5- and 2.3-fold, respectively, that of the age-matched control mice, and 4.3-fold that of the 3-month–old mice in our previous studies. Analysis of mutation spectra showed that both G:C to A:T transitions and G:C to T:A transversions were predominant in the lungs of control mice at all ages. In benzo [*a*] pyrene-treated mice in our previous studies, G:C to T:A transversions were the predominant type of mutation (55 %) at 3 months. Here we found that their frequency was dramatically reduced to 18 % by 24 months, and the G:C to A:T transitions became the predominant type of mutation in 24-month–old mice (41 % [16 % at CpG sites]). Our findings suggest that susceptibility to benzo[*a*]pyrene is highest in young mice and is elevated again in old age. The elevation of G:C to A:T transitions was observed following benzo [*a*] pyrene administration in the lungs of aged mice, and accelerated cytidine deamination is

speculated to contribute to this elevation.
Keywords: benzo [*a*] pyrene, mutation spectrum, age

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Sugiyama K, Yamada M, Awogi T*1, Hakura A*2: The strains recommended for use in the bacterial reverse mutation test (OECD guideline 471) can be certified as non-genetically modified organisms.
Genes and Environment 2016;38:1.

The bacterial reverse mutation test, called Ames test, is used worldwide. In Japan, the genetically modified organisms (GMOs) are regulated under the Cartagena Domestic Law, and organisms obtained by self-cloning and/or natural occurrence would be exempted from the law case by case. The strains recommended for use in the bacterial reverse mutation test (OECD guideline 471), have been considered as non-GMOs because they can be constructed by self-cloning or naturally occurring bacterial strains, or do not disturb the biological diversity. The present article explains the reasons why these strains should be classified as non-GMOs.
Keywords: Ames test, genetically modified organisms, Cartagena Domestic Law

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Matsuda T*, Matsuda S*, Yamada M: Mutation assay using single-molecule real-time (SMRTTM) sequencing technology.
Genes and Environment 2015;37:15.

Introduction: We present here a simple, phenotype-independent mutation assay using a PacBio RSII DNA sequencer employing single-molecule real-time (SMRT) sequencing technology. *Salmonella typhimurium* YG7108 was treated with the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) and grown through several generations to fix the induced mutations, the DNA was extracted and the mutations were analyzed by using the SMRT DNA sequencer. Results: The ENU-induced base-substitution frequency was 15.4 per megabase pair, which is highly consistent with our previous results based on colony isolation and next-generation sequencing. The induced mutation spectrum (95% G:C to A:T, 5% A:T to G:C) is also consistent with the known ENU signature. The base-substitution frequency of the control was calculated to be less than 0.12 per megabase pair. Conclusions: Ultra-low frequency base-substitution mutations can be detected directly by using the SMRT DNA sequencer, and this technology provides a phenotype-independent mutation assay.
Keywords: PacBio RSII DNA sequencer, Single-molecule real-time (SMRT) sequencing technology, Mutation assay

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Yasui M, Kamoshita N, Nishimura T*, Honma M: Mechanism of induction of binucleated cells by multiwalled carbon nanotubes as revealed by live-cell imaging analysis.
Genes and Environment 2015;37:6.

Introduction: Asbestos-induced formation of mesothelioma has been attributed to phenotypic and morphological changes in cells caused by polyploidization and aneuploidization, and multiwalled carbon nanotubes (MWCNTs) are suspected to have similar adverse effects due to the similarity in their physical form. MWCNTs and crocidolite, a kind of asbestos, show similar genotoxicity characteristics *in vitro*, including induction of binucleated cells. We here focused on the mechanisms underlying polyploidization during cell division on exposure to MWCNTs and conducted confocal live-cell imaging analysis using MDA-435 human breast cancer cells in which chromosomes and centromeres were visualized using fluorescent proteins. Findings: During anaphase, relatively short MWCNT fibers (approximately 5 μm) migrated rapidly to either of the daughter cells, whereas some long MWCNT fibers (approximately 20 μm) remained inside the contractile ring and induced the formation of binucleated cells through impairment of cytokinesis. This toxicity mechanism has also been observed with crocidolite. Conclusions: Our findings indicate that the mechanism of polyploidization by MWCNTs is very similar to that observed with crocidolite.
Keywords: Polyploidization, Crocidolite, Cytokinesis

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Sassa A, Kamoshita N, Kanemaru Y, Honma M, Yasui M: Nucleotide excision repair suppresses mutagenesis caused by clustered oxidative DNA adducts in human genome.
PLOS One 2015;10:e0142218.

