Tanabe S, Aoyagi K[*1], Yokozaki H[*2], Sasaki H[*1]: Regulated genes in mesenchymal stem cells and gastric cancer.

World J Stem Cells. 2015;7:208-22.

AIM: To investigate the genes regulated in mesenchymal stem cells (MSCs) and diffuse-type gastric cancer (GC), gene expression was analyzed. METHODS: Gene expression of MSCs and diffuse-type GC cells were analyzed by microarray. Genes related to stem cells, cancer and the epithelial-mesenchymal transition (EMT) were extracted from human gene lists using Gene Ontology and reference information. Gene panels were generated, and messenger RNA gene expression in MSCs and diffuse-type GC cells was analyzed. Cluster analysis was performed using the NCSS software. RESULTS: The gene expression of regulator of G-protein signaling 1 (RGS1) was up-regulated in diffuse-type GC cells compared with MSCs. A panel of stem-cell related genes and genes involved in cancer or the EMT were examined. Stem-cell related genes, such as growth arrest-specific 6, musashi RNA-binding protein 2 and hairy and enhancer of split 1 (Drosophila), NOTCH family genes and Notch ligands, such as delta-like 1 (Drosophila) and Jagged 2, were regulated. CONCLUSION: Expression of RGS1 is up-regulated, and genes related to stem cells and NOTCH signaling are altered in diffuse-type GC compared with MSCs.

Keywords: Epithelial-mesenchymal transition, Gastric cancer, Gene

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Amakura Y[*1], Yamakami S[*1], Yoshimura M[*1], Yoshida T[*1], Fuchino H[*2], Goda Y, Kawahara N[*2]: High-performance thin layer chromatography data of representative crude drugs available on the Japanese market.

Pharmaceutical and Medical Device Regulatory Science 2014;45:510-8.

As a part of a project to create a Comprehensive Medicinal Plant Database of crude drugs, a high-performance thin layer chromatography (HPTLC) analysis of 19 representative crude drugs was performed according to the identification test in the sixteenth edition of the Japanese Pharmacopoeia(JP16). The crude drugs included in this study were: Angelicae Radix, Astragali Radix, Bupleuri Radix, Cinnamomi Cortex, Cnidii Rhizoma, Coptidis Rhizoma, Ephedrae Herba, Evodiae Fructus, Gardeniae Fructus, Ginseng Radix, Glycyrrhizae Radix, Moutan Cortex, Paeoniae Radix, Perillae Herba, Persicae Semen, Plantaginis Semen, Puerariae Radix, Rhei Rhizoma, and Zingiberis Rhizoma. More than five (5-25) products for each crude drug available on the market in Japan were compared in their HPTLC features imaging composition of the constituents. Crude drugs, for which a TLC confirmatory method has not been included in JP16, were investigated suitable analytical conditions. Thus, we were able to obtain HPTLC image data with good separation for all samples. The HPTLC data, which can be visually verified, would not only provide useful material in the database, but also constitute a rare example of estimation of chemical equivalence of a large number of crude drug products from the Japanese market.

Keywords: Crude drug, Japanese Pharmacopoeia, HPTLC

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Wakana D*, Itabashi T*, Kawai K-i*, Yaguchi, T*, Fukushima K*, Goda Y, Hosoe T*: Cytotoxic anthrasteroid glycosides, malsterosides A-C, from *Malbranchea filamentosa*.

Journal of Antibiotics 2014;67:585-8.

Malbranchea species belong to the family *Onygenaceae* and are taxonomically close to human and animal pathogenic fungi.1 The fact prompted us to investigate the chemical constituents of *Malbranchea* fungi. We already have reported the isolation and structural characterization of 4-benzyl-3-phenyl-5H-furan-2-one as a vasodilator, malfilanols A and B as antifungal and cytotoxic sesquiterpenes, malbrancheosides A–D as triterpene glycosides and malfilamentosides A and B as furanone glycosides, from the fungus *Malbranchea filamentosa* IFM41300. Further purification of extracts of rice cultivated by the above fungus allowed us to

isolate three new cytotoxic anthrasteroid glycosides, designated malsterosides A, B and C.
Keywords: *Malbranchea filamentosa*, cytotoxic anthrasteroid glycoside, Onygenaceae

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花岡信義*1，石塚康弘*1，林克彦*1，Tu P*2，神本敏弘*3，袴塚高志，合田幸広：生薬「肉蓯蓉」と「和肉蓯蓉」の基原植物の成分比較.
生薬学雑誌 2015;60:1-9.

Cistanche salsa（C. A. Meyer）G. Beck, *Cistanche deserticola* Y. C. Ma and *Cistanche tubulosa*（Schrenk）Wight（*Orobanchaseae*）, the sources of crude drug called Cistanche Herb（肉蓯蓉）, and *Boschniakia rossica*（Cham. et Schltdl.）B. Fedtsch. ex Fedtsch. et Flerov, the source of crude drug called Boschniakia Herb（和肉蓯蓉）were analyzed by high performance liquid chromatography（HPLC）systems attached the photo-diode array（PDA）detector and the charged aerosol detector（CAD）. Based on the HPLC chromatograms, *Boschniakia rossica* samples were easily distinguished from the *Cistanche* samples. About three *Cistanche* samples, the principal component analysis（PCA）based on the quantities of the 10 constituents; cistanoside F, echinacoside, cistanoside A, acteoside, tubuloside A, acteoside isomer, syringalide A 3'-α-L-rhamnopyranoside, cistanoside C, 2'-acetylacteoside and tubuloside B which of those having pharmacologically activities were carried out. The samples were not divided according to the plant species.
Keywords: Cistanche Herb, Boschniakia Herb, principal component analysis

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Wakana D, Kato H*, Momose T*, Sasaki N*, Ozeki Y*, Goda Y: NMR-based characterization of a novel yellow chlorophyll catabolite Ed-YCC isolated from *Egeria densa*.
Tetrahedron Letters 2014;55:2982-5.

A novel yellow chlorophyll catabolite, Ed-YCC, was isolated from leaves detached from Egeria densa shoots, in which chlorophyll degradation and anthocyanin synthesis were induced in 0.1 M fructose solution under light illumination as a plant senescence process, a model of autumnal leaf coloration. Structure elucidation was accomplished by various NMR techniques including 2D-INADEQUATE.
Keywords: chlorophyll, *Egeria densa*, yellow chlorophyll catabolite

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Hashimoto M*, Wakana D, Ueda M*, Kobayashi D*, Goda Y, Fujii I*: Product identification of non-reducing polyketide synthases with C-terminus methyltransferase domain from *Talaromyces stipitatus* using *Aspergillus oryzae* heterologous expression.
Bioorganic & Medicinal Chemistry Letters 2015;25:1381-4.

Talaromyces stipitatus ATCC 10500 possesses 17 non-reducing polyketide synthase（NR-PKS）genes. During the course of our functional analysis of PKS genes with a C-terminus methyltransferase domain from *T. stipitatus*, we expressed tspks2, tspks3 and tspks4 genes in the heterologous host *Aspergillus oryzae*, respectively. Although the tspks4 transformant gave no apparent product in HPLC analysis, a novel azaphilone pentaketide was identified along with two known related products from the tspks2 transformant. Of four hexaketide products from the tspks3 transformant, two new compounds were identified to be 2-acetyl-7-methyl-3,6,8-trihydroxynaphthalene and its derivative fused with α-methyl-α, β-unsaturated γ-lactone.
Keywords: polyketide biosynthesis, fungi, non-reducing polyketide synthase

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Izutsu K, Shibata H, Yoshida H, Goda Y: Miscibility as a determining factor for component crystallization in Multi-solute frozen solutions.
J Pharm Sci. 2014;103:2139-46.

The relationship between the miscibility of formulation ingredients and their crystallization during the freezing segment of the lyophilization process was studied. The thermal properties of frozen solutions containing myo-inositol and cosolutes were obtained by performing heating scans from -70 ℃ before and after heat treatment at -20 ℃ to -5 ℃ . Addition of dextran

40,000 reduced and prevented crystallization of myo-inositol. In the first scan, some frozen solutions containing an inositol-rich mixture with dextran showed single broad transitions (T_g's: transition temperatures of maximally freeze-concentrated solutes) that indicated incomplete mixing of the concentrated amorphous solutes. Heat treatment of these frozen solutions induced separation of the solutes into inositol-dominant and solute mixture phases (T_g' splitting) following crystallization of myo-inositol (T_g' shifting). The crystal growth involved myo-inositol molecules in the solute mixture phase. The amorphous-amorphous phase separation and resulting loss of the heteromolecular interaction in the freeze-concentrated inositol-dominant phase should allow ordered assembly of the solute molecules required for nucleation. Some dextran-rich and intermediate concentration ratio frozen solutions retained single T_g's of the amorphous solute mixture, both before and after heat treatments. The relevance of solute miscibility on the crystallization of myo-inositol was also indicated in the systems containing glucose or recombinant human albumin.

Keywords: freeze-drying, crystallization, protein formulation

Izutsu K, Yomota C, Okuda H, Kawanishi T, Yamaki T[*1], Ohdate R[*1], Yu Z[*1], Yonemochi E[*1,2], Terada K[*1]: Effects of formulation and process factors on the crystal structure of Freeze-dried *Myo*-Inositol.

J Pharm Sci. 2014;103:2347-55

The objective of this study was to elucidate effects of formulation and process variables on the physical forms of freeze-dried *myo*-inositol. Physical properties of *myo*-inositol in frozen solutions, freeze-dried solids, and cooled heat-melt solids were characterized by powder X-ray diffraction (PXRD), thermal analysis (differential scanning calorimetry [DSC] and thermogravimetric), and simultaneous PXRD-DSC analysis. Cooling of heat-melt *myo*-inositol produced two forms of metastable anhydrate crystals that change to stable form (melting point 225 ℃ -228 ℃) with transition exotherms at around 123 ℃ and 181 ℃, respectively. Freeze-drying of single-solute aqueous *myo*-inositol solutions after rapid cooling induced crystallization of *myo*-inositol as metastable anhydrate (transition at 80 ℃ -125 ℃) during secondary drying segment. Contrarily, post-freeze heat treatment (i.e., annealing) induced crystallization of *myo*-inositol dihydrate. Removal of the crystallization water during the secondary drying produced the stable-form *myo*-inositol anhydrate crystal. Shelf-ramp slow cooling of *myo*-inositol solutions resulted in the stable and metastable anhydrous crystal solids depending on the solute concentrations and the solution volumes. Colyophilization with phosphate buffer retained *myo*-inositol in the amorphous state. Crystallization in different process segments varies crystal form of freeze-dried *myo*-inositol solids.

Keywords: freeze-drying, crystal polymorphism, amorphous solids

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Izutsu K, Yonemochi E[*], Yomota C, Goda Y, Okuda H: Studying the morphology of lyophilized protein solids using X-Ray micro CT: effect of Post-freeze annealing and controlled nucleation.

AAPS PharmSciTech 2014;15:1181-8.

The objective of this study was to determine how different techniques used during the freezing step of lyophilization affect morphology of the dried protein solids. Aqueous solutions containing recombinant human albumin, trehalose, and sodium phosphate buffer were dried after their freezing by shelf-ramp cooling, immersion in liquid nitrogen, or controlled ice nucleation. Some shelf-frozen solutions were heat treated (annealed) before the vacuum drying. We used three-dimensional (3D) X-ray micro-computed tomography (micro-CT) and scanning electron microscopy (SEM) to study the morphology of solids. The X-ray micro-CT images of the lyophilized microporous solids showed traces of varied size and structure ice crystals that were comparable to corresponding SEM images. A post-freeze heat treatment and a controlled nucleation both induced larger ice crystal ghosts in the solids. The variations in the structure of walls surrounding ice crystals, formed by the different freezing procedures, should affect the water vapor transition during the primary and secondary drying. Some solids also showed higher-density layer in the upper surface. Overall, the simple sample preparation procedures and the ample morphological information make the X-ray micro-CT

appropriate for analyzing lyophilized pharmaceuticals.
Keywords: controlled nucleation, freeze-drying, X-ray micro-CT

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吉田寛幸，伊豆津健一，柴田寛子，桑名明美，合田幸広：リザーバー式吸入粉末剤における振とう操作と薬物放出量に関する検討.
医療薬学 2015;41:50-5.

リザーバー式吸入粉末剤の振とう操作が薬物放出量に及ぼす影響について，薬物サンプリング器具を用いて検討した．振とう操作を行なわなかったデバイスからのPH放出量は，表示量に対して著しく低く，またバラつきが大きかったことから，吸入粉末剤からの十分な薬物放出には，振とう操作は必須であると考えられた．振とう操作に代わり，デバイスをタッピングする方法を試みたところ，主薬の効率的な放出が可能であった．同製剤に充填されている薬物粒子のサイズは，他の振とう操作を要しないリザーバー式吸入粉末剤と比較して小さく，デバイスの振とう操作が重要となる一因として考えられた．振とう操作を要するリザーバー式吸入粉末剤を使用するにあたっては，医療従事者から患者に対し適切な吸入指導を行なうことの重要性が確認された.
Keywords: 吸入粉末剤，薬物放出量，振とう操作

宮崎玉樹，阿曽幸男，奥田晴宏：アルファー化デンプンと部分アルファー化デンプンの識別に関する研究.
医薬品医療機器レギュラトリーサイエンス 2014;45:519-28.

Monograph of pregelatinized starch is under discussion for harmonization among the Japanese, United States and European pharmacopeia（JP, USP and EP）. In JP, two individual monographs, “Pregelatinized Starch” and “Partly Pregelatinized Starch” are listed according to the degree of gelatinization, while in USP and EP, these two types of pregelatinized starch are listed as one monograph. As a matter of policy of JP, monograph of“ Pregelatinized Starch” and “Partly Pregelatinized Starch” should be harmonized individually, because the two types of pregelatinized starch are used with different purpose depending on their degree of gelatinization. Therefore, identification tests which can discriminate among partly pregelatinized starches, “totally” pregelatinized starches and starch definitely are required. In this paper, we propose identification tests for this purpose: First, microscopic observation under polarized light was done. Obvious birefringent feature could be seen for starch granules without gelatinization, and the granules showed a distinct black cross intersecting at the hilum. Most of the partly pregelatinized starch granules also showed the birefringent feature. On the contrary, “totally” pregelatinized starch granules did not indicate detectable level of birefringence. Next, sample powder-water slurry（0.5 g in 25 mL）was centrifuged at 3500 rpm for 15 minutes, and the color reaction of the supernatant liquid was observed when the iodine solution was added. Starches without gelatinization showed no apparent reaction, while both partly and “totally” pregelatinized starches gave a deep blue or reddish-violet color. Starches in three levels of gelatinization（without, partly and totally）could be discriminated by judging from the both results of the microscopic observation with polarized light and the starch-iodine test of the supernatant.
Keywords: Pregelatinized starch, Identification, Starch-iodine test

香取典子，坂本知昭，小出達夫：日本薬局方における品質試験と製造工程管理：プロセス解析工学（PAT）と新たな品質パラダイム.
レギュラトリーサイエンス学会誌 2014;4:177-87.