Clustered DNA damage is defined as multiple sites of DNA damage within one or two helical turns of the duplex DNA. This complex damage is often formed by exposure of the genome to ionizing radiation and is difficult to repair. The mutagenic potential and repair mechanisms of clustered DNA damage in human cells remain to be elucidated. In this study, we investigated the involvement of nucleotide excision repair (NER) in clustered oxidative DNA adducts. To identify the *in vivo* protective roles of NER, we established a human cell line lacking the NER gene xeroderma pigmentosum group A (*XPA*). *XPA* knockout (KO) cells were generated from TSCER122 cells derived from the human lymphoblastoid TK6 cell line. To analyze the mutagenic events in DNA adducts in vivo, we previously employed a system of tracing DNA adducts in the targeted mutagenesis (TATAM), in which DNA adducts were site-specifically introduced into intron 4 of thymidine kinase genes. Using the TATAM system, one or two tandem 7,8-dihydro-8-oxoguanine (8-oxoG) adducts were introduced into the genomes of TSCER122 or *XPA* KO cells. In *XPA* KO cells, the proportion of mutants induced by a single 8-oxoG (7.6%) was comparable with that in TSCER122 cells (8.1%). In contrast, the lack of *XPA* significantly enhanced the mutant proportion of tandem 8-oxoG in the transcribed strand (12%) compared with that in TSCER122 cells (7.4%) but not in the non-transcribed strand (12% and 11% in *XPA* KO and TSCER122 cells, respectively). By sequencing the tandem 8-oxoG-integrated loci in the transcribed strand, we found that the proportion of tandem mutations was markedly increased in *XPA* KO cells. These results indicate that NER is involved in repairing clustered DNA adducts in the transcribed strand in vivo.
Keywords: clustered DNA damage, ionizing radiation, xeroderma pigmentosum group A

Horibata K, Kono S[*1], Ishigami C[*1], Zhang X[*1], Aizawa M[*2], Kako Y[*2], Ishii T[*3], Kosaki R[*4], Saijo M[*1], Tanaka K[*1]: Constructive rescue of TFIIH instability by an alternative isoform of XPD derived from a mutated XPD allele in mild but not severe XP-D/CS.
J Hum Genet. 2015;60:259-65.

Mutations in XPD cause xeroderma pigmentosum (XP), XP and Cockayne syndrome (CS) crossover syndrome (XP/CS), trichothiodystrophy and cerebro-oculo-facio-skeletal syndrome (COFS). COFS represents the most severe end of the CS spectrum. This study reports two Japanese patients, COFS-05-135 and COFS-Chiba1, who died at ages of <1 year and exhibited typical COFS manifestations caused by XPD mutations p.[I619del];[R666W] and p.[G47R];[I619del], respectively. Two other cases of severe XP-D/CS (XP group D/CS), XP1JI (p.[G47R];[0]) and XPCS1PV (p.[R666W];[0]), died at ages <2 years. On the other hand, two cases of mild XP-D/CS, XP1NE (p.[G47R];[L461V;V716_R730del]) and XPCS118LV (p.[L461V;V716_R730del];[R666W]), lived beyond 37 years of age. p.I619Del and p.[L461V;V716_R730del] are functionally null; therefore, despite the differences in clinical manifestations, the functional protein in all of these patients was either p.G47R or p.R666W. To resolve the discrepancies in these XPD genotype-phenotype relationships, the p.[L461V;V716_R730del] allele was analyzed and we found that p.[L461V;A717G] was expressed from the same allele as p.[L461V;V716_R730del] by authentic splicing. Additionally, p.[L461V;A717G] could partially rescue the loss of XPD function, resulting in the milder manifestations observed in XP1NE and XPCS118LV.
Keywords: Cockayne syndrome, cerebro-oculo-facio-skeletal syndrome, xeroderma pigmentosum

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Kato H, Fujii S[*], Takahashi M, Matsumoto M, Hirata-Koizumi M, Ono A, Hirose A: Repeated dose and reproductive/developmental toxicity of perfluorododecanoic acid in rats.
Environ Toxicol. 2015;30:1235-43.

Perfluoroalkyl carboxylic acids (PFCAs) are a series of environmental contaminants that have received attention because of their possible adverse effects on wildlife and human health. Although many toxicological studies have been performed on perfluorooctanoic

acid with carbon chain length C8, available toxicity data on PFCAs with longer chains are still insufficient to evaluate their hazard. A combined repeated dose and reproductive/developmental toxicity screening study for perfluorododecanoic acid (PFDoA; C12) was conducted in accordance with OECD guideline 422 to fill these toxicity data gaps. PFDoA was administered by gavage to male and female rats at 0.1, 0.5, or 2.5 mg/kg/day. The administration of PFDoA at 0.5 and 2.5 mg/kg/day for 42–47 days mainly affected the liver, in which hypertrophy, necrosis, and inflammatory cholestasis were noted. Body weight gain was markedly inhibited in the 2.5 mg/kg/day group, and a decrease in hematopoiesis in the bone marrow and atrophic changes in the spleen, thymus, and adrenal gland were also observed. Regarding reproductive/developmental toxicity, various histopathological changes, including decreased spermatid and spermatozoa counts, were observed in the male reproductive organs, while continuous diestrous was observed in the females of the 2.5 mg/kg/day group. Seven of twelve females receiving 2.5 mg/kg/day died during late pregnancy while four other females in this group did not deliver live pups. No reproductive or developmental parameters changed at 0.1 or 0.5 mg/kg/day. Based on these results, the NOAELs of PFDoA were concluded to be 0.1 mg/kg/day for repeated dose toxicity and 0.5 mg/kg/day for reproductive/developmental toxicity.

Keywords: perfluorododecanoic acid, repeated dose toxicity, reproductive toxicity

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Morita T, Uno Y[*1], Honma M, Kojima H, Hayashi M[*2], Tice RC[*3], Corvi R[*4], Schechtman L[*5]: The JaCVAM International Validation Study on the in vivo Comet Assay: Selection of Test Chemicals.
Mutat Res. 2015;786-788:14-44.

The Japanese Center for the Validation of Alternative Methods (JaCVAM) sponsored an international prevalidation and validation study of the in vivo rat alkaline pH comet assay. Based on existing carcinogenicity and genotoxicity data and chemical class information, 90 chemicals were identified as primary candidates for use in the validation study. From these 90 chemicals, 46 secondary candidates and then 40 final chemicals were selected based on a sufficiency of carcinogenic and genotoxic data, differences in chemical class or genotoxic or carcinogenic mode of action (MOA), availability, price, and ease of handling.

Keywords: JaCVAM Validation study, *In vivo* comet assay, Chemical selection

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Uno Y[*1], Kojima H, Omori T[*2], Corvi R[*3], Honma M, Schechtman LM[*4], Tice RR[*5], Burlinson B[*6], Escobar P[*7], Kraynak AR[*8], Nakagawa Y[*9], Nakajim M[*10], Pant K[*11], Asano N[*12], Lovell D[*13], Morita T, Ohno Y, Hayashi M[*14]: JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens: I. Summary of pre-validation study results.
Mutat Res. 2015;786-788:3-13.

The in vivo rodent alkaline comet assay (comet assay) is used internationally to investigate the in vivo genotoxic potential of test chemicals. This assay, however, has not previously been formally validated. The Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an international validation study to evaluate the reliability and relevance of the assay for identifying genotoxic carcinogens, using liver and stomach as target organs. The ultimate goal of this validation effort was to establish an Organisation for Economic Co-operation and Development (OECD) test guideline. The purpose of the pre-validation studies (i.e., Phase 1 through 3), conducted in four or five laboratories with extensive comet assay experience, was to optimize the protocol to be used during the definitive validation study.

Keywords: Comet assay, Validation study, JaCVAM

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Uno Y*1, Kojima H, Omori T*2, Corvi R*3, Honma M, Schechtman LM*4, Tice RR*5, Beevers C*6, Boeck MB*7, Burlinson B*8, Hobbs CH*9, Kitamoto S*10, Kraynakl AR*11, McNamee J*12, Nakagawa Y*13, Pant K*14, Plappert-Helbig U*15, Priestley C*16, Takasawa H*17, Wada K*18, Wirnitzer U*19, Asano N*20, Escobar P*21, Lovell D*22, Morita T, Nakajima M*23, Ohno Y, Hayashi M*24: JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results.

Mutat Res. 2015;786-788:47-56.

The in vivo rodent alkaline comet assay (comet assay) is used internationally to investigate the in vivo genotoxic potential of test chemicals. The Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an international validation study. The study protocol was optimized in the pre-validation studies, and then the definitive (4th phase) validation study was conducted in two steps. In the 1st step, assay reproducibilitywasconfirmedamonglaboratories using four coded reference chemicals and the positive control ethyl methanesulfonate. In the 2nd step, the predictive capabilitywasinvestigated using 40 coded chemicals with known genotoxic and carcinogenic activity (i.e., genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens). Based on the results obtained, the in vivo comet assay is concluded to be highly capable of identifying genotoxic chemicals and therefore can serve as a reliable predictor of rodent carcinogenicity.