The basic quality concept in Pharmacopoeias has been “Quality by Test”, a quality assurance with the test standard. However, recently, the new concept in quality assurance for pharmaceuticals called QbD（Qality by Design）is widely used. QbD is a systematic approach based on scientific principles. New analytical technologies such as Near Infrared Spectroscopy（NIR）, terahertz and Raman spectroscopy enable to measure a lot of products rapidly without destroying products in manufacturing process. In this article, we introduce these new technologies for Process analytical technology（PAT）and indicate situation that Pharmacopoeias facing with the need to incorporate a more advanced concept such as QbD also the way of quality assurance.
Keywords: Pharmaceutical Quality System（PQS）, PAT, QbD

Fluhler E[*1], Hayes R[*2], Garofolo F[*3], Dumont I[*3], Blaye OL[*4], Arnold M[*5], Bansal S[*6], Verhaeghe T[*7], Wilson A[*8], Stevenson L[*9], Myler H[*5], Bauer R[*10], Bergeron A[*3], Bustard M[*11], Cai XY[*12], Carbone M[*3], Cojocaru L[*13], Desai-Krieger D[*14], Duggan J[*28], Haidar S[*15], Ho S[*16], Ingelse B[*17], Katori N, Lévesque A[*18],

Lowes S[*19], Ma M[*20], Mettke K[*21], Michon J[*22], Musuku A[*23], Olah T[*5], Patel S[*23], Rose M[*20], Schultz G[*19], Smeraglia J[*24], Spooner N[*25], Stouffer B[*5], Vazvaei F[*6], Wakelin-Smith J[*26], Wang J[*5], Welink J[*27], Whale E[*26], Woolf E[*28], Xue L[*29], Yang TY[*23]: 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis（Part 1 – small molecules by LCMS）.
Bioanalysis 2014;6:3039-49.

The 2014 8th Workshop on Recent Issues in Bioanalysis（8th WRIB）, a 5-day full immersion in the evolving field of bioanalysis, took place in Universal City, California, USA. Close to 500 professionals from pharmaceutical and biopharmaceutical companies, contract research organizations and regulatory agencies worldwide convened to share, review, discuss and agree on approaches to address current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS, LBA approaches and immunogenicity. From the prolific discussions held during the workshop, specific recommendations are presented in this 2014 White Paper. As with the previous years' editions, this paper acts as a practical tool to help the bioanalytical community continue advances in scientific excellence, improved quality and better regulatory compliance. Due to its length, the 2014 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication（Part 1）covers the recommendations for small molecule bioanalysis using LCMS. Part 2（Hybrid LBA/LCMS, Electronic Laboratory Notebook and Regulatory Agencies' input）and Part 3（Large molecules bioanalysis using LBA and Immunogenicity）will be published in the upcoming issues of Bioanalysis.

Keywords: LC-MS, BMV Guideline, Regulated Bioanalysis

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Dufield D[*1], Neubert H[*1], Garofolo F[*2], Kirkovsky L[*3], Stevenson L[*4], Dumont I[*2], Kaur S[*5], Xu K[*5], Alley SC[*6], Szapacs M[*7], Arnold M[*8], Bansal S[*9], Haidar S[*10], Welink J[*11], Le Blaye O[*12], Wakelin- Smith J[*13], Whale E[*13], Ishii-Watabe A, Bustard M[*14], Katori N, Amaravadi L[*4], Aubry AF[*8], Beaver C[*15], Bergeron A[*2], Cai XY[*16], Cojocaru L[*17], DeSilva B[*8], Duggan J[*18], Fluhler E[*19], Gorovits B[*1], Gupta S[*20], Hayes R[*21], Ho S[*22], Ingelse B[*23], King L[*24], Lévesque A[*25], Lowes S[*26], Ma M[*27], Musuku A[*28], Myler H[*8], Olah T[*8], Patel S[*29], Rose M[*27], Schultz G[*26], Smeraglia J[*30], Swanson S[*27], Torri A[*31], Vazvaei F[*9], Wilson A[*32], Woolf E[*33], Xue L[*1], Yang TY[*29]: 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis（Part 2 – hybrid LBA/LCMS, ELN & regulatory agencies' input）.
Bioanalysis 2014;6:3237-49.

The 2014 8th Workshop on Recent Issues in Bioanalysis（8th WRIB）, a 5-day full immersion in the evolving field of bioanalysis, took place in Universal City, California, USA. Close to 500 professionals from pharmaceutical and biopharmaceutical companies, contract research organizations and regulatory agencies worldwide convened to share, review, discuss and agree on approaches to address current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS, LBA approaches and immunogenicity. From the prolific discussions held during the workshop, specific recommendations are

presented in this 2014 White Paper. As with the previous years' editions, this paper acts as a practical tool to help the bioanalytical community continue advances in scientific excellence, improved quality and better regulatory compliance. Due to its length, the 2014 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication（Part 2）covers the recommendations for Hybrid LBA/LCMS, Electronic Laboratory Notebook and Regulatory Agencies' Input. Part 1（Small molecules bioanalysis using LCMS）was published in the Bioanalysis issue 6（22）and Part 3（Large molecules bioanalysis using LBA and Immunogenicity）will be published in the Bioanalysis issue 6(24).

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

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*15 inVentiv Health Clinical
*16 Merck, Kenilworth
*17 Tandem Labs
*18 Boehringer-Ingelheim
*19 Pfizer, Pearl River
*20 Allergan Inc.
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*24 Pfizer, Groton
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Un K, Sakai-Kato K. Goda Y: Intracellular trafficking mechanism of cationic phospholipids including cationic liposomes in HeLa cells.
Pharmazie. 2014;69:525-31.

The development of gene delivery methods is essential for the achievement of effective gene therapy. Elucidation of the intracellular transfer mechanism for cationic carriers is in progress, but there are few reports regarding the intracellular trafficking processes of the cationic phospholipids taken up into cells. In the present work, the trafficking processes of a cationic phospholipid（1,2-dioleoyl-3-trimethylammonium-propane, DOTAP）were investigated from intracellular uptake to extracellular efflux using cationic liposomes *in vitro*. Following intracellular transport of liposomes *via* endocytosis, DOTAP was localized in the endoplasmic reticulum, Golgi apparatus, and mitochondria. Moreover, the proteins involved in DOTAP intracellular trafficking and extracellular efflux were identified. In addition, helper lipids of cationic liposomes were found to partially affect this intracellular trafficking. These findings might provide valuable information for designing cationic carriers and avoiding unexpected toxic side effects derived from cationic liposomal components.

Keywords: liposome, intracellular trafficking, Cationic phospholipids

Inoue M*1, Kamada H*1,3, Abe Y, Higashisaka K*2, Nagano K*1,2, Mukai Y*1,2, Yoshioka Y*1,3, Tsutsumi Y*1-3, Tsunoda S*1-3: Aminopeptidase P3, a new member of the TNF-TNFR2 signaling complex, induces phosphorylation of JNK1 and JNK2.
J Cell Sci. 2015;128:656-69.

Tumor necrosis factor（TNF）is an important mediator that triggers onset of autoimmune diseases and exerts its biological effects by interacting through two receptors, TNFR1（also known as TNFRSF1A）and TNFR2（also known as TNFRSF1B）. TNFR2 signaling has significant potential to exert pro-survival and protective roles in several diseases. Unlike TNFR1 signaling, however, the mechanism of TNFR2 signal transduction is poorly understood, and few of its adaptor molecules are known. The present study utilized a proteomics approach to search

for adaptor molecules in the TNFR2 signaling complex and identified aminopeptidase P3（APP3, also known as XPNPEP3）to be a key molecule. One of its two isoforms, mitochondrial APP3（APP3m）but not cytosolic APP3（APP3c), was recruited to TNFR2 and shown to regulate TNF-TNFR2-dependent phosphorylation of JNK1（also known as MAPK8）and JNK2（also known as MAPK9). Furthermore, APP3m was released from mitochondria upon TNF stimulation in the absence of mitochondrial outer membrane permeabilization（MOMP). The observation of increased cell death upon downregulation of APP3m also suggested that APP3m exerts an anti-apoptotic function. These findings reveal that APP3m is a new member of the TNF-TNFR2 signaling complex and characterize an APP3-mediated TNFR2 signal transduction mechanism that induces activation of JNK1 and JNK2.

Keywords: Aminopeptidase P3, JNK, TNFR2

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Takakura D, Harazono A, Hashii N, Kawasaki N: Selective glycopeptide profiling by acetone enrichment and LC/MS.

Journal of Proteomics 2014;101:17-30.

LC/MS is commonly used for site-specific glycosylation analysis of glycoproteins in cells and tissues. A limitation of this technique is the difficulty in acquiring reliable mass spectra for glycopeptides, mainly due to their high heterogeneity and poor hydrophobicity. Here, we establish a versatile method for efficient glycopeptide enrichment to acquire reliable mass spectra. Several lines of evidence using model glycoproteins suggest that our method is based on the different solubility between non-glycosylated and glycosylated peptides in acetone. We also provide data showing that the acetone-precipitated glycopeptide enrichment was successful in acquiring a more comprehensive MS/MS data set for the various glycoforms of each glycopeptide in crude human serum. We propose that this method is a powerful tool for the acquisition of reliable mass spectra from trace amounts of glycopeptides and an alternative to lectin affinity enrichment.

Keywords: Glycopeptides, Acetone enrichment, Glycoproteomics

橋井則貴，蛭田葉子，渡部沙木絵*，森岡知子*，海老澤亜樹子*，中川ゆかり*，川崎ナナ：日本薬局方ヘパリンナトリウム各条のエンドトキシン試験法に関する研究.

医薬品医療機器レギュラトリーサイエンス 2014;45(4):345-54.

The bacterial endotoxins test is adopted in Japanese Pharmacopoeia（JP）Heparin Calcium and Heparin Sodium Injection monographs as well as United States Pharmacopeia and European Pharmacopoeia Heparin Sodium monographs, whereas the pyrogen test is still adopted in the JP XVI Heparin Sodium monograph. The replacement for the pyrogen test to bacterial endotoxins test is required on the basis of an alternative to animal experiments and for international harmonization. In this study, we evaluated the applicability of JP bacterial endotoxins test <4.01> to JP Heparin Sodium monograph by examining the interference effect of heparin sodium on the bacterial endotoxins test with commercially available lysate reagents.

Keywords: heparin sodium, Japanese Pharmacopoeia, bacterial endotoxin test

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Tada M, Ishii-Watabe A, Suzuki T, Kawasaki N: Development of a cell-based assay measuring the activation of FcγRIIa for the characterization of therapeutic monoclonal antibodies.

PLOS ONE 2014;9(4):e95787.

Antibody-dependent cellular cytotoxicity（ADCC）is one of the important mechanisms of action of the targeting of tumor cells by therapeutic monoclonal antibodies（mAbs). Among the human Fcγ receptors（FcγRs), FcγRIIIa is well known as the only receptor expressed in natural killer（NK）cells, and it plays a pivotal role in ADCC by IgG1-subclass mAbs. In addition, the contributions of FcγRIIa to mAb-mediated cytotoxicity have been reported. FcγRIIa is expressed in myeloid effector cells including neutrophils and macrophages, and it is involved in the activation of these effector cells. However, the measurement of the cytotoxicity via FcγRIIa-expressing effector cells is

complicated and inconvenient for the characterization of therapeutic mAbs. Here we report the development of a cell-based assay using a human FcγRIIa-expressing reporter cell line. The FcγRIIa reporter cell assay was able to estimate the activation of FcγRIIa by antigen-bound mAbs by a very simple method in vitro. The usefulness of this assay for evaluating the activity of mAbs with different abilities to activate FcγRIIa was confirmed by the examples including the comparison of the activity of the anti-CD20 mAb rituximab and its Fc-engineered variants, and two anti-EGFR mAbs with different IgG subclasses, cetuximab (IgG1) and panitumumab (IgG2). We also applied this assay to the characterization of a force-oxidized mAb, and we observed that oxidation significantly decreased the FcγRIIa activation by EGFR-bound cetuximab. These results suggest that our FcγRIIa reporter assay is a promising tool for the characterization of therapeutic mAbs, including Fc-engineered mAbs, IgG2-subclass mAbs, and their product-related variants.
Keywords: monoclonal antibody, ADCC, FcγRIIa

Hashii N, Harazono A, Kuribayashi R, Takakura D, Kawasaki N: Characterizations of N-Glycan heterogeneities of erythropoietin products by liquid chromatography/mass spectrometry and multivariate analysis.
Rapid Commun Mass Spectrum. 2014;28(8):921-32.

Glycan heterogeneity on recombinant human erythropoietin (rEPO) product is considered to be one of the critical quality attributes, and similarity tests of glycan heterogeneities are required in the manufacturing process changes and developments of biosimilars. A method for differentiating highly complex and diverse glycosylations is needed to evaluate comparability and biosimilarity among epoetin lots and products manufactured by different processes. The glycan heterogeneities of 9 rEPO products (4 innovator products and 5 biosimilar products) were distinguished by multivariate analysis (MVA) using the peak area ratios of each glycan to the total peak area of glycans in mass spectra obtained by liquid chromatography/mass spectrometry (LC/MS) of N-glycans from rEPOs. Principal component analysis (PCA) using glycan profiles obtained by LC/MS of N-glycans from rEPOs proved to be a useful method for differentiating glycan heterogeneities among 9 rEPOs. Using PC values as indices, we were able to visualize and digitalize the glycan heterogeneities of each rEPO. The characteristic glycans of each rEPO were also successfully identified by orthogonal partial least squares discrimination analysis (OPLS-DA), an MVA method, using the mass spectrometric data. PCA values were useful for evaluating the relative differences among the glycan heterogeneities of rEPOs. The characteristic glycans that contributed to the differentiation were also successfully identified by OPLS-DA. PCA and OPLS-DA based on mass spectrometric data are applicable for distinguishing glycan heterogeneities, which are virtually indistinguishable on rEPO products.
Keywords: multivariate analysis, glycan heterogeneity, erythropoietin

小林哲，遊佐敬介，川崎ナナ：抗体医薬品及び免疫抑制作用を有する各種薬剤の投与症例におけるウイルス感染プロファイルの比較とこれを利用したウイルス感染のリスク分析.
医薬品医療機器レギュラトリーサイエンス 2014;45(5):436-41.

In order to compare viral infection profiles during treatment with various immunosuppressants, viral infection case reports were extracted from open-source data available in the form of spontaneous reports published on the homepage of the Pharmaceutical and Medical Devices Agency on February 1, 2013. Among a total of 1920 cases extracted, cytomegalovirus (CMV) infection was reported in 761 cases, and varicella-zoster virus (VZV) infection was reported in 690 cases. CMV was common after treatment with basiliximab or micophenolate mofetil (77% and 62%, respectively), and VZV was predominant after treatment with adalimumab, infliximab, etanercept, or tocilizumab (97%, 72%, 86%, and 82%, respectively). In addition, BK virus, Epstein-Barr virus (EBV), herpes simplex virus, JC virus, parvovirus B19, adenovirus, and RS virus infections were reported in 167, 108, 62, 52, 37, 32, and 11cases, respectively. Risk analysis of each virus was performed based on the likelihood of infection (reported number of cases) and the severity of outcome (percentage of serious outcomes). JCV, EBV, and CMV received the high scores in this risk analysis.
Keywords: viral infection, immunosuppressant drugs, risk analysis

Isaji T[*1], Im S[*1], Gu W[*1], Wang Y[*1], Hang Q[*1], Lu J[*1], Fukuda T, Hashii N, Takakura D, Kawasaki N, Miyoshi

H[*2], Gu J[*1]: An oncogenic protein golgi Phosphoprotein 3 Up-regulates cell migration via sialylation.
J Biol Chem. 2014;289(30):20694-705.