Keywords: Comet assay, Validation study, JaCVAM

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Uno Y*1, Morita T, Luijten M*2, Beevers C*3, Hamada S*4, Itoh S*5, Ohyama W*6, Takasawa H*4: Recommended protocols for the liver micronucleus test: report of the IWGT working group.

Mutat Res. 2015;783:13-8.

At the 6th International Workshop on Genotoxicity Testing (IWGT), the liver micronucleus test working group discussed practical aspects of the in vivo rodent liver micronucleus test (LMNT). The group members focused on the three methodologies currently used, i.e., a partial hepatectomy (PH) method, a juvenile/young rat (JR) method, and a repeated-dose (RD) method in adult rodents. Since the liver is the main organ that metabolizes chemicals, the LMNT is expected to detect clastogens, especially those that need metabolic activation in the liver, and aneugens. Based on current data the three methods seem to have a high sensitivity and specificity, but more data, especially on non-genotoxic but toxic substances, would be needed to fully evaluate the test performance. The working group concluded that the LMNT could be used as a second in vivo test when a relevant positive result in in vitro mammalian cell genotoxicity tests is noted (especially under the condition of metabolic activation),

and a negative result is observed in the in vivo BM/PB-MNT.

Keywords: Micronucleus test, Liver, IWGT

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Uno Y*1, Morita T, Luijten M*2, Beevers C*3, Hamada S*4, Itoh S*5, Ohyama W*6, Takasawa H*4: Micronucleus test in rodent tissues other than liver or erythrocytes: Report of the IWGT working group. *Mutat Res.* 2015;783:19-22.

At the 6th International Workshop on Genotoxicity Testing, the liver micronucleus test (MNT) working group briefly discussed the MNT using tissues other than liver/erythrocytes. Many tissues other than liver/erythrocytes have been studied, primarily for research purposes. They have included the colon and intestinal epithelium, skin, spleen, lung, stomach, bladder, buccal mucosa, vagina, and fetal/neonatal tissues. Recently, there has been particular focus on the gastrointestinal (GI) tract as it is a contact site associated with high exposure following oral gavage. Based on limited data currently available, the rodent MNT using the glandular stomach and/or colon seems to detect genotoxic carcinogens with GI tract target-organ specificity. The working group concluded that the GI tract MNT would be a promising method to examine clastogenicity or aneugenicity of test chemicals in the stomach and/or colon. Further data will be needed to fully establish the methods, and to identify the sensitivity and specificity of the GI tract MNT.

Keywords: Micronucleus test, Stomach, Colon

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MacGregor JT*1, Frötschl R*2, White PA*3, Crump KS*4, Eastmond DA*5, Fukushima S*6, Guérard M*7, Hayashi M*8, Soeteman-Hernandez L*9, Kasamatsu T*10, Levy D*11, Morita T, Müller L*7, Schoeny R*12, Schuler MJ*13, Thybaud V*14, Johnson GE*15: IWGT Report on Quantitative Approaches to Genotoxicity Risk Assessment I. Methods and metrics for defining exposure-response relationships and points of departure (PoDs).
Mutat Res. 2015;783:55-65.

This report summarizes the discussion, conclusions, and points of consensus of the IWGT Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (QWG). Topics addressed included (1) the need for quantitative dose–response analysis, (2) methods to analyze exposure–response relationships & derive point of departure (PoD) metrics, (3) points of departure (PoD) and mechanistic threshold considerations, (4) approaches to define exposure-related risks, (5) empirical relationships between genetic damage (mutation) and cancer, and (6) extrapolations across test systems and species. The QWG recognizes that scientific evidence suggests that thresholds below which genotoxic effects do not occur likely exist for both DNA-reactive and DNA-nonreactive substances, but notes that small increments of the spontaneous level cannot be unequivocally excluded either by experimental measurement or by mathematical modeling. Therefore, rather than debating the theoretical possibility of such low-dose effects, emphasis should be placed on determination of PoDs from which acceptable exposure levels can be determined by extrapolation using available mechanistic information and appropriate uncertainty factors.

Keywords: Genotoxicity, Quantitative risk assessment, Points of departure

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Mutat Res. 2015;783:66-78.