Recently, the Golgi phosphoprotein 3 (GOLPH3) and its yeast homolog Vps74p have been characterized as essential for the Golgi localization of glycosyltransferase in yeast. GOLPH3 has been identified as a new oncogene that is commonly amplified in human cancers to modulate mammalian target of rapamycin signaling. However, the molecular mechanisms of the carcinogenic signaling pathway remain largely unclear. To investigate whether the expression of GOLPH3 was involved in the glycosylation processes in mammalian cells, and whether it affected cell behavior, we performed a loss-of-function study. Cell migration was suppressed in GOLPH3 knockdown (KD) cells, and the suppression was restored by a re-introduction of the GOLPH3 gene. HPLC and LC/MS analysis showed that the sialylation of N-glycans was specifically decreased in KD cells. The specific interaction between sialyltransferases and GOLPH3 was important for the sialylation. Furthermore, overexpression of α2,6-sialyltransferase-I rescued cell migration and cellular signaling, both of which were blocked in GOLPH3 knockdown cells. These results are the first direct demonstration of the role of GOLPH3 in N-glycosylation to regulate cell biological functions.

Keywords: Phosphoprotein 3, cell migration, sialylation

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Kawasaki N, Okumoto T[*1], Yamaguchi Y[*1,2], Takahashi N[*1], Fridman WH[*3], Sautès-Fridman C[*3], Yagi H[*1], Kato K[*1,4-6]: Site-specific classification of N-linked oligosaccharides of the extracellular regions of Fcγ receptor IIIb expressed in baby hamster kidney cells.
J Glycomics Lipidomics. 2014;4(2):1000116.

Human Fcγ receptor III (FcγRIII) consists of two isoforms that are encoded by two individual genes: transmembrane FcγRIIIa and glycosylphosphatidylinositol-linked FcγRIIIb. Both isoforms can exist as a soluble form (sFcγRIII), which is composed of their extracellular region produced by proteolytic cleavage. FcγRIII-mediated immunological functions such as antibody-dependent cell-mediated cytotoxicity and phagocytosis critically depend on the N-glycosylation of FcγRIII molecules. In our previous study, high-performance liquid chromatography-based profiling indicated that N-linked oligosaccharides released from the NA2 allele of human sFcγRIIIb expressed in baby hamster kidney cells are composed of high-mannose-type oligosaccharides and core-fucosylated complex-type oligosaccharides. Here we successfully classified the N-glycans of this glycoprotein into these two types at each of the six N-glycosylation sites by liquid chromatography (LC)-electrospray tandem mass spectrometry analysis combined with endoglycosidase treatments. Our results indicated that four sites of sFcγRIIIb, Asn38, Asn74, Asn162, and Asn169, expressed only complex-type oligosaccharides, while the remaining two sites, Asn45 and Asn64 (both are not conserved in the NA1 allele), were occupied by not only complextype oligosaccharides but also high-mannose-type oligosaccharides, which are thought to be involved in the interaction of FcγRIIIb with complement receptor type 3. Together with the previously reported site-specific N-glycosylation profiling of recombinant sFcγRIIIa, this study underlines that both sFcγRIIIa and sFcγRIIIb produced in different production vehicles express core-fucosylated complex-type oligosaccharides as the major glycoforms at Asn74 and Asn162. These finding provide insights into the design and development of therapeutic antibodies because the Asn162 N-glycan significantly contributes to immunoglobulin G binding.

Keywords: Site-specific classification, N-linked oligosaccharides, Fcγ receptor IIIb

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Kitazume S[*1], Imamaki R[*1], Kurimoto A[*1], Ogawa K[*1], Kato M[*2], Yamaguchi Y[*2], Tanaka K[*3], Ishida H[*4], Ando H[*4], Kiso M[*4], Hashii N, Kawasaki N, Taniguchi N[*1]: Interaction of PECAM with α2,6-sialylated glycan regulates its cell surface residency and anti-apoptotic role.
J Biol Chem. 2014;289(40):27604-13.

The luminal sides of vascular endothelial cells are heavily covered with a so-called glycocalyx, but the precise role of the endothelial glycocalyx remains unclear. Our previous study showed that N-glycan α2,6-sialylation

regulates the cell surface residency of an anti-apoptotic molecule, platelet endothelial cell adhesion molecule (PECAM), as well as the sensitivity of endothelial cells toward apoptotic stimuli. As PECAM itself was shown to be modified with biantennary N-glycans having α2,6-sialic acid, we expected that PECAM would possess lectin-like activity toward α2,6-sialic acid to ensure its homophilic interaction. To verify this, a series of oligosaccharides were initially added to observe their inhibitory effects on the homophilic PECAM interaction in vitro. We found that a longer α2,6-sialylated oligosaccharide exhibited strong inhibitory activity. Furthermore, we found that a cluster-type α2,6-sialyl N-glycan probe specifically bound to PECAM-immobilized beads. Moreover, the addition of the α2,6-sialylated oligosaccharide to endothelial cells enhanced the internalization of PECAM as well as the sensitivity to apoptotic stimuli. Collectively, these findings suggest that PECAM is a sialic acid binding lectin and that this binding property supports endothelial cell survival. Notably, our findings that α2,6-sialylated glycans influenced the susceptibility to endothelial cell apoptosis shed light on the possibility of using a glycan-based method to modulate angiogenesis.

Keywords: PECAM, α2,6-sialylated glycan, surface residency and anti-apoptotic role

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Li X*1, Iida M*1, Tada M, Watari A*1, Kawahigashi M*1, Kimura Y*1, Yamashita T*1, Ishii-Watabe A, Uno T*1, Fukasawa M*2, Kuniyasu H*3, Yagi K*1, Kondoh M*1: Development of an Anti–Claudin-3 and -4 bispecific monoclonal antibody for cancer diagnosis and therapy. *J Pharmacol Exp Ther*. 2014;351:206-13.

Most malignant tumors are derived from epithelium, and claudin (CLDN)-3 and CLDN-4 are frequently overexpressed in such tumors. Although antibodies have potential in cancer diagnostics and therapy, development of antibodies against CLDNs has been difficult because the extracellular domains of CLDNs are too small and there is high homology among human, rat, and mouse sequences. Here, we created a monoclonal antibody that recognizes human CLDN-3 and CLDN-4 by immunizing rats with a plasmid vector encoding human CLDN-4. A hybridoma clone that produced a rat monoclonal antibody recognizing both CLDN-3 and -4 (clone 5A5) was obtained from a hybridoma screen by using CLDN-3- and -4-expressing cells; 5A5 did not bind to CLDN-1-, -2-, -5-, -6-, -7-, or -9-expressing cells. Fluorescence-conjugated 5A5 injected into xenograft mice bearing human cancer MKN74 or LoVo cells could visualize the tumor cells. The human-rat chimeric IgG1 monoclonal antibody (xi5A5) activated FcγRIIIa in the presence of CLDN-3- or -4-expressing cells, indicating that xi5A5 may exert antibody-dependent cellular cytotoxicity. Administration of xi5A5 attenuated tumor growth in xenograft mice bearing MKN74 or LoVo cells. These results suggest that 5A5 shows promise in the development of a diagnostic and therapeutic antibody for cancers.

Keywords: Claudin, monoclonal antibody, cancer therapy

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Lu J*, Isaji T*, Im S*, Fukuda T*, Hashii N, Takakura D*, Kawasaki N, Gu J*: β-Galactoside α2,6-sialyltranferase 1 promotes transforming growth factor-β-mediated epithelial-mesenchymal transition. *J Biol Chem*. 2014;289(50):34627-41.

β-Galactoside α2,6-sialyltranferase 1 (ST6GAL1) catalyzes the addition of terminal α2,6-sialylation to N-glycans. Increased expression of ST6GAL1 has been reported in diverse carcinomas and highly correlates with tumor progression. Here, we report that St6gal1 transcription and α2,6-sialylated N-glycans are up-regulated during TGF-β-induced epithelial-mesenchymal transition (EMT) in GE11 cells, requiring the Sp1 element within the St6gal1 promoter. Knockdown of St6gal1 strongly suppressed TGF-β-induced EMT with a concomitant increase in E-cadherin expression, a major determinant of epithelial cell adherens junctions. Conversely, overexpression of ST6GAL1 increased the turnover of cell surface E-cadherin and promoted TGF-β-induced EMT. Overexpressing β-galactoside α2,3-sialyltranferase 4 had little influence on EMT, indicating specificity for α2,6-sialylation. The basal mesenchymal phenotype of MDA-MB-231 human breast cancer cells was partially reversed by ST6GAL1 silencing. Moreover, ST6GAL1 knockdown inhibited the phosphorylation of

Akt, but not Smad2, suggesting that ST6GAL1 contributes to EMT through a non-Smad signaling pathway. Taken together, our data indicate that ST6GAL1 promotes TGF-β-dependent EMT as well as maintenance of the mesenchymal state by growth signaling, providing a plausible mechanism whereby up-regulated ST6GAL1 may promote malignant progression.
Keywords: β-Galactoside α2,6-Sialyltranferase 1, Transforming Growth Factor-β, Epithelial-Mesenchymal Transition

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Zaima K, Wakana D, Demizu Y, Kumeta Y, Kamakura H, Maruyama T, Kurihara M, Goda Y: Isoheleproline: a new amino acid-sesquiterpene adduct from *Inula helenium*.
J Nat Med. 2014;68:432-5.

A new amino acid-sesquiterpene adduct, isoheleproline (**1**), was isolated from the roots of *Inula helenium* (elecampane), together with four known sesquiterpene lactones (**2–5**). The planar configuration of **1** was elucidated on the basis of spectroscopic data analysis, and the relative configuration of **1** was determined by performing a detailed analysis of NOESY correlations and comparing its physicochemical data with $_{D}$- and $_{L}$-proline adducts of **2** obtained by Michael addition. This is the first report of a new amino acid-sesquiterpene adduct from *Inula* plants.
Keywords: *Inula helenium*, Amino acid-sesquiterpene adduct, Sesquiterpene lactone

Oshima N, Zaima K, Kamakura H, Hamato A[*1], Yamamoto Y[*1], Kang DH[*1], Yokokura T[*2], Goda Y, Hakamatsuka T, Maruyama T: Identification of marker compounds for Japanese Pharmacopoeia non-conforming jujube seeds from Myanmar.
J Nat Med. 2015;69:68-75.

Jujube seed is a crude drug defined as the seed of *Ziziphus jujuba* Miller var. *spinosa* Hu ex H.F. Chou (Rhamnaceae) in the Japanese Pharmacopoeia (JP). Most of the jujube seed in the Japanese markets is imported from China, with the rest obtained from other Asian countries. Here we confirmed the botanical origins of jujube seeds from both China and Myanmar by a DNA sequencing analysis. We found that the botanical origins of the crude drugs from China and Myanmar were *Z. jujuba* and *Z. mauritiana*, respectively. Although the jujube seed from China conforms to the JP, that from Myanmar does not. A method for discriminating jujube seeds from China and Myanmar using a chemical approach is thus desirable, and here we sought to identify a compound specific to *Z. jujuba*. Jujuboside A (1) was identified as a compound specific to *Z. jujuba*. To establish a purity test of Jujube Seed in the JP against *Z. mauritiana*, we fractionated the extract of *Z. mauritiana* seeds and identified frangufoline (2) and oleanolic acid (4) as the marker compounds specific to *Z. mauritiana*. Thin-layer chromatography (TLC) and gas chromatography-mass spectrometry analyses revealed that the latter compound was useful for testing by TLC analysis. The established TLC conditions were as follows: chromatographic support, silica gel; developing solvent, *n*-hexane:EtOAc:HCOOH = 10:5:1; developing length, 7 cm; visualization, diluted sulfuric acid; R_f value, 0.43 (oleanolic acid).
Keywords: Jujube Seed, *Ziziphus jujuba* var. *spinosa*, *Ziziphus mauritiana*

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若菜大悟[*1]，丸山卓郎，在間一将，武田尚[*1]，杉村康司[*2]，安食菜穂子[*2]，飯田修[*2]，川原信夫[*2]，合田幸広，細江智夫[*1]：^{1}H-NMR-メタボロミクスによるショウガ抽出エキスの規格化.
日食化誌 2014;21:135-8.

Ginger (*Zingiber officinale*) is well-known spice and cultured on a temperate region. We attempted to standardize the ginger using ^{1}H-NMR-metabolomics because the standardization of the ginger has been provided by the quantity of one or a small number of compounds detected in ginger. The score plot of principal component analysis (PCA) using ^{1}H-NMR of the ginger aqueous extract showed some outliers. The results of conducting PCA to the ginger extract except the outliers showed that there are differences between Kintoki species and Amami native species, and between China L5 species and Sanshu Kochi species, China L4 species. Furthermore, we tried to OPLS-DA to evaluate the varietal variation in chemical components. The results showed that sucrose, glucose, alanine, arginine, asparagine, malic acid and gingerol are important factors for the classification of the ginger.
Keywords: ginger, metabolomics, ^{1}H-NMR

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Horii H*, Okonogi A*, Okubo T*, Kamakura H, Goda Y: Studies on bioequivalence of Shoseiryuto decoction and its extract preparation (I).
Shoyakugaku Zasshi 2014;68:65-9.

In a previous report (Horii, C., *et al*., *Shoyakugaku Zasshi*, 68 (1), 9-12, 2014), we studied the bioequivalence of Kakkonto decoction and its extract preparation and the result suggested that ephedrine (E) and pseudoephedrine (PE) from Ephedrae Herba might be used as marker compounds for a bio-equivalence judgment between preparations. In this study, we deal with Shoseiryuto, the formulation of which also involves Ephedrae Herba. A crossover study was performed involving 6 healthy adult males as study participants randomly divided into 2 groups. A change in concentrations of the two marker compounds, E and PE, in human blood plasma was observed after their oral administration. As the results, no significant differences in the plasma levels between the decoction and the product were noted at any sampling times. Variance analysis of the maximum plasma concentration (C_{max}) and the area under the plasma concentration-time curve (AUC) on both E and PE revealed no significant differences between the decoction and the product or between the administration days. The statistical power (1-β) is determined to be insufficient (less than 80%) for both C_{max} and AUC on E and PE. However, assuming that the standard deviation is the same as our result for E, when the number of the study participants is 10 it is revealed that its statistical power becomes sufficient (more than 80%) for both C_{max} and AUC on E. Since E and PE are known to be important biologically active components in Shoseiryuto preparations as well as Kakkonto ones, these results also suggest that E and PE may be used as the marker compounds for their bio-equivalence judgment, although further studies on bio-marker compounds derived from other crude drugs than Ephedrae Herba are needed to discuss this issue.

Keywords: bio-equivalence, Shoseiryuto, blood plasma level

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Suzuki M*, Miyahara T*, Tokumoto H, Hakamatsuka T, Goda Y, Ozeki Y*, Sasaki N*: Transposon-mediated mutation of CYP76AD3 affects betalain synthesis and produces variegated flowers in four o'clock (*Mirabilis jalapa*).
Journal of Plant Physiology 2014;171:1586-90.

The variegated flower colors of many plant species have been shown to result from the insertion or excision of transposable elements into genes that encode enzymes involved in anthocyanin synthesis. To date, however, it has not been established whether this phenomenon is responsible for the variegation produced by other pigments such as betalains. During betalain synthesis in red beet, the enzyme CYP76AD1 catalyzes the conversion of $_{L}$-dihydroxyphenylalanine (DOPA) to *cyclo*-DOPA. RNA sequencing (RNA-seq) analysis indicated that the homologous gene in four o'clock (*Mirabilis jalapa*) is *CYP76AD3*. Here, we show that in four o'clock with red perianths, the *CYP76AD3* gene consists of one intron and two exons; however, in a mutant with a perianth showing red variegation on a yellow background, a transposable element, *dTmj1*, had been excised from the intron. This is the first report that a transposition event affecting a gene encoding an enzyme for betalain synthesis can result in a variegated flower phenotype.

Keywords: Betalain, *En/Spm* (CACTA) transposable element, Four o'clock

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Kammoto T*1,2, Yomura K*2, Kikuchi Y*2, Hirakura K*2, Makino B*2, Hashimoto K*2, Nishimura H*2, Usui K*2, Hakamatsuka T, Goda Y, Kawahara N*3, Kiuchi F*1: Discrimination between Prepared Glycyrrhiza and Glycyrrhiza by TLC.
Shoyakugaku Zasshi 2014;68:70-7.

Prepared Glycyrrhiza is an important crude drug used in Kampo products and formulae such as Shakanzoto. However, no identification test evaluating a characteristic maker constituent for quality control of this crude drug has been established. In this paper, we compared the constituent of Prepared Glycyrrhiza and those of Glycyrrhiza by TLC and found three spots which exist in Prepared Glycyrrhiza but not in Glycyrrhiza. These spots were formed when Glycyrrhiza was heated above 130°C for more than 30 min. Among these three spots, two originated

from sugars and were also found in heat-treated Astragalus Root. However, the other spot was characteristic for Prepared Glycyrrhiza and suitable as an indicator spot for an identification test of Prepared Glycyrrhiza. We developed a method to detect this spot by TLC which can be used as an identification test of this crude drug in the Japanese Pharmacopoeia.

Keywords: Prepared Glycyrrhiza, Identification test, TLC

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Fukahori M*, Kobayashi S*, Naraki Y*, Sasaki T*, Oka H*, Seki M*, Masada-Atsumi S, Hakamatsuka T, Goda Y: Quality evaluation of medicinal products and health foods containing Chaste Berry (*Vitex agnus-castus*) in Japanese, European and American markets. *Chem Pharm Bull*. 2014;62:379-85.

We evaluated the qualities of chaste berry (fruit of *Vitex agnus-castus* L.) preparations using HPLC fingerprint analysis. Seven medicinal products and 17 health foods were analyzed and HPLC profile and 26 authentic peaks were compared medicinal products and health foods. This study clearly demonstrated that a combination of HPLC fingerprints and the amount ratios of the marker compounds of chaste berry preparations serves as a useful tool to evaluate the qualities of these preparations.

Keywords: Chaste berry extract, quality evaluation, HPLC fingerprint

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Kikura-Hanajiri R, Uchiyama N, Kawamura M, Goda Y: Changes in the prevalence of new psychoactive substances before and after the introduction of the generic scheduling of synthetic cannabinoids in Japan. *Drug Testing and Analysis* 2014;6:832-9.

To counter the spread of the many analogs of psychoactive substances, the Pharmaceutical Affairs Law in Japan was amended in 2006 to establish a new category, "Designated Substances" in order to more promptly control these drugs. As of March 2013, 106 substances (including one plant, *Salvia divinorum*) were listed in the category of Designated Substances, and 13 of them had had their category changed from Designated Substances into the much stricter category, Narcotics. However, new analogs of controlled substances, especially synthetic cannabinoids, appeared one-by-one since the new category was introduced. To avoid a "cat-and-mouse game" between regulators and illicit drug manufacturers, a comprehensive system (generic scheduling) for designating naphthoylindole-type synthetic cannabinoids, with particular substituents, was introduced into the Designated Substances in 2013. Since late 2012, the naphthoylindole-type compounds have been gradually replaced by other types of synthetic cannabinoids, such as cyclopropylmethanones, cannabimimetic carboxamide derivatives, adamanthoyl indoles and cannabimimetic quinolinyl carboxylates. After the enforcement of the generic scheduling for designating naphthoylindoles in March 2013, these natphthoylindoles have been completely replaced by other types and have rarely been detected in the products. New types of psychoactive substances, including opioid receptor agonists (e.g., AH-7921, MT-45), hallucinogenic phenethylamines (e.g., NBOMe-type compounds) and thiophene derivatives (e.g., methiopropamine, α-PVT) have also appeared. The almost infinite possibilities of altered structures of chemicals make it difficult to carry out effective and exhaustive scheduling. To prevent the widespread distribution and abuse of these new psychoactive substances, continuous and dedicated monitoring for the emergence of these substances is necessary.

Keywords: new psychoactive substances, synthetic cannabinoids, generic scheduling

Takayama T*, Suzuki M*, Inoue K*, Todoroki K*, Min JZ*, Kikura-Hanajiri R, Goda Y, Toyo'oka T*: UPLC-ESI-MS/MS based determination of metabolism of several new designated substances, ADB-FUBINACA, AB-FUBINACA, AB-PINACA, QUPIC, 5F-QUPIC and α-PVT, by human liver microsome.
Biomed Chromatgr. 2014;28:831-8.

The metabolism by human liver microsomes of several new illicit drugs, that is, *N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (ADB-FUBINACA), *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA), *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide (AB-PINACA), quinolin-8-yl 1-pentyl-(1*H*-indole)-3-carboxylate (QUPIC), quinolin-8-yl 1-(5-fluoropentyl)-(1*H*-indole)-3-carboxylate (5F-QUPIC) and α-pyrrolidinovalerothiophenone (α-PVT), which have indole, indazole, quinolinol ester and

thiophene structures, was investigated using reversed-phase chromatography and mass spectrometry. The present method is based upon the oxidation by cytochrome p450 superfamily enzymes in the microsomes. The oxidation of ADB-FUBINACA and AB-FUBINACA mainly occurred on the *N*-(1-amino-alkyl-1-oxobutan) moiety. However, the oxidation of AB-PINACA seemed to occur on the 1-pentyl moiety. On the other hand, QUPIC and 5F-QUPIC, which have a quinolinol ester structure, predominantly underwent a cleavage reaction to produce indoleacetic acid type metabolites. In contrast, the metabolism reaction of α-PVT was different from that of the other tested drugs, and various oxidation products were observed on the chromatograms. The obtained metabolites are not in conflict with the results predicted by MetaboLynx software. However, the exact structures of the metabolites, except for 1-pentyl-1*H*-indole-3-carboxylic acid（QUPIC metabolite）and 1-(5-fluoropentyl)-1*H*-indole-3-carboxylic acid（5F-QUPIC metabolite), are currently not proven, because we have no authentic compounds for comparison. The proposed approach using human liver microsome seems to provide a new technology for the prediction of possible metabolites occuring in humans.

Keywords: LC/ESI-MS/MS, human liver microsome, illicit drugs

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Uchiyama N, Matsuda S, Kawamura M, Shimokawa Y, Kikura-Hanajiri R, Aritake K*, Urade Y*, Goda Y: Characterization of four new designer drugs, 5-chloro-NNEI, NNEI indazole analog, α-PHPP and α-POP, with 11 newly distributed designer drugs in illegal products. *Forensic Sci Int.* 2014;243:1-13.

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, 3,4-Dichloromethylphenidate, Synthetic cannabinoid

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Uchiyama N, Shimokawa Y, Kawamura M, Kikura-Hanajiri R, Hakamatsuka T: Chemical analysis of a benzofuran derivative, 2-(2-Ethylaminopropyl) benzofuran（2-EAPB), eight synthetic cannabinoids, five cathinone derivatives and five other designer drugs newly detected in illegal products. *Forensic Toxicol.* 2014;32:266-81.

During November 2013 and May 2014, 19 newly distributed designer drugs were identified in 104 products in our ongoing survey of illegal products in Japan. Eight synthetic cannabinoids, i.e., FUB-PB-22（**1**), 5-fluoro-NNEI indazole analog（5-fluoro-MN-18, **2**), AM-2201 indazole analog（THJ-2201, **3**), XLR-12（**4**), 5-fluoro-AB-PINACA（**5**), 5-chloro-AB-PINACA（**6**), AB-CHMINACA（**7**）and 5-fluoro-AMB（**8**), five cathinone derivatives, i.e., DL-4662（**9**), α-PHP（**10**), 4-methoxy-α-POP（**11**), 4-methoxy-α-PHPP（**12**）and 4-fluoro-α-PHPP（**13**), and six other substances, i.e., the benzofuran derivative 2-(2-ethylaminopropyl)benzofuran（2-EAPB, **14**), nitracaine（**15**), diclofensine（**16**), diphenidine（**17**), 1-benzylpiperidine（**18**）and acetylfentanyl（**19**), were identified. To our knowledge, this is the first report on the chemical properties of compounds **9** – **11** and **14**. A total of 34 designer drugs, including compounds **1** – **19**, were detected in the 104 illegal products, in 60 different combination patterns. The numbers of detected compounds per product ranged from one to seven. Additionally, several products contained three different types of compounds, such as synthetic cannabinoids, cathinone derivatives and phenethylamine derivatives per product. Therefore, not only the types of compounds emerging but also their combinations in illegal products seem to be increasing in diversity.

Keywords: 2-(2-Ethylaminopropyl) benzofuran（2-EAPB), Synthetic cannabinoid, Cathinone

内山奈穂子，花尻（木倉）瑠理，袴塚高志：薬物簡易スクリーニングキットを用いた危険ドラッグ成分である合成カンナビノイドの識別法の検討.

薬学雑誌 2015;135:535-41.

Recently, illegal herbal or liquid products containing psychoactive compounds have been a serious problem damaging human health and causing numerous traffic accidents. Reports indicate that most of those herbal products contain various types of synthetic cannabinoids. There are many on-site drug-testing devices; however, synthetic cannabinoids are not targeted compounds for such devices. In this study, we evaluated the on-site drug-testing device "K2/Spice Test" for the detection of 12 different types of 38 synthetic cannabinoids (including 13 naphthoylindole-type synthetic cannabinoids) and a natural cannabinoid (Δ9-tetrahydrocannabinol). Although this device is primarily used for the detection of metabolites of naphthoylindole-type synthetic cannabinoids in urine samples, we applied it to detect synthetic cannabinoids in illegal herbal products for rapid screening analyses. As a result of the on-site examination of synthetic cannabinoids, 10 naphthoylindole-type synthetic cannabinoids [five narcotics (JWH-018, JWH-073, AM-2201, MAM-2201, and JWH-122); five designated substances (JWH-015, JWH-200, AM-1220, JWH-019, and JWH-020)], and two other types of synthetic cannabinoid [designated substances (a benzoylindole AM-694 and a naphthoylnaphthalene CB-13)]showed positive results (the limit of detection ranged from 50 to 250 μg/mL). Furthermore, MeOH extracts of illegal herbal products containing naphthoylindole-type synthetic cannabinoids also showed positive results (the limit of detection ranged from 2.5 to 10 mg herbal products/mL). Therefore, we found that this device may be useful for the on-site examination of some naphthoylindole-type synthetic cannabinoids not only in urine samples but also in illegal herbal products.

Keywords: Synthetic cannabinoid, drug-screening device, immune assay

内田恵理子，古田美玲，菊池裕，窪崎敦隆，遊佐精一，宮原美知子，佐々木裕子[*1]，小原有弘[*2]，大谷梓[*2]，松山晃文[*3]，大倉華雪[*3]，山口照英：細胞基材に対するマイコプラズマ否定試験のPCR法の見直しに関する研究.

医薬品医療機器レギュラトリーサイエンス 2014;45:42-51.

日本薬局方（日局）参考情報「バイオテクノロジー応用医薬品/生物起源由来医薬品の製造に用いる細胞基材に対するマイコプラズマ否定試験」では，PCR法（C法）は培養法（A法）及び指標細胞を用いたDNA染色法（B法）を補完する二次的試験と位置づけられている．しかし，バイオ医薬品製造での工程管理や再生医療製品の試験としては，迅速試験であるPCR法等の核酸増幅検査（NAT）の利用が望まれている．既に欧州薬局方（EP）では，適切なバリデーションの実施により，NATをA法又はB法に代替可能である．そこで，日局C法を改正し，EPに準じてマイコプラズマ否定試験としてNATを適用するためのバリデーションの条件を示すため，4施設からなる共同研究班を組織して検討を行った．EPに適合するとされる市販の複数のPCRキットのうち，プライマーが公開されているものをモデルとし，日局PCR法と検出感度等を比較する共同検定を行うと共に，EPのバリデーション手法の妥当性の検証とNAT実施上の注意点を検討した．共同検定結果を基に，日局改正案作成に向けた提言をまとめた.

Keywords: マイコプラズマ，NAT，日本薬局方

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Sakurai M[*], Watanabe T[*], Suzuki T, Furihata C[*]: Time-course comparison of gene expression profiles induced by the genotoxic hepatocarcinogen, chrysene, in the mouse liver.

Genes and Environment 2014;36:54-64.

Changes in gene expression profile in rodent liver at the acute stage within 48 h after administration of a hepatocarcinogen have not been extensively reported. In the present study we examined changes in gene expression in mouse liver within 48 h induced by chrysene, a polycyclic aromatic hydrocarbon and genotoxic hepatocarcinogen, by quantitative real-time PCR (qPCR). We quantified 50 candidate genes which discriminated genotoxic hepatocarcinogens from non-genotoxic hepatocarcinogens as determined from our previous DNA microarray studies. Chrysene (100 mg/kg bw) was injected intraperitoneally into male 9-week-old B6C3F$_1$ mice, and at 4, 16, 20, 24 and 48 h after chrysene administration, livers were dissected and processed for gene expression. A total of 35 genes exhibited statistically significant increases at least once within 48 h after chrysene administration. *Cyp1a1* and *Cyp1a2* showed remarkably consistent increases in gene expression during 4 to 48 h. Fifteen genes (*Bhlhe40*, *Btg2*, *Casp4*, *Ccng2*, *Cdkn1a*, *Crp*, *Cyp1a1*, *Cyp1a2*,

Fkbp5, *Gadd45b*, *Gadd45g*, *Hmox1*, *Igfbp1*, *Lcn2* and *Ly6a*) at 4 h, 6 genes at 16 h,7 genes at 20 h, 7 genes at 24 h, and 10 genes (*Bhlhe40*, *Ccnf*, *Cyp1a1*, *Cyp1a2*, *Ephx1*, *Hhex*, *Hmox1*, *Rcan1*, *Tubb2a* and *Tubb4b*) at 48 h exhibited statistically significant increases of more than two-fold. At 4 h, 10 of 15 expression-increased genes were associated with DNA damage, DNA repair, cell cycle, cell proliferation and apoptosis. The expression-increased genes at 16 to 48 h were associated with a variety of biological processes. In conclusion three time-dependent patterns in gene expression were observed within 48 h after chrysene administration in mouse liver: *Cyp1a1* and *Cyp1a2* exhibited consistent increases; the highest number of genes (15 genes) increased in expression at 4 h; and 6 different genes expressed at 4 h increased at 48 h.

Keywords: chrysene, gene expression profile, mouse liver

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Shimo T*[1], Tachibana K*[1], Saito K*[1], Yoshida T, Tomita E*[1], Waki R*[1], Yamamoto T*[1], Doi T*[1], Inoue T, Kawakami J*[2], Obika S*[1]: Design and evaluation of locked nucleic acid-based splice-switching oligonucleotides in vitro.

Nucleic Acids Res. 2014;42:8174-887.

Antisense-mediated modulation of pre-mRNA splicing is an attractive therapeutic strategy for genetic diseases. Currently, there are few examples of modulation of pre-mRNA splicing using locked nucleic acid (LNA) antisense oligonucleotides, and, in particular, no systematic study has addressed the optimal design of LNA-based splice-switching oligonucleotides (LNA SSOs). Here, we designed a series of LNA SSOs complementary to the human dystrophin exon 58 sequence and evaluated their ability to induce exon skipping in vitro using reverse transcription-polymerase chain reaction. We demonstrated that the number of LNAs in the SSO sequence and the melting temperature of the SSOs play important roles in inducing exon skipping and seem to be key factors for designing efficient LNA SSOs. LNA SSO length was an important determinant of activity: a 13-mer with six LNA modifications had the highest efficacy, and a 7-mer was the minimal length required to induce exon skipping. Evaluation of exon skipping activity using mismatched LNA/DNA mixmers revealed that 9-mer LNA SSO allowed a better mismatch discrimination. LNA SSOs also induced exon skipping of endogenous human dystrophin in primary human skeletal muscle cells. Taken together, our findings indicate that LNA SSOs are powerful tools for modulating pre-mRNA splicing.