This is the second of two reports from the International Workshops on Genotoxicity Testing (IWGT) Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (the QWG). This report summarizes the QWG discussions and recommendations. Recommendations include the selection of appropriate genetic endpoints and target tissues, uncertainty factors and extrapolation methods to be considered, the importance and use of information on mode of action, toxicokinetics, metabolism, and exposure biomarkerswhenusing quantitative exposure-response data to determine acceptable exposure levels in human populations or to assess the risk associated with knownor anticipated exposures. It was concluded that there is a general correlation between cancer induction and mutagenic and/or clastogenic damage for agents thought to act via a genotoxic mechanism, but that the correlation is limited due to an inadequate number of cases in which mutation and cancer can be compared at a sufficient number of doses in the same target tissues of the same species and strain exposed under directly comparable routes and experimental protocols.
Keywords: Benchmark dose, Extrapolation, Low-dose risk

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Hamada S*1, Ohyama W*2, Takashima R*3, Shimada K*4, Matsumoto K*5, Kawakami S*6, Uno F*7, Sui H*8, Shimada Y*9, Imamura T*10, Matsumura S*11, Sanada H*12, Inoue K*13, Muto S*14, Ogawa I*15, Hayashi A*16, Takayanagi T*17, Ogiwara Y*18, Maeda A*19, Okada E*2, Terashima Y*20, Takasawa H*1, Narumi K*2, Wako Y*1, Kawasako K*1, Sano M*6, Ohashi N*6, Morita T, Kojima H, Honma M, Hayashi M*6: Evaluation of the repeated-dose liver and gastrointestinal tract micronucleus assays with 22 chemicals using young adult rats: Summary of the collaborative study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/The Japanese Environmental Mutagen Society (JEMS) – Mammalian Mutagenicity Study Group (MMS).
Mutat Res. 2015;780-781:2-17.

The repeated-dose liver micronucleus (RDLMN) assay using young adult rats has the potential to detect hepatocarcinogens. We conducted a collaborative study to assess the performance of this assay and to evaluate the possibility of integrating it into general toxicological studies. Twenty-two model chemicals, including some hepatocarcinogens, were tested in 14- and/or 28-day RDLMN assays. As a result, 14 out of the 16 hepatocarcinogens were positive, including 9 genotoxic hepatocarcinogens, which were reported negative in the bone marrow/peripheral blood micronucleus (MN) assay by a single treatment. These outcomes show the high sensitivity of the RDLMN assay to hepatocarcinogens. Regarding the specificity, 4 out of the 6 non-liver targeted genotoxic carcinogens gave negative responses. This shows the high organ specificity of the RDLMN assay. The outcomes of our collaborative study indicated that the new techniques to detect chromosomal aberrations in vivo in several tissues worked successfully.
Keywords: Micronucleus, Liver, Repeated-dose

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Hirata-Koizumi M, Fujii S*, Hina K, Matsumoto M, Takahashi M, Ono A, Hirose A: Repeated dose and reproductive/developmental toxicity of long-chain perfluoroalkyl carboxylic acids in rats: perfluorohexadecanoic acid and perfluorotetradecanoic acid.
Fundam Toxicol Sci. 2015;2:177-90.

Perfluoroalkyl carboxylic acids (PFCAs) are global environmental contaminants that are the cause of concern due to their possible effects on wildlife and human health. Since few studies have investigated the toxicity of long-chain PFCAs, we have performed combined repeated dose toxicity studies with the reproduction/developmental toxicity screening tests. We previously examined perfluoroundecanoic acid (C11), perfluorododecanoic acid (C12), and perfluorooctadecanoic acid (C18). We herein reported our results for perfluorotetradecanoic acid (PFTeDA; C14) and perfluorohexadecanoic acid (PFHxDA: C16). Male and female rats were administered PFTeDA at 1, 3 or 10 mg/kg/day or PFHx- DA at 4, 20 or 100 mg/kg/day by gavage, and each female was then mated with a male in the same dose group after 14 days. Males were dosed for a total of 42 days and females were dosed throughout the gestation period until day 5 after parturition. PFTeDA and PFHxDA caused hepatocyte hypertrophy and/or fatty changes in the liver at the middle and high doses. PFTeDA also induced follicular cell hypertrophy in the thyroid at the middle and high doses. The only reproductive/developmental effect observed was an inhibited postnatal body weight gain in pups in the 10 mg/kg/day PFTeDA group. Based on these results, the NOAELs for the repeated dose and reproductive/developmental toxicity were concluded to be 1 and 3 mg/kg/day for PFTeDA and 4 and 100 mg/kg/day for PFHxDA, respectively. Our current and previous results indicate that the toxicity of PFCAs decreases with increases in the carbon chain length from 12 to 18.

Keywords: Perfluorotetradecanoic acid, Perfluorohexadecanoic acid, Reproductive and developmental toxicity

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Ono A, Kobayashi K, Serizawa H*, Kawamura T, Kato H, Matsumoto M, Takahashi M, Hirata-Koizumi M, Matsushima Y, Hirose A: A repeated dose 28-day oral toxicity study of β-bromostyrene in rats.
Fundam Toxicol Sci. 2015;2:191–200.

To obtain information on the possible repeated-dose oral toxicity of β-bromostyrene and its reversibility, Crl: CD (SD) rats were administered β-bromostyrene through gavage at 0, 30, 125, and 500 mg/kg/day once for 28 days, followed by a 14-day recovery period. In the 500 mg/kg group, decrease in spontaneous movement was observed in all males and females on the first dosing day, and one female rat died on Day 3. There were no significant changes in body weight or food consumption. An increase in urine volume and decrease in urine osmolality were observed in males receiving 125 mg/kg and above, and an increase in urine volume was observed in females receiving 500 mg/kg. On blood biochemical examination, increases in total cholesterol, phospholipids, triglycerides, total protein, albumin, inorganic phosphorus, and/or chlorine were observed in the 125 and/or 500 mg/kg groups. Histopathologically, eosinophilic bodies of tubular cells and/or renal tubular degeneration were observed in the kidneys of males in the 125 and 500 mg/kg

groups. In the thyroid, hypertrophy of follicular cells was observed in females receiving 125 mg/kg and above and males receiving 500 mg/kg. Furthermore, centrilobular hepatocellular hypertrophy was observed in both sexes receiving 500 mg/kg. These changes observed at the end of the dosing period disappeared or were reduced after the recovery period. Based on these results, the no-observed-adverse-effect-level of β-bromostyrene was judged to be 30 mg/kg/day for both sexes.

Keywords: β-bromostyrene, OECD TG407, Repeated dose toxicity

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Okamura H*1, Abe H*2, Hasegawa-Baba Y*2, Saito K*1, Sekiya F*1, Hayashi S*1, Mirokuji Y*1, Maruyama S*1, Ono A, Nakajima M*3, Degawa M*3, Ozawa S*4, Shibutani M*2, Maitani T: The Japan Flavour and Fragrance Materials Association's (JFFMA) safety assessment of acetal food flavouring substances uniquely used in Japan.

Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2015;32:1384-96.

Using the procedure devised by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), we performed safety evaluations on five acetal flavouring substances uniquely used in Japan: acetaldehyde 2,3-butanediol acetal, acetoin dimethyl acetal, hexanal dibutyl acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal. As no genotoxicity study data were available in the literature, all five substances had no chemical structural alerts predicting genotoxicity. Using Cramer's classification, acetoin dimethyl acetal and hexanal dibutyl acetal were categorised as class I, and acetaldehyde 2,3-butanediol acetal, hexanal glyceryl acetal and 4-methyl-2-pentanone propyleneglycol acetal as class III. The estimated daily intakes for all five substances were within the range of 1.45-6.53 μg/person/day using the method of maximised survey-derived intake based on the annual production data in Japan from 2001, 2005, 2008 and 2010, and 156-720 μg/person/day using the single-portion exposure technique (SPET), based on the average use levels in standard portion sizes of flavoured foods. The daily intakes of the two class I substances were below the threshold of toxicological concern (TTC) - 1800 μg/person/day. The daily intakes of the three class III substances exceeded the TTC (90 μg/person/day). Two of these, acetaldehyde 2,3-butanediol acetal and hexanal glyceryl acetal, were expected to be metabolised into endogenous products after ingestion. For 4-methyl-2-pentanone propyleneglycol acetal, one of its metabolites was not expected to be metabolised into endogenous products. However, its daily intake level, based on the estimated intake calculated by the SPET method, was about 1/15 000th of the no observed effect level. It was thus concluded that all five substances raised no safety concerns when used for flavouring foods at the currently estimated intake levels. While no information on in vitro and in vivo toxicity for all five substances was available, their metabolites were judged as raising no safety concerns at the current levels of intake.

Keywords: acetals, Cramer's decision tree, food flavours

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Hashiguchi S*, Yoshida H*, Akashi T*, Komemoto K*, Ueda T*, Ikarashi Y, Miyauchi A*, Konno K*, Yamanaka S*, Hirose A, Kurokawa M*, Watanabe W*: Titanium dioxide nanoparticles exacerbate pneumonia in respiratory syncytial virus (RSV)-infected mice. Environ.

Toxicol Pharmacol. 2015;39:879-86.