Keywords: locked nucleic acid (LNA), LNA-based splice-switching oligonucleotides (LNA SSOs), pre-mRNA splicing

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Kuroda T, Yasuda S, Sato Y: In vitro detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human induced pluripotent stem cells.

Methods Mol Biol. 2014;1210:183-92.

Human pluripotent stem cells (hPSCs) such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are a leading candidate for regenerative medicine/cell therapies because of their capacity for pluripotency and unlimited self-renewal. However, there are significant obstacles preventing the clinical use of hPSCs. A significant safety issues is the presence of residual undifferentiated cells that have the potential to form tumors in vivo. Here, we describe the highly sensitive qRT-PCR methods for detection of residual undifferentiated cells in retinal pigment epithelial (RPE) cells derived from hiPSCs. qRT-PCR using probes and primers targeting *LIN28A* (*LIN28*) transcripts can detect residual undif- ferentiated cell levels as low as 0.002 % in hiPSC-derived RPE cells. We expect this method to contribute to process validation and quality control of hiPSC-derived cell therapy product.

Keywords: Human induced pluripotent stem cells, Tumorigenicity, *LIN28*

Tano K*, Yasuda S, Kuroda T, Saito H*, Umezawa A*, Sato Y: A novel in vitro method for detecting undifferentiated human pluripotent stem cells as impurities in cell therapy products using a highly efficient culture system.

PLoS ONE 2014;9:e110496.

We showed a novel approach for direct and sensitive detection of a trace amount of undifferentiated human induced pluripotent stem cells (hiPSCs) using a highly

efficient amplification method in combination with laminin-521 and Essential 8 medium. Essential 8 medium allowed robust hiPSC proliferation plated on laminin-521 at low cell density, whereas mTeSR1 did not enhance the cell growth. This highly efficient culture system detected hiPSCs spiked into primary human mesenchymal stem cells（hMSCs）or human neurons at the ratio of 0.001%–0.01% as formed colonies. Moreover, this assay method was demonstrated to detect residual undifferentiated hiPSCs in cell preparations during the process of hMSC differentiation from hiPSCs. These results indicate that our highly efficient amplification system is able to detect a trace amount of undifferentiated hPSCs contained as impurities in CTPs and would contribute to quality assessment of hPSC-derived CTPs during the manufacturing process.

Keywords: residual undifferentiated hiPSCs, tumorigenicity, laminin-521

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Kusakawa S*1, Machida K*2, Yasuda S, Takada N*3, Kuroda T, Sawada R, Okura H*3, Tsutsumi H*2, Kawamata S*1, Sato Y: Characterization of in vivo tumorigenicity tests using severe immunodeficient NOD/Shi-scid IL2Rg^{null} mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products.

Regenerative Therapy 2015;1:30-7.

We examined tumor formation after subcutaneous transplantation of HeLa cells, as a model of tumorigenic cells, in NOD/Shi-scid IL2Rg^{null} NOG mice and nude mice. Sixteen weeks after inoculation, the 50% tumor-producing dose（TPD_{50}）values of HeLa cells were stable at $1.3x10^4$ and $4.0x10^5$ cells in NOG and nude mice, respectively, indicating a 30-fold higher sensitivity of NOG mice compared to that of nude mice. Transplanting HeLa cells embedded with Matrigel in NOG mice further decreased the TPD_{50} value to 7.9x10 cells, leading to a 5000-fold higher sensitivity, compared with that of nude mice. Additionally, when HeLa cells were mixed with 10^6 or 10^7 human mesenchymal stem cells as well as Matrigel, the TPD_{50} values in NOG mice were comparable to those of HeLa cells alone with Matrigel. These results suggest that the in vivo tumorigenicity test using NOG mice with Matrigel is a highly sensitive and quantitative method to detect a trace amount of tumorigenic cellular impurities in human somatic cells, which can be useful in the quality assessment of hCTPs.

Keywords: Tumorigenicity test, NOG mice, Cellular therapy

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Maeda Y*1, Terasawa T*1, Tanaka Y*2, Mitsuura C*1, Nakashima K*1, Yusa K, Harada S*1: 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, GLUT1, Env-mediated fusion

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Kono K, Takada N, Yasuda S, Sawada R, Niimi S, Matsuyama A*, Sato Y: Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells.
Biologicals. 2015;43:146-9.

The analysis of in vitro cell senescence/growth after serial passaging can be one of ways to show the absence of immortalized cells, which are frequently tumorigenic, in human cell-processed therapeutic products (hCTPs). However, the performance of the cell growth analysis for detection of the immortalized cellular impurities has never been evaluated. In the present study, we examined the growth rates of human mesenchymal stem cells (hMSCs, passage 5 (P = 5)) contaminated with various doses of HeLa cells, and compared with that of hMSCs alone. The growth rates of the contaminated hMSCs were comparable to that of hMSCs alone at P = 5, but significantly increased at P = 6 (0.1% and 0.01% HeLa) or P = 7 (0.001% HeLa) within 30 days. These findings suggest that the cell growth analysis is a simple and sensitive method to detect immortalized cellular impurities in hCTPs derived from human somatic cells.
Keywords: Cellular therapy, Tumorigenicity, Mesenchymal stem cell

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Takeda E*1, Kono K, Hulme AE*2, Hope TJ*2, Nakayama EE*1, Shioda T*1: Fluorescent image analysis of HIV-1 and HIV-2 uncoating kinetics in the presence of old world monky TRIM5α.
PLoS ONE 2015;10:e0121199.

In the present study, we re-evaluated uncoating kinetics of HIV-1 in the presence of OWM TRIM5α by using an in situ uncoating assay, which allowed us to differentiate productive HIV-1 entry from simple (non-productive) endocytosis. Results showed that the uncoating kinetics of HIV-1 was indeed accelerated in the presence of OWM TRIM5α. Furthermore, we adapted an in situ uncoating assay to HIV-2, which showed wide variations in TRIM5α sensitivity among different isolates. HIV-2 isolate GH123, whose infectivity was suppressed by cynomolgus monkey (CM) TRIM5α, showed accelerated uncoating in the presence of CM TRIM5α. In contrast, mutant HIV-2 ASA, whose infectivity was unaltered by CM TRIM5α, showed no change in uncoating kinetics in the presence of CM TRIM5α. These results confirmed and further extended the previous notion that accelerated uncoating is associated with restriction activity of TRIM5α against lentiviruses.
Keywords: uncoating kinetics, TRIM5α

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Ohoka N, Nagai K*, Hattori T, Okuhira K, Shibata N, Cho N*, Natio M: Cancer cell death induced by novel small molecules degrading the TACC3 protein via the ubiquitin-proteasome pathway.
Cell Death Dis. 2014;5:e1513.

The selective degradation of target proteins with small molecules is a novel approach to the treatment of various diseases, including cancer. We have developed a protein knockdown system with a series of hybrid small compounds that induce the selective degradation of target proteins via the ubiquitin-proteasome pathway. In this study, we designed and synthesized novel small molecules called SNIPER(TACC3)s, which target the spindle regulatory protein transforming acidic coiled-coil-3(TACC3). SNIPER(TACC3)s induce poly-ubiquitylation and proteasomal degradation of TACC3 and reduce the TACC3 protein level in cells. Mechanistic analysis indicated that the ubiquitin ligase APC/C(CDH1) mediates the SNIPER(TACC3)-induced degradation of TACC3. Intriguingly, SNIPER(TACC3) selectively induced cell death in cancer cells expressing a larger amount of TACC3 protein than normal cells. These results suggest that protein knockdown of TACC3 by SNIPER(TACC3) is a potential strategy for treating cancers overexpressing the TACC3 protein.
Keywords: TACC3, ubiquitin, proteasome

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Hashimoto Y*, Takeshita Y*, Naito M, Uchino H*, Matsuoka M*: Apollon/Bruce is upregulated by Humanin.

Cell Biochem. 2014;397:147-55.

Humanin, a short bioactive peptide, inhibits a variety of cell deaths. Humanin-mediated inhibition of neuronal cell death, caused by an Alzheimer's disease（AD）-linked mutant gene occurs via binding of Humanin to its heterotrimeric Humanin receptor（htHNR）, which results in the activation of the Janus-associated kinases（JAKs）and signal transducer and activator and transcription 3（STAT3）signaling pathway. A previous study demonstrated that the Humanin-induced activation of the htHNR/JAK2/STAT3 signaling pathway leads to increased expression of SH3 domain-binding protein 5（SH3BP5）, which is an essential effector of Humanin's anti-cell death activity in some cultured neuronal cells. However, it remains unknown whether SH3BP5 is the sole effector of the Humanin signaling pathway via htHNR/JAKs/STAT3. Here we show that the Humanin signaling pathway via htHNR/JAKs/STAT3 increased the expression levels of mRNA and protein of Apollon/Bruce, an unusual member of the inhibitors of apoptosis proteins, and that overexpression of Apollon/Bruce inhibits neuronal death, caused by a London-type familial AD-linked mutant（V642I）of amyloid β precursor protein. Overall, the results indicate that expression of Apollon/Bruce is upregulated by Humanin, and Apollon/Bruce could be an effector of Humanin in a context-dependent manner.
Keywords: Apollon, Humanin, Gene expression

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Haishima Y, Hasegawa C, Nomura Y, Kawakami T, Yuba T[*1], Shindo T[*2], Sakaguchi K[*3], Tanigawa T[*3], Inukai K[*3], Takenouchi M[*3], Isama K, Matsuoka A, Niimi S: Development and performance evaluation of a positive reference material for hemolysis testing.
J Biomed Mater Res Part B. 2014;102B:1809-16.

This study deals with the development and performance evaluation of a positive reference material for hemolysis testing, which is used for evaluating the biological safety of medical devices. Genapol X-080, a non-ionic detergent, was selected as a candidate hemolytic substance in a survey of 23 chemical compounds; it showed significant hemolytic activity against rabbit defibrinated blood at concentrations more than 20 μg/mL. A polyvinyl chloride（PVC）sheet spiked with 0.6%（w/w）of the compound exhibited weak hemolytic activity in direct contact and/or extract-based assays after 4 h incubation at 37℃. A PVC sheet containing 5.8%（w/w）Genapol X-080 induced complete hemolysis in both assays. The amount of Genapol X-080 eluted from each PVC sheet during hemolysis testing using the direct contact method increased time-dependently and reached 25.6（former sheet）or 1154（later sheet）μg/mL after 4 h incubation, which was similar to or much higher than the critical micelle concentration（CMC）, respectively. Similar elution behavior was observed using the extract-based method, and the Genapol X-080 content in test solutions prepared by autoclave extraction of both sheets was 22.5 and 358 μg/mL, respectively, indicating a clear relationship between the degree of hemolytic activity and the eluted amount of Genapol X-080. Thus, a PVC sheet spiked with a compound exhibiting different hemolytic activity depending on its concentration may be useful as a positive reference material to validate the hemolysis tests.
Keywords: hemolysis test, positive control, biological safety evaluation

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迫田秀行，京本政之*，井上祐貴*，石原一彦*，新見伸吾：人工関節摺動面材料の形状変化に基づく新規摩耗量評価法の開発.
臨床バイオメカニクス 2014;35:207-10.

Although ultra-high molecular weight polyethylene（UHMWPE）has been used as a load bearing material of artificial joints, the frequent occurrence of wear-related failure has promoted searches for more wear-resistant materials. To evaluate the amount of wear, most studies in this field have employed a gravimetric method developed for UHMWPE. However, we considered that the gravimetric method is not suitable to evaluate very low wear of materials. In this study, we proposed a new geometric wear evaluation method and compared it to the gravimetric method. Wear of carbon-fi ber-reinforced polyetheretherketone（CFR-PEEK）was evaluated with the gravimetric and geometric methods. For geometric wear evaluation, fi ve indents per specimen were made on the surface of wear test pins with a micro-hardness tester. The shape of each indent was measured by a three-dimensional measurement laser microscope before and after the wear tests. The wear depth was estimated

from the changes in the size of each indent. The wear volume was calculated from the average wear depth and area of the wear surface. The gravimetric and geometric methods generated similar wear factors for CFR-PEEK, which were as low as 1/46 of those of UHMWPE. The results of the geometric method tended to show a smaller deviation than those of the gravimetric method irrespective of the length of the pre-soaking period. Marked weight gain due to water uptake and minimal weight loss due to wear were considered to have resulted in the large error in the results using the gravimetric method. The geometric method could be used to successfully evaluate the very low wear of CFRPEEK, and is considered to be more useful than the gravimetric method for evaluating the very low wear of many novel materials.

Keywords: artificial joint, wear, geometric method

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Yoda I*, Koseki H*, Tomita M*, Shida T*, Horiuchi H*, Sakoda H, Osaki M*: Effect of surface roughness of biomaterials on *Staphylococcus epidermidis* adhesion. *BMC Microbiology* 2014;14:234.

Implant-related infections are caused by adhesion of bacteria to the surface of biomaterials. In this in vitro research, we evaluated the ability of *Staphylococcus epidermidis*（ATCC35984）to adhere to the surface of solid biomaterials at different levels of roughness below 30 nm Ra and investigated the minimum level of roughness required to promote bacterial adhesion on five kinds of biomaterials: oxidized zirconium-niobium alloy（Oxinium）, cobalt-chromium-molybdenum alloy（Co-Cr-Mo）, titanium alloy（Ti-6Al-4V）, commercially pure titanium（Cp-Ti）and stainless steel（SUS316L）, samples of which were categorized into a fine group and a coarse group according to surface roughness. The test specimens were physically analyzed and the viable bacterial density of the adhered bacteria was quantitatively determined（n=20）.

The amount of bacteria that adhered to the biomaterials in the coarse group was higher than those in the fine group. Oxinium, Ti-6Al-4V and SUS316L in particular demonstrated statistically significant differences between the two groups（$P<0.05$）. Of the materials, the Co-Cr-Mo specimens exhibited significantly lower amounts of adhered bacteria than the Ti-6Al-4V, Cp-Ti and SUS316L specimens in the fine group. Similarly, the Co-Cr-Mo specimens in the coarse group exhibited significantly lower values than the other four materials.

These results suggest that minimum level of roughness affecting initial bacterial adherence activity differs according to the type of biomaterial used, and that even a surface roughness of below 30 nm Ra in Oxinium, Ti-6Al-4V and SUS316L can promote bacterial adhesion. Relative hydrophobic Co-Cr-Mo surfaces were less susceptible to bacterial adherence.

Keywords: bacterial adhesion, biomaterials, roughness

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Koseki H*1, Yonekura A*1, Shida T*1, Yoda I*1, Horiuchi H*1, Morinaga Y*2, Yanagihara K*2, Sakoda H, Osaki M*1, Tomita M*1: Early Staphylococcal biofilm formation on solid orthopaedic implant materials: In vitro study. *PLoS ONE* 2014;9:e107588.