To reveal the effects of TiO2 nanoparticles, used in cosmetics and building materials, on the immune response, a respiratory syncytial virus (RSV) infection mouse model was used. BALB/c mice were exposed once intranasally to TiO2 at 0.5mg/kg and infected intranasally with RSV five days later. The levels of IFN-γ and chemokine CCL5, representative markers of pneumonia, in the bronchoalveolar lavage fluids of RSV-infected mice had increased significantly in TiO2-exposed mice compared with the control on day 5 post-infection, but not in uninfected mice. While pulmonary viral titers were not affected by TiO2 exposure, an increase in the infiltration of lymphocytes into the alveolar septa in lung tissues was observed. Immunohistochemical analysis revealed aggregation of TiO2 nanoparticles near inflammatory cells in the

severely affected region. Thus, a single exposure to TiO2 nanoparticles affected the immune system and exacerbated pneumonia in RSV-infected mice.
Keywords: TiO2, Immune response, Pneumonia

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Watanabe H*1, Tamura I*1,2, Abe R*1, Takanobu H*1, Nakamura A*1, Suzuki T*3, Hirose A, Nishimura T*4, Tatarazako N*1: Chronic toxicity of an environmentally relevant mixture of pharmaceuticals to three aquatic organisms (alga, daphnid, and fish).
Environ Toxicol Chem. 2015;35:996-1006.

Principles of concentration addition and independent action have been used as effective tools to predict mixture toxicity based on individual component toxicity. The authors investigated the toxicity of a pharmaceutical mixture composed of the top 10 detected active pharmaceutical ingredients (APIs) in the Tama River (Tokyo, Japan) in a relevant concentration ratio. Both individual and mixture toxicities of the 10 APIs were evaluated by 3 short-term chronic toxicity tests using the alga Pseudokirchneriella subcapitata, the daphnid Ceriodaphnia dubia, and the zebrafish Danio rerio. With the exception of clarithromycin toxicity to alga, the no-observed-effect concentration of individual APIs for each test species was dramatically higher than the highest concentration of APIs found in the environment. The mixture of 10 APIs resulted in toxicity to alga, daphnid, and fish at 6.25 times, 100 times, and 15 000 times higher concentrations, respectively, than the environmental concentrations of individual APIs. Predictions by concentration addition and independent action were nearly identical for alga, as clarithromycin was the predominant toxicant in the mixture. Both predictions described the observed mixture toxicity to alga fairly well, whereas they slightly underestimated the observed mixture toxicity in the daphnid test. In the fish embryo test, the observed toxicity fell between the predicted toxicity by concentration addition and independent action. These results suggested that the toxicity of environmentally relevant pharmaceutical mixtures could be predicted by individual toxicity using either concentration addition or independent action.
Keywords: Active pharmaceutical ingredients, Chronic Ecotoxicity, Environmental concentration

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Sewell F*1, Ragan I*2, Marczylo T*3, Anderson B*4, Braun A*5, Casey W*6, Dennison N*7, Griffiths D*8, Guest R*8, Holmes T*9, van Huygevoort T*10, Indans I*11, Kenny T*12, Kojima H, Lee K*13, Prieto P*14, Smith P*15, Smedley J*16, Stokes WS*17, Wnorowski G*18, Horgan G*19: A global initiative to refine acute inhalation studies through the use of 'evident toxicity' as an endpoint: Towards adoption of the fixed concentration procedure.
Regul Toxicol Pharmacol. 2015;73:770-9.

Acute inhalation studies are conducted in animals as part of chemical hazard identification and characterisation, including for classification and labelling purposes. Current accepted methods use death as an endpoint (OECD TG403 and TG436), whereas the fixed concentration procedure (FCP) (draft OECD TG433) uses fewer animals and replaces lethality as an endpoint with 'evident toxicity.' Evident toxicity is defined as clear signs of toxicity that predict exposure to the next highest concentration will cause severe toxicity or death in most animals. A global initiative including 20 organisations, led by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has shared data on the clinical signs recorded during acute inhalation studies for 172 substances (primarily dusts or mists) with the aim of making evident toxicity more objective and transferable between laboratories. Pairs of studies (5 male or 5 female rats) with at least a two-fold change in concentration were analysed to determine if there are any signs at the lower dose that could have predicted severe toxicity or death at the higher concentration. The results show that signs such as body weight loss (>10% pre-dosing weight), irregular respiration, tremors and hypoactivity, seen at least once in at least one animal after the day of dosing are highly predictive (positive predictive value > 90%) of severe toxicity or death at the next highest concentration. The working group has used these data to propose changes to TG433 that incorporate a

clear indication of the clinical signs that define evident toxicity.

Keywords: 3Rs, Acute inhalation studies, Regulatory toxicology

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*10 WIL Research
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*12 Huntingdon Life Sciences
*13 Korea Institute of Toxicology
*14 EURL ECVAM
*15 Charles River Laboratories
*16 Charles River Laboratories
*17 U.S. Department of Agriculture
*18 Product Safety Laboratories
*19 BioSS

Yamaguchi H*1,2, Kojima H, Takezawa T*1: Predictive performance of the Vitrigel-eye irritancy test method using 118 chemicals.

J Appl Toxicol. 2015;doi:10.1002/jat.3254.

We recently developed a novel Vitrigel-eye irritancy test (EIT) method. The Vitrigel-EIT method is composed of two parts, i.e., the construction of a human corneal epithelium (HCE) model in a collagen vitrigel membrane chamber and the prediction of eye irritancy by analyzing the time-dependent profile of transepithelial electrical resistance values for 3 min after exposing a chemical to the HCE model. In this study, we estimated the predictive performance of Vitrigel-EIT method by testing a total of 118 chemicals. The category determined by the Vitrigel-EIT method in comparison to the globally harmonized system classification revealed that the sensitivity, specificity and accuracy were 90.1%, 65.9% and 80.5%, respectively. Here, five of seven false-negative chemicals were acidic chemicals inducing the irregular rising of transepithelial electrical resistance values. In case of eliminating the test chemical solutions showing pH 5 or lower, the sensitivity, specificity and accuracy were improved to 96.8%, 67.4% and 84.4%, respectively. Meanwhile, nine of 16 false-positive chemicals were classified irritant by the US Environmental Protection Agency. In addition, the disappearance of ZO-1, a tight junction-associated protein and MUC1, a cell membrane-spanning mucin was immunohistologically confirmed in the HCE models after exposing not only eye irritant chemicals but also false-positive chemicals, suggesting that such false-positive chemicals have an eye irritant potential. These data demonstrated that the Vitrigel-EIT method could provide excellent predictive performance to judge the widespread eye irritancy, including very mild irritant chemicals. We hope that the Vitrigel-EIT method contributes to the development of safe commodity chemicals.

Keywords: collagen vitrigel membrane, corneal epithelium, eye irritation test

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Kojima H: The use of 3-D models as alternatives to animal testing.

Altern Lab Anim. 2015;43(4):P40-3.

A number of three-dimensional in vitro models are now available, but significant further developments are needed before their routine and widespread use as alternatives to animal testing will be possible.

Keywords: alternative, 3-D, toxicity

Speit G*1, Kojima H, Burlinson B*2, Collins AR*3, Kasper P*4, Plappert-Helbig U*5, Uno Y*6, Vasquez M*7, Beevers C*8, De Boeck M*9, Escobar PA*10, Kitamoto S*11, Pant K*12, Pfuhler S*13, Tanaka J*14, Levy DD*15: Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT).

Mutat Res Genet Toxicol Environ Mutagen. 2015; 783:6-12

As a part of the 6th IWGT, an expert working group on the comet assay evaluated critical topics related to the use of the in vivo comet assay in regulatory genotoxicity testing. The areas covered were: identification of the domain of applicability and regulatory acceptance, identification of critical

parameters of the protocol and attempts to standardize the assay, experience with combination and integration with other in vivo studies, demonstration of laboratory proficiency, sensitivity and power of the protocol used, use of different tissues, freezing of samples, and choice of appropriate measures of cytotoxicity. The standard protocol detects various types of DNA lesions but it does not detect all types of DNA damage. Modifications of the standard protocol may be used to detect additional types of specific DNA damage (e.g., cross-links, bulky adducts, oxidized bases). In addition, the working group identified critical parameters that should be carefully controlled and described in detail in every published study protocol. In vivo comet assay results are more reliable if they were obtained in laboratories that have demonstrated proficiency. This includes demonstration of adequate response to vehicle controls and an adequate response to a positive control for each tissue being examined. There was a general agreement that freezing of samples is an option but more data are needed in order to establish generally accepted protocols. With regard to tissue toxicity, the working group concluded that cytotoxicity could be a confounder of comet results. It is recommended to look at multiple parameters such as histopathological observations, organ-specific clinical chemistry as well as indicators of tissue inflammation to decide whether compound-specific toxicity might influence the result. The expert working group concluded that the alkaline in vivo comet assay is a mature test for the evaluation of genotoxicity and can be recommended to regulatory agencies for use.

Keywords: Genotoxicity testing, Test protocol, Tissue toxicity

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