Biofilms forming on the surface of biomaterials can cause intractable implant-related infections. Bacterial adherence and early biofilm formation are influenced by the type of biomaterial used and the physical characteristics of implant surface. In this in vitro research, we evaluated the ability of *Staphylococcus epidermidis*, the main pathogen in implant-related infections, to form biofilms on the surface of the solid orthopaedic biomaterials, oxidized zirconium-niobium alloy, cobalt-chromium-molybdenum alloy（Co-Cr-Mo）, titanium alloy（Ti-6Al-4V）, commercially pure titanium（cp-Ti）and stainless steel. A bacterial suspension of *Staphylococcus epidermidis* strain RP62A（ATCC35984）was added to the surface of specimens and incubated. The stained biofilms were imaged with a digital optical microscope and the biofilm coverage rate（BCR）was calculated. The total amount of biofilm was determined with the crystal violet assay and the number of viable cells in the biofilm was counted using the plate count method. The BCR of all the biomaterials rose in proportion to culture duration. After culturing for 2-4 hours, the BCR was similar for all materials. However, after culturing for 6 hours, the BCR for Co-Cr-Mo alloy was significantly lower than for Ti-6Al-4V, cp-Ti and stainless steel（$P<0.05$）. The absorbance value determined in the crystal violet assay and the number of viable cells on Co-Cr-Mo were not significantly lower than for the other materials（$P>0.05$）. These results suggest that surface properties, such as hydrophobicity

or the low surface free energy of Co-Cr-Mo, may have some influence in inhibiting or delaying the two-dimensional expansion of biofilm on surfaces with a similar degree of smoothness.

Keywords: bacterial adhesion, biomaterials, surface properties

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迫田秀行，新見伸吾，菅野伸彦*：抜去した股関節インプラントの超高分子量ポリエチレンコンポーネントに含まれる脂質の測定.

日本人工関節学会誌 2014;44:609-10.

人工関節摺動面に使用される超高分子量ポリエチレン（UHMWPE）には，関節液中の脂質が浸入することが報告されている．脂質の浸入によるUHMWPEの力学特性への影響や，UHMWPEの劣化の可能性の報告があるため，抜去されたUHMWPEコンポーネントに含まれる脂質量を測定し，脂質の浸入量に影響する因子の解明を試みた．特に，脂質の浸入量には関節液のアクセスのしやすさ，埋植期間，接触圧力が関係するという仮説を立て，その妥当性について検討した．その結果，UHMWPEコンポーネントに浸入する脂質量と，関節液のアクセス量，時間，荷重との関係が示唆された．摺動面やリムの表面には早期から脂質の浸入が認められた．脂質による材料特性への影響が報告されていることから，今後さらなる検討を進める予定である．

Keywords: artificial joint, UHMWPE, lipids

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Kono K, Niimi S, Sawada R: Cyclin D2 promotes the proliferation of human mesenchymal stem cells.

Journal of Bone Marrow Research 2014;2(1)2:136

Human mesenchymal stem cells（hMSCs）hold promise for use in cell-based therapies and tissue engineering. Although hMSCs are thought to be stable *ex vivo*, it is possible that they undergo an undesirable transformation to a phenotype of unlimited proliferation during *ex vivo*. In this study, we searched for the factor required for unlimited proliferation of hMSCs. Methods: Changes in gene expression were evaluated between hMSCs and Ewing's sarcoma cell lines, which may be derived from hMSCs, using GeneChip Human Genome U133 plus 2.0 Array. A gene up-regulated by at least 10-fold in Ewing's sarcoma cell lines, Cyclin D2, was overexpressed in hMSCs by a lentiviral vector. Results: Overexpression of Cyclin D2 in hMSCs altered cell morphology and promoted cell proliferation. Expression of transforming growth factor-β2（TGF-β2）, which induces senescence in hMSCs, was down-regulated in Cyclin D2-overexpressing hMSCs. Furthermore, Gene Ontology analysis revealed that Cyclin D2 overexpression activated expression of genes associated with proliferation and interphase. Conclusions: Cyclin D2 promotes hMSC proliferation and is a candidate biomaker for hMSC transformation.

Keywords: hMSC, Ewing's sarcoma, Cyclin D2

Sasaki H*1, Takeuchi I*2, Okada M*3, Sawada R, Kanie K*1,3, Kiyota Y*4, Honda H*1, Kato R*1,3: Label-free morphology-based prediction of multiple differentiation potentials of human mesenchymal stem cells for early evaluation of intact cells.

PLoS One 2014;9(4):e93952.

Precise quantification of cellular potential of stem cells, such as human bone marrow-derived mesenchymal stem cells（hBMSCs）, is important for achieving stable and effective outcomes in clinical stem cell therapy. Here, we report a method for image-based prediction of the multiple differentiation potentials of hBMSCs. This method has four major advantages:（1）the cells used for potential prediction are fully intact, and therefore directly usable for clinical applications;（2）predictions of potentials are generated before differentiation cultures are initiated;（3）prediction of multiple potentials can be provided simultaneously for each sample; and（4）predictions of potentials yield quantitative values that correlate strongly with the experimental data. Our results show that the collapse of hBMSC differentiation potentials, triggered by in vitro expansion, can be quantitatively predicted far in advance by predicting multiple potentials, multi-lineage differentiation potentials（osteogenic, adipogenic, and chondrogenic）and population doubling potential using morphological features apparent during the first 4 days of expansion culture. In order to understand how such morphological features can be effective for advance predictions, we measured gene-expression profiles of the same early undifferentiated cells. Both senescence-related genes（p16 and p21）and cytoskeleton-related genes（PTK2, CD146, and CD49）already correlated to

the decrease of potentials at this stage. To objectively compare the performance of morphology and gene expression for such early prediction, we tested a range of models using various combinations of features. Such comparison of predictive performances revealed that morphological features performed better overall than gene-expression profiles, balancing the predictive accuracy with the effort required for model construction. This benchmark list of various prediction models not only identifies the best morphological feature conversion method for objective potential prediction, but should also allow clinicians to choose the most practical morphology-based prediction method for their own purposes.

Keywords: image-based prediction, differentiation potentials, hBMSCs

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小林憲弘，久保田領志，高玲華*，安藤正典*，五十嵐良明：液体クロマトグラフィータンデム質量分析（LC/MS/MS）による水道水中農薬類の一斉分析法の妥当性評価.

水道協会雑誌 2014;83(4):3-14.

標準検査法の定められていない農薬類76物質を対象とした液体クロマトグラフィータンデム質量分析（LC/MS/MS）による一斉分析法の妥当性評価を実施した．7機関において，共通の標準作業手順書に従って，各物質の目標値の1/100超1/10以下および1/100以下に相当する濃度になるように混合標準溶液を添加した水道水を分析したところ，実施機関全てで概ね良好な結果が得られた.

Keywords: agricultural chemicals, validation test, LC/MS/MS

* （特非）水・環境分析技術支援ネットワーク

田原麻衣子，杉本直樹，大槻崇，多田敦子，穐山浩，合田幸広，五十嵐良明：定量NMRによる多環芳香族炭化水素市販試薬の純度決定.

環境科学会誌 2014;27:142-50.

物質量の絶対値は国際単位系（SI）にトレーサブルな測定によって得られると定義されている．しかし，環境分析において測定対象となる化合物は多種多様であり，計量計測学的に純度が証明された標準物質はほとんど流通していない．環境分析に応用されているクロマトグラフィーで正確な定量値を求めるためには，計量計測トレーサビリティが確保された純度値が決定された測定対象の標準物質が必須である．本研究では，環境中の多環芳香族炭化水素（PAH）類についてSIにトレーサブルな分析法を構築するため，定量核磁気共鳴法（定量NMR: quantitative NMR（qNMR））の一つであるAQARI（Accurate quantitative NMR with internal reference substance）法を応用した．AQARI法を応用することにより，科学的な根拠に基づいた，且つ，計量計測学的に信頼性を確保した純度値が求められる．定量用標準物質の代用品として使用される市販試薬製品18種のPAH および水酸化PAH（OH-PAH）について，計量計測学的に信頼性の高い純度値を測定した．その結果，各市販試薬製品の純度は90.2 ± 0.04～101.6 ± 0.9 %（arithmetic mean ± RSD）と算出された．このことから，メーカー成績書の純度値より最大6.6 %下回るものが認められ，メーカー成績書記載の純度値を質量%純度とし定量用標準物質として扱うことは適切ではない場合があることが示唆された．また，市販試薬製品の品質管理や使用時の純度が定量分析値の精度に大きく影響を及ぼすため，標準物質として使用する市販試薬製品の精確な純度の把握が重要であることが明らかとなった.

Keywords: polycyclic aromatic hydrocarbons, qNMR, purity

Kawakami T, Isama K, Ikarashi Y: Analysis of isothiazolinone preservatives in polyvinyl alcohol cooling towels used in Japan.

J Environ Sci Health Part A. 2014;49:1209-17.

Recently, cases of contact dermatitis that were related to the use of polyvinyl alcohol (PVA) cooling towels containing isothiazolinone preservatives were reported in Japan. The aim of this investigation was to analyze the concentrations of five different isothiazolinone compounds present in PVA towels and to assess the effectiveness of washing in removing the preservatives from new towels prior to being used for the first time. Twenty-seven PVA towels were used in this study. Two groups (i.e., laboratory-simulation and volunteer) of washing experiments were conducted to evaluate the effect of washing procedures. Qualitative and quantitative analyses were performed by LC/MS/MS, which detected 2-methyl-4-isothiazolin-3-one (MI) and

5-chloro-2-methyl-4-isothaizolin-3-one（CMI）in 23 samples（MI: 0.29–154 μg/g-wet, CMI: 2.2–467 μg/g-wet），2-*n*-octyl-4-isothiazolin-3-one（OIT）in one sample（478 μg/g-wet）. 4,5-Dichloro-2-*n*-octyl-4-isothiazolin-3-one（2Cl-OIT）and 1,2-benzisothiazolin-3-one（BIT）were not detected in all samples. We confirmed the presence of residual MI, CMI, and OIT in the washed towels, and the residual to original content ratio of OIT was higher than that of MI and CMI in PVA towels owing to the higher hydrophobicity of OIT than MI and CMI. A concern has been raised about the occurrence of contact dermatitis being caused by the use of PVA towels. It is suggested that a detailed description of isothiazolinone preservatives in PVA towels and an effective washing procedure for the removal of these preservatives should be provided by the manufacturer. Further, alternative non-sensitizing preservatives might be considered for the manufacture of PVA cooling towels in future.

Keywords: isothiazolinone preservatives, contact dermatitis, polyvinyl alcohol cooling towel

小林憲弘，久保田領志，木村謙治[*1]，金田智[*2]，茶木哲[*3]，天満一倫[*3]，田中美奈子[*4]，三枝慎一郎[*5]，小林利男[*6]，舟洞健二[*6]，齋藤信裕[*7]，杉本智美[*8]，古谷智仁[*9]，小嶋和博[*9]，平林達也[*10]，五十嵐良明：水道水中11農薬を対象とした固相抽出－GC/MS一斉分析法の妥当性評価.

水道協会雑誌 2014;83(9):11-22.

水道水中農薬を対象とした固相抽出-GC/MSによる一斉分析法の妥当性を評価するため，水道事業体10機関において11農薬（ウニコナゾールP，シプロジニル，チアメトキサム，チフルザミド，テブコナゾール，トリフルミゾール，ピリミホスメチル，プロパニル（DCPA），プロメトリン，ベンフセレート，およびメトミノストロビン）の添加回収試験を行った．その結果，トリフルミゾールを除く10農薬については概ね良好な結果が得られ，本分析法の妥当性を検証することができた.

Keywords: agricultural chemicals, validation test, GC/MS

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Akiyama T, Yamazaki T[*1], Tada A, Ito Y[*2], Otsuki N, Akiyama H: Classification of microbial α-amylases for food manufacturing using proteinase digestion.

Food Sci Nutri. 2014;2:571-7.

Enzymes produced by microorganisms and plants are used as food additives to aid the processing of foods. Identification of the origin of these enzyme products is important for their proper use. Proteinase digestion of α-amylase products, followed by HPLC analysis, was applied to α-amylase from the mold Aspergillus species, the bacteria Bacillus species, and the actinomycetes Saccharomonospora species. Eighteen commercial products of α-amylase were digested with trypsin and endoproteinase Lys-C and HPLC analyzed. For some proteinase/sample combinations, the area of the intact α-amylase peak decreased and new peaks were detected after digestion. The presence and retention times of the novel peaks were used to group the products. The results from this method, called the proteinase digestion–HPLC method, allowed the classification of the α-amylase products into 10 groups, whereas the results from SDS-PAGE allowed their classification into 7 groups.

Keywords: α-amylase, proteinase, HPLC

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Kawakami T, Isama K, Ikarashi Y: Analysis of 19 preservatives in polyvinyl alcohol cooling towels used in Japan.

J Environ Anal Chem. 2015;2:122.

The cases of contact dermatitis due to using polyvinyl alcohol（PVA）towel containing isothiazolinone preservatives have been reported in Japan. Thus, we had investigated the concentrations of these preservatives and the removal of isothiazolinone preservatives from PVA towels by washing before initial use. In the summer of 2013, clinical information regarding contact dermatitis due to using PVA cooling towels containing other preservatives was provided from the supplier of PVA towel. Thus, we analyzed 19 preservatives in 21 PVA towels. 2-Methyl-4-isothiazolin-3-one（MI）and 5-chloro-2-methyl-4-

isothiazolin-3-one（CMI）were detected in 16 samples, including the sample which was sold in a dry condition; the concentrations of these substances ranged from 7.9-84 μg/g-wet and 9.5-173 μg/g-wet, respectively（2.9 μg/g-dry and 9.3 μg/g-dry, respectively）. 2-*n*-Octyl-4-isothiazolin-3-one（OIT）was detected in one sample（484 μg/g-wet）. 2-Bromo-2-nitropropane-1,3-diol（BP）was detected in 15 samples, including the sample which was sold in a dry condition; its concentration ranged from 68-2303 μg/g-wet（160 μg/g-dry）. 2-Phenoxyethanol（PE）and benzoic acid（BA）were detected in 3 and 2 samples, and their concentrations ranged from 99-3171 μg/g-wet and 1896-23043 μg/g-wet. Other preservatives were not detected. Although isothiazolinone preservatives were detected in 17 samples, the product notes of 10 products, including the product with clinical information, did not describe about the use of isothiazolinone preservatives. Since PVA cooling towels in contact with human skin for a long time, the PVA cooling towels for the patients who allergic sensitive to isothiazolinone preservatives. Furthermore, we evaluated the effectiveness of the washing process on the removal of BP, PE, and BA from the PVA towels before their initial use. The results of this laboratory-simulated washing procedure suggest that contact dermatitis is likely not related to the presence of BP, PE, and BA in washed PVA towels.
Keywords: preservatives, contact dermatitis, polyvinyl alcohol cooling towel

小林憲弘，久保田領志，佐々木俊哉*，五十嵐良明：水道水中のイミノクタジン・ジクワット・パラコートのLC/MS/MS一斉分析法の開発.
環境科学会誌 2015;28:117–25.

パラコートは，水道水質検査の対象農薬に選定されているが標準検査法が設定されていない．また，同様に検査対象とされているイミノクタジンおよびジクワットは，現在の標準検査法では農薬類の検査で原則達成すべき定量下限値（目標値の1/100の濃度）が得られない．本研究では，これら3農薬に共通する強塩基性に着目し，弱陽イオン交換基と逆相の二つの保持能を併せ持つミックスモード固相カラムを用いた新たな前処理法と，HILICモードの分離カラムを用いたLC/MS/MSによる一斉分析法を開発した.
Keywords: iminoctadine, diquat, paraquat

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久保田領志，小林憲弘，五十嵐良明：固相抽出－液体クロマトグラフ－質量分析計によるハロアセトアミド類の分析法の開発及び水道水中の存在実態.
環境科学会誌 2015;28:143–52.

含窒素消毒副生成物のハロアセトアミド類を対象に，固相抽出－液体クロマトグラフ－質量分析計（LC/MS）による分析法の検討を行った．LC/MS条件については，移動相は5mmol/L酢酸アンモニウム水溶液：5mmol/L酢酸アンモニウムメタノール溶液（95：5，v/v）とし，アイソクラティック法で流速0.25mL/minで送液することで，ハロアセトアミド類を高感度，かつ，水道水中の夾雑成分による分析時の影響を軽減できることがわかった．固相抽出は，3種のC18固相カラム及び1種の活性炭固相カラムで検討した結果，C18では一旦保持されるが保持は弱く，固相カラムの溶出時まで保持されなかったが，活性炭では固相カラムへの保持や，固相カラムからの溶出ともに良好であった．確立した分析法について，精製水及び水道水を用いた添加回収試験を実施し，厚生労働省健康局水道課発出の妥当性評価ガイドラインに従い，分析法の妥当性を評価した．その結果，真度及び併行精度について目標を満たし，分析精度が良好であることが示された．本分析法を用いて国内の複数の浄水場の浄水及び給水栓水を冬季（2月）に採水して存在実態調査を行った結果，全て定量下限値未満であった.
Keywords: nitrogenous disinfection by-products, solid phase extraction, validation test

齊藤静夏，根本了，松田りえ子：LC-MS/MSを用いた茶中の残留農薬一斉分析法～厚生労働省通知一斉試験法の改良～.
日本食品化学学会誌 2014;21(1):27-36.

An LC-MS/MS method for the simultaneous determination of pesticide residues in tea was developed by modifying the Japanese official multiresidue method. In the optimal sample preparation procedure, the following sequence of steps was adopted: (1) swelling of the sample in water; (2) extraction with acetonitrile; (3) removal of water by salting-out; (4) cleanup on an ODS column and then on a tandem graphitized carbon/PSA column. The resulting test solution was subjected to LC-MS/MS and determined by external solvent standard calibration. The recoveries for 135 pesticides from fortified green tea, black tea, oolong tea, and matcha (powdered green tea) after spiking at the Japanese maximum residue limits were mostly within the range 70–120%, with relative standard deviations of <20%. The test solutions obtained by the modified method

were cleaner than those obtained by the original multiresidue method and contained relatively smaller amounts of pigments and other matrix components. No interfering peak was observed in the blank chromatograms, indicating the high selectivity of the modified method. Therefore, the developed method is considered to be highly efficient and suitable for the quantitative analysis of pesticide residues in tea.

Keywords: pesticide, multiresidue method, LC-MS/MS

Amakura Y[*1], Yoshimura M[*1], Takaoka M[*1], Toda H[*1], Tsutsumi T, Matsuda R, Teshima R, Nakamura M[*2], Handa H[*2], Yoshida T[*1]: Characterization of natural aryl hydrocarbon receptor agonists from cassia seed and rosemary.

Molecules 2014;19(4):4956-66.

Many recent studies have suggested that activation of the aryl hydrocarbon receptor（AhR）reduces immune responses, thus suppressing allergies and autoimmune diseases. In our continuing study on natural AhR agonists in foods, we examined the influence of 37 health food materials on the AhR using a reporter gene assay, and found that aqueous ethanol extracts of cassia seed and rosemary had particularly high AhR activity. To characterize the AhR-activating substances in these samples, the chemical constituents of the respective extracts were identified. From an active ethyl acetate fraction of the cassia seed extract, eight aromatic compounds were isolated. Among these compounds, aurantio-obtusin, an anthraquinone, elicited marked AhR activation. Chromatographic separation of an active ethyl acetate fraction of the rosemary extract gave nine compounds. Among these compounds, cirsimaritin induced AhR activity at 10–10^2 μM, and nepitrin and homoplantagenin, which are flavone glucosides, showed marked AhR activation at 10–10^3 μM.

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

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Nakamura M[*1,2], Yagami A[*1], Hara K[*2], Sano A[*1], Kobayashi T[*1], Aihara M[*3], Hide M[*4], Chinuki Y[*5], Morita E[*5], Teshima R, Matsunaga K[*1]: A new reliable method for detecting specific IgE antibodies in the patients with immediate type wheat allergy due to hydrolyzed wheat protein: correlation of its titer and clinical severity.

Allergol Int. 2014;63(2):243-9.

We developed quantitative and high-throughput test method for HWP-IWA（Immediate-type wheat allergy caused by a specific hydrolyzed wheat protein）. An enzyme-linked immunosorbent assay（ELISA）-based GP19S-specific IgE assay was tested using sera from 14 HWP-IWA and five conventional wheat-dependent exercise-induced anaphylaxis（CO-WDEIA）patients, as well as five healthy subjects. Then a validation study at five different institutions was carried out using these sera. The mean unit values converted from measured absorbance of ELISA were 68.3, 1.3 and 1.1 respectively. Furthermore, the validation study revealed reproducible results across all five institutions, with the standard deviation（SD）being 0.3-0.4 for the healthy group, 0.2-0.6 for the CO-WDEIA group, and 3.8-9.6 for HWP-IWA group except for one case. One case of HWP-IWA was excluded from analysis due to the high SD of 53.3 units, indicating that samples with a unit value > 100.0 will affect inter-laboratory reproducibility. Our findings suggest that the ELISA-based GP19S-specific IgE assay can be used to test HWP-IWA using venous blood samples, except for those with a unit value > 100.0.

Keywords: hydrolyzed wheat protein, IgE, ELISA

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Saito-Shida S, Nemoto S, Matsuda R: Multiresidue analysis of pesticides in vegetables and fruits by supercritical fluid extraction and liquid chromatography-tandem mass spectrometry.

Food Hyg Saf Sci. 2014;55:142-51.

A multiresidue method for analyzing pesticides in vegetables and fruits by supercritical fluid extraction（SFE）and LC-MS/MS was developed. The sample preparation and SFE parameters were optimized for extracting LC-amenable polar and medium-polarity pesticides. High recoveries were achieved for most of the tested pesticides by extracting a 1:1:1 sample–Celite–anhydrous magnesium sulfate mixture with supercritical carbon dioxide at 16.4 MPa at 40 ℃ for 30

min with methanol added as a modifier. The recoveries of 117 pesticides fortified with 0.01 mg/kg of each pesticide were 70–120%, and the relative standard deviations were less than 25% for 112 pesticides in tomato and 103 pesticides in cucumber. No significant differences were observed in the residue concentrations determined in real samples by the SFE method and the liquid extraction method (the modified Japanese official method). Higher recoveries of polar pesticides, such as acephate and methamidophos, were achieved by the developed SFE method than a liquid extraction method.

Keywords: pesticide, supercritical fluid extraction, LC-MS/MS

植草義徳，鍋師裕美，堤智昭，蜂須賀暁子，松田りえ子，手島玲子：トータルダイエット試料による食品を介した放射性物質の摂取量の推定.

食品衛生学雑誌 2014;55(4):177-82.

本研究では，食品を介した放射性物質の摂取量の実態を把握することを目的とし，マーケットバスケット（MB）試料（平成24～25年）および陰膳試料（平成24年）を用いて，放射性セシウムの一日摂取量（Bq/day）および1年当たりの預託実効線量（mSv/year）を推定した．MB試料および陰膳試料から推定された放射性セシウムの年当たり預託実効線量の最大値は，それぞれ0.0094および0.027mSv/yearであり，福島県近辺地域においてやや高い値を示す傾向が見られた．しかしながら，いずれの試料においても，放射性セシウムによる年当たり預託実効線量は，平成24年4月より施行された新基準値を定める根拠となった1mSv/yearと比較して極めて小さい値であることが明らかとなった．

Keywords: radioactive cesium, daily intake, annual committed effective dose

Knipping K*[1,2], Simons PJ*[3], Buelens-Sleumer LS*[1], Cox L*[3], den Hartog M*[3], de Jong N*[3], Teshima R, Garssen J*[1,2], Boon L*[3], Knippels Leon MJ*[1,2]: Development of : β-lactogloblin-specific chimeric human IgEκ monoclonal antibodies for in vitro safety assessment of whey hydrolysates.

PLOS ONE 2014;9(8):e106025.

Cow's milk-derived whey hydrolysates are nutritional substitutes for allergic infants. Safety or residual allergenicity assessment of these whey hydrolysates is crucial. Currently, rat basophilic leukemia RBL-2H3 cells expressing the human IgE receptor α-chain (huFcεRIα- RBL-2H3), sensitized with serum IgE from cow's milk allergic children, are being employed to assess in vitro residual allergenicity of these whey hydrolysates. An oligoclonal pool of chimeric human (chu)IgE antibodies against bovine β-lactoglobulin (a major allergen in whey) was generated to increase sensitivity, specificity, and reproducibility of existing degranulation assays. Mice were immunized with bovine β-lactoglobulin, and subsequently the variable domains of dissimilar anti-β-lactoglobulin mouse IgG antibodies were cloned and sequenced. Six chimeric antibodies were generated comprising mouse variable domains and human constant IgE/κ domains.

After sensitization with this pool of anti-β-lactoglobulin chuIgEs, huFcεRIα-expressing RBL-2H3 cells demonstrated degranulation upon cross-linking with whey, native 18 kDa β-lactoglobulin, and 5-10 kDa whey hydrolysates, whereas a 3 kDa whey hydrolysate and cow's milk powder (mainly casein) showed no degranulation. Usage of our 'unlimited' source and well-defined pool of β-lactoglobulin-specific recombinant chuIgEs to sensitize huFcεRIα on RBL-2H3 cells showed to be a relevant and sensitive alternative for serum IgEs from cow's milk allergic patients to assess safety of whey-based non-allergic hydrolyzed formula.

Keywords: chimeric human IgE antibodies, whey hydrolysates, in vitro safety assessment

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亀谷宏美*，高附巧，松田りえ子，堤智昭，等々力節子*：放射線照射した甲殻類（エビおよびカニ）の検知への電子スピン共鳴分光法の適用.

食品衛生学雑誌 2014;55(5):193-204.

日本で流通するエビ，カニの多くは輸入品が占めているため，国内向けの品種を対象とした照射検知法開発が重要である．本研究では，輸入量の多い品種のエビとカニを対象に，電子スピン共鳴（ESR）分光法による照射誘導ラジカルの同定と照射判定の可能性を検討した．エビ（ブラックタイガー，バナメイエビ）は腹節の殻と尾扇を，カニ（ズワイガニ，タラバガニ，ワタリガニ）は脚と螯（ハサミ）の殻を使用した．エビの腹節の殻と尾扇，カニの脚の殻は照射によって特異的に誘導されるラジカルは検出されず，ESRによる照射判定は不可能で

あった．カニの螯の殻はヒドロキシアパタイト由来の照射誘導ラジカルが強く認められ，照射判定できる可能性が示された．

Keywords: electron spin resonance spectroscopy, irradiated food, crustacean

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Higashisaka K*1, Fujimura M*1, Taira M*1, Yoshida T*1, Tsunoda S*2, Baba T*1, Yamaguchi N*1, Nabeshi H, Yoshikawa T*1, Nasu M*1, Yoshioka Y*1, Tsutsumi Y*1: Asian dust particles induce macrophage inflammatory responses via mitogen-activated protein kinase activation and reactive oxygen species production.

J Immunol Res. 2014;856154.

Asian dust is a springtime meteorological phenomenon that originates in the deserts of China and Mongolia. The dust is carried by prevailing winds across East Asia where it causes serious health problems. Most of the information available on the impact of Asian dust on human health is based on epidemiological investigations, so from a biological standpoint little is known of its effects. To clarify the effects of Asian dust on human health, it is essential to assess inflammatory responses to the dust and to evaluate the involvement of these responses in the pathogenesis or aggravation of disease. Here, we investigated the induction of inflammatory responses by Asian dust particles in macrophages. Treatment with Asian dust particles induced greater production of inflammatory cytokines interleukin-6 and tumor necrosis factor-α（TNF-α）compared with treatment with soil dust. Furthermore, a soil dust sample containing only particles ≤10 μm in diameter provoked a greater inflammatory response than soil dust samples containing particles >10 μm. In addition, Asian dust particles-induced TNF-α production was dependent on endocytosis, the production of reactive oxygen species, and the activation of nuclear factor-κ B and mitogen-activated protein kinases. Together, these results suggest that Asian dust particles induce inflammatory disease through the activation of macrophages.

Keywords: Asian dust, macrophage inflammatory responses, mitogen-activated protein kinase

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Yoshida T*1, Yoshioka Y*1, Takahashi H*1, Misato K*1, Mori T*1, Hirai T*1, Nagano K*2, Abe Y, Mukai Y*2, Kamada H*2, Tsunoda S*2, Nabeshi H, Yoshikawa T*1, Higashisaka K*1, Tsutsumi Y*1: Intestinal absorption and biological effects of orally administered amorphous silica particles.

Nanoscale Res Lett. 2014;9(1):532.

Although amorphous silica nanoparticles are widely used in the production of food products（e.g., as anticaking agents）, there is little information available about their absorption and biological effects after oral exposure. Here, we examined the in vitro intestinal absorption and in vivo biological effects in mice of orally administered amorphous silica particles with diameters of 70, 300, and 1,000 nm（nSP70, mSP300, and mSP1000, respectively）and of nSP70 that had been surface-modified with carboxyl or amine groups（nSP70-C and nSP70-N, respectively）. Analysis of intestinal absorption by means of the everted gut sac method combined with an inductively coupled plasma optical emission spectrometer showed that the intestinal absorption of nSP70-C was significantly greater than that of nSP70. The absorption of nSP70-N tended to be greater than that of nSP70; however, the results were not statistically significant. Our results indicate that silica nanoparticles can be absorbed through the intestine and that particle diameter and surface properties are major determinants of the degree of absorption. We also examined the biological effects of the silica particles after 28-day oral exposure in mice. Hematological, histopathological, and biochemical analyses showed no significant differences between control mice and mice treated with the silica particles, suggesting that the silica nanoparticles evaluated in this study are safe for use in food production.

Keywords: amorphous silica nanoparticles, oral exposure, intestinal absorption

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堤智昭，足立利華，高附巧，根井大介*1，亀谷宏美*1，等々力節子*1，菊地正博*2，小林泰彦*2，松田りえ子，手島玲子：加工食品を対象としたアルキルシクロブタノン法（EN1785）の性能評価．

食品照射 2014;49(1):9-15.

2-アルキルシクロブタノン（ACB）法は，食品中の脂

質から放射線照射に特異的に生じる2-ドデシルシクロブタノン（DCB）と2-テトラデシルシクロブタノン（TCB）を検知指標として，照射の有無を判定する定性試験法である．本研究では，我々が既に報告しているACB法の単一試験室における性能評価方法を用いて，汎用されているACB法であるヨーロッパ標準分析法（EN1785）の液卵，カマンベールチーズ，ソーセージ，及びウナギ白焼きに対する適用性を評価した．未照射の各食品から抽出した脂肪を陰性試料，陰性試料にDCB及びTCBを0.05μg/g lipid添加した脂肪を陽性試料とした．各食品について4個の陰性試料，及び16個の陽性試料を分析し，本法の検知性能を評価した．本法は各食品の陰性及び陽性試料を全て正しく判定でき，これらの食品への適用が妥当であると判断できた．次に妥当性評価した本法の検知性能を確認するため，未照射及びガンマ線照射（0.5～4kGy）した上記と同種の食品を本法により分析した．その結果，全ての試料について照射の有無を正しく判定することができ，実用されている線量で照射された食品に対して十分な検知性能を有していた．

Keywords: irradiated food, 2-alkylcyclobutanone, EN1785

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Saito-Shida S, Nemoto S, Matsuda R: Simultaneous determination of acidic pesticides in vegetables and fruits by liquid chromatography–tandem mass spectrometry. *J Environ Sci Health B*. 2015;50:151-62.

A sensitive and efficient method has been developed for the simultaneous determination of 73 multiclass acidic pesticides, such as phenoxy acid and sulfonylurea herbicides, in vegetables and fruits. The sample preparation procedure was carefully optimized for the efficient removal of co-extracted matrix components. The method involves extraction of the acidic pesticides with acetonitrile containing hydrochloric acid, removal of water from the crude extract by salting out, and sequential cleanup by ODS and silica gel columns. For samples containing high amounts of pigments such as spinach, additional cleanup using a graphitized carbon column was performed prior to LC-MS/MS analysis. Recovery tests were performed five times for each sample of cabbage, spinach, potato, eggplant, orange, and apple fortified at 0.01 mg/kg. Out of the 73 tested pesticides, 70 for cabbage, 67 for spinach, 69 for potato, 67 for eggplant, 64 for orange, and 70 for apple were within 70–120%, with relative standard deviations below 25%. Nitenpyram and pyrasulfotole showed low recoveries for all the samples tested, probably due to low recoveries from silica gel column. The developed method effectively removed co-extracted matrix components and was highly selective, with no interfering peaks found in the chromatograms of blank samples. The overall results indicate that the developed method is suitable for the quantitative analysis of acidic pesticide residues in vegetables and fruits.

Keywords: acidic pesticide, multi-residue method, LC-MS/MS

Tsutsumi T, Watanabe T, Matsuda R, Teshima R: Dietary intake of dioxins in Japan, fiscal year 1998-2013. *Organohalogen Compounds* 2014;76:1325-8.

Food is generally recognized as the main source of human intake of dioxins. A total diet study（TDS), also known as a market basket study, is a useful method of estimating the average dietary intake of contaminants. Here, we report the nationwide TDS results for fiscal year（FY）2013 and also discuss the time trend of dietary intake of dioxins from TDS results obtained over the last 16 years（FY 1998-2013). The average dietary intake calculated at ND = 0 in FY 2013 was 0.58 pg TEQ/kg bw/day for an adult weighing 50 kg. The intake was about one-seventh of the tolerable daily intake（TDI）of 4 pg TEQ/kg bw/day set by the Japanese government. Overall, the average intakes appeared to be decreasing slowly between FY 1998 and 2013. We also conducted a Monte Carlo simulation using our surveillance data to obtain information on the distribution of intake of dioxins from fish and shellfish in the general Japanese population. The estimated average dioxin intake was 1.3 pg TEQ/kg bw/day. The average dioxin intake was well below the Japanese TDI but about twice the intake estimated by the TDS in FY 2013.

Keywords: dioxins, total diet study, dietary intake

Uekusa Y, Takatsuki S, Watanabe T, Kataoka Y, Tsutsumi T, Matsuda R, Hachisuka A, Teshima R: Concentrations of polychlorinated biphenyls in commercially available fish obtained from tsunami-stricken areas of Japan.
Organohalogen Compounds 2014;76:1074-7.

The contamination of foods by chemical pollutants including radioactive materials, heavy metals, and hazardous organic compounds has been highly concerned after the Great East Japan Earthquake in 2011. Here, we focused

on the contamination of marine fish by polychlorinated biphenyls (PCBs). To determine whether fresh PCB contamination has occurred, we investigated not only the total concentration of PCBs but also the proportions of 209 congeners of PCBs in fish obtained from markets in tsunami-stricken areas, by using a high-resolution gas chromatography–high-resolution mass spectroscopy (HRGC-HRMS). PCBs were detected in all 101 fish samples. Total PCB concentrations in about 90% of samples were below 15 ng/g (wet weight). The minimum and maximum concentrations were 0.45 and 83 ng/g, respectively; these levels were lower than the provisional regulation value (500 ng/g in oceans) in Japan. Our results revealed that it was unlikely marine fish obtained from markets in tsunami-stricken areas were contaminated with PCBs at high concentrations.

Keywords: PCBs, marine fish, HRGC-HRMS

Kataoka Y, Watanabe T, Hayashi T, Matsuda R, Hachisuka A, Teshima R: Surveillance of concentrations of harmful elements in foods purchased in areas affected by the Great East Japan Earthquake.
Organohalogen Compounds 2014;76:1092-5.

Serious damage occurred as a result of the Great East Japan Earthquake in 2011. Therefore, it is possible that foods of the disaster area have been contaminated with harmful elements such as heavy metal elements dispersed widely into the environment by the tsunami. Here, we examined the possibility of food contamination with potentially harmful trace elements and heavy metal elements as a result of the tsunami. We used ICP-MS to survey the concentrations of 15 elements (Pb, Hg, Ba, Sb, Sn, Cd, Mo, Se, As, Ni, Co, Cr, V, Al, B) in 510 food products purchased from markets located in the disaster area. When an individual food contaminated with harmful elements at clearly high levels was found frequently in the same food group, we considered that the contamination was a result of the tunami. However, we found here that the concentrations of harmful elements in the various food products purchased from markets located in tunami-striken areas were not clearly high.

Keywords: trace element, heavy metal element, ICP-MS

Akiyama H, Matsuoka H, Okuyama T[*1], Higashi K[*1], Toida T[*1], Komatsu H[*2], Sugita-Konishi Y, Kobori S, Kodama Y, Yoshida M, Endou H[*3]: The acute encephalopathy induced by intake of Sugihiratake mushroom in the patients with renal damage might be associated with the intoxication of cyanide and thiocyanate.
Food Safety 2015;3:16-25.

A novel type of encephalopathy associated with the ingestion of Sugihiratake mushroom (Pleurocybella porrigens) occurred in patients with chronic renal failure treated on hemodialysis in fall, 2004 in Japan. To clarify the mechanism of encephalopathy onset, we, for the first time, purified the cyanogen glycoside fraction (CG) from Sugihiratake mushroom using reversed phase high-performance liquid chromatography and hydrophilic interaction chromatography. Furthermore, we investigated single dose toxicity of the CG in an adenine-induced rat model of chronic renal damage (CRD). Pathological examination of kidneys indicates the development of CRD. Oral administration of the CG induces the accumulation of thiocyanate in the hemolyzed blood and brain in CRD rats, although no morphological changes were found in the brain. No further enhancement of kidney damage is observed after the oral administration of the CG in CRD rats. This is the first experimental report to suggest that acute encephalopathy, induced by Sugihiratake mushroom intake in the patients with chronic renal failure, is associated with intoxication of cyanide and thiocyanate, presumably produced metabolically produced after the ingestion of Sugihiratake mushroom.

Keywords: Sugihiratake, cyanogen glycoside, encephalopathy

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Minegishi Y[*1,2], Mano J[*3], Takabatake T[*3], Nakamura K, Kondo K, Kato Y[*2], Kitta K[*3], Akiyama H: Development of pBT63, a positive control plasmid for qualitative detection of genetically modified rice.
Jpn J Food Chem Safety. 2014;21:48-56.

Plasmids containing polymerase chain reaction (PCR) target sequences are widely used as positive controls for analyses of genetically modified food. To eliminate amplification of false positives due to plasmid contamination, we developed a qualitative PCR control plasmid containing amplicons with an additional internal restriction enzyme recognition site. We designed a control plasmid template

using the detection method of genetically modified rice line Shanyou 63 that had a BamHI site added（pBT63), and amplicons derived from it were digested, whereas amplicons derived from the unaltered Bt63 genomic DNA template could not be digested. Thus, our control plasmid enables distinction between detection of false positives（caused by amplicons derived from the plasmid）and true positives（due to the presence of Bt63 rice genomic DNA）in qualitative PCR testing of genetically modified rice products.

Keywords: positive control plasmid, genetically modified, qualitative PCR

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大月典子，杉本理恵*，佐藤恭子，杉本直樹，秋山卓美，豊田正武*，穐山浩：化粧品・医薬部外品中の乳アレルゲンタンパク質の分析.

日本食品化学学会誌 2014;21:155-62.

牛乳アレルギーの発症と乳アレルゲンタンパク質による経皮感作との関連性について考察するために，スキンケア用の化粧品，医薬部外品に含まれる乳由来のアレルゲンタンパク質の調査を行った．国産の化粧品および医薬部外品29製品について，αS1-caseinおよびβ-lactoglobulinをイムノクロマトグラフィーとELISAで測定した．29製品中9製品より，7.1 μg/gから18,810 μg/gのαS1-casein，あるいは検出限界以上から10,429 μg/gのβ-lactoglobulinが定量された．ヨーグルト・脱脂粉乳など，全乳から加工された成分を表示に記載した5製品からは，7.1 μg/gから18,810 μg/gのαS1-caseinと4.4 μg/gから10,429 μg/gのβ-lactoglobulinの両方が検出された．一方，乳清画分を原材料とした15製品からは2製品のみ，6.6 μg/gと6.9 μg/gのβ-lactoglobulin が検出された．加えて，乳清画分，カゼイン画分，乳脂，乳糖などの非タンパク質画分を原材料とした23製品のαS1-caseinは検出限界以下であった．乳アレルゲンタンパク質の含有量は，原材料の乳成分の種類に影響される傾向にあった．結果として，無作為に抽出した国産化粧品および医薬部外品中，31%の製品よりαS1-caseinあるいはβ-lactoglobulinが検出された．さらに乳アレルゲンタンパク質陽性であった9製品のうち石鹸，ローション，入浴剤など4製品が乳幼児用の製品だった．本研究は，市販化粧品に含まれる食品由来アレルゲンタンパク質を定量した初めての報告である．これらの結果により，牛乳由来成分を含む化粧品の使用による乳アレルゲンタンパク質の経皮感作が懸念された.

Keywords: 牛乳アレルギー，イムノクロマトグラフィー，αs1-casein

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山川有子*1，山野朋子*2，相原道子*2，穐山浩，池澤善郎*3：フランス製赤色マカロンに含まれるコチニール色素が原因と思われるアナフィラキシーの1例.

皮膚臨床 2014;56:1241-5.

30歳，女性．フランス製赤色マカロンを摂食中から，即時型アレルギー反応が出現．赤色マカロンにはカルミンあるいはコチニール色素が含有されており，皮膚プリックテストにてカルミンおよびコチニール色素に陽性を示し，コチニール色素によるⅠ型アレルギーと診断した．近年コチニール色素のアレルギーの原因物質としてフランス製赤色マカロンが数例に報告されている．またこれらが経皮感作後に発症している症例もあり，今後も注意が必要である.

Keywords: コチニール色素，カルミン，経皮感作

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原田晋*1，穐山浩，杉本直樹，山川有子*2：ドイツ製ブラッドオレンジジュースに含まれていたコチニール色素によるアナフィラキシーの1例.

皮膚臨床 2014;56:1247-51.

29歳，女性．ドイツ滞在中にブラッドオレンジジュースなどを摂取後に全身性膨疹，眼瞼浮腫，咳嗽などのアナフィラキシー症状が出現．プリックテスト等の結果より，ブラッドオレンジジュース中に含まれたコチニール色素によるアナフィラキシーと診断した．コチニール色素を含む食品の経口摂取による即時型アレルギーの近年の報告はすべて日本人症例であり，日本人でフランス製赤色マカロンやブラッドオレンジジュースなどを原因食物としたコチニールアレルギーを発症しやすい要因が潜在している可能性が疑われる．そのため特に本邦では今後コチニールアレルギーの発症に留意する必要がある.

Keywords: コチニール，カルミン，アナフィラキシー

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山内良子*1，深水さやか*1，小浜友紀子*1，島村智子*2，

柏木丈拡[*2]，受田浩之[*2]，穐山浩，松井利郎[*3]，石川洋哉[*1]：酸化防止剤力価評価を目的としたDPPHおよびABTSラジカル消去能評価法の特性比較．
日本食品保蔵科学会誌 2014;40:55-63.

食品添加物として使用されている天然酸化防止剤の品質評価を行うため，抗酸化能に基づいた新たな評価法の策定が望まれている．我々は評価法の候補として，DPPHラジカル消去能測定法およびABTSラジカル消去能測定法を選択し，抗酸化能測定法の検証と特徴づけを行った．フラボノイド類，ポリフェノール類，ビタミン，アミノ酸，ペプチドなど計21種類の抗酸化物を用い，DPPH法およびABTS法による抗酸化能の測定を行った．DPPH法およびABTS法による活性値は，カテコール構造およびピロガロール構造と密接に関連していることが明らかとなった．さらに，フラボノイド類におけるC環3位の水酸基およびカテキン類の没食子酸エステル構造も同様に重要な活性発現要因であった．DPPH法およびABTS法による抗酸化活性値は，カテコール構造を持たない化合物では同程度であるものの，カテコール構造持つ化合物ではABTS法よりもDPPH法による活性値がおよそ1.3 μmol TE/μmol高い結果となった．この結果は，DPPH法における活性値に，抗酸化物カテコール構造の再生反応が反映されている可能性を示唆するものであった．DPPH法およびABTS法とFRAP法との相関を求めた結果，ABTS法とFRAP法による相関と比較してDPPH法とFRAP法による相関が極めて高く，DPPH法が鉄イオン還元能をより正確に反映していることが示された．以上のことから，DPPH法は食品添加物として利用されている天然酸化防止剤を評価するための評価法として有力であることが示唆された．
Keywords: 酸化防止剤，ポリフェノール，DPPH法

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Sato K, Suzuki I, Kubota H, Furusho N, Inoue T[*], Yasukouchi Y[*], Akiyama H: Estimation of daily aluminum intake in Japan based on food consumption inspection results: impact of food additives.
Food Science & Nutrition 2014;2:389-97.

Dietary aluminum (Al) intake by young children, children, youths, and adults in Japan was estimated using the market basket method. The Al content of food category (I–VII) samples for each age group was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The Al content in processed foods and unprocessed foods ranged from 0.40 to 21.7 mg/kg and from 0.32 to 0.54 mg/kg, respectively. For processed foods in all age groups, the Al content in food category VI samples, sugar and confections/savories, was the highest, followed by those in category II, cereals. The daily dietary Al intake from processed foods was much larger than that from unprocessed foods. The mean weekly percentages of the provisional tolerable weekly intake (PTWI, established by the joint FAO/WHO Expert Committee on Food Additives in 2011) from processed foods for all age groups are 43.1, 22.4, 17.6 and 15.1%, respectively. Only the highest consumer Al exposure value (>P95) of the young children group exceeded the PTWI.
Keywords: Aluminum, dietary intake, ICP-AES

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Tatebe C, Zhong X, Ohtsuki T, Kubota H, Sato K, Akiyama H: A simple and rapid chromatographic method to determine unauthorized basic colorants (rhodamine B, auramine O, and pararosaniline) in processed foods.
Food Science & Nutrition 2014;2:547-56.

A simple and rapid high-performance liquid chromatography (HPLC) method to determine basic colorants such as pararosaniline (PA), auramine O (AO), and rhodamine B (RB) in various processed foods was developed. Linearity of the calibration curves ranged from 0.05 to 50 μg/mL for PA and 0.05–100 μg/mL for AO and RB. The detection and quantification limits (LOD and LOQ) of the basic colorants, which were evaluated as signal-to-noise ratios of 3 for LOD and 10 for LOQ, ranged from 0.0125 to 0.05 and 0.025 to 0.125 μg/g, respectively. The recoveries and relative standard deviations of three basic colorants in six processed foods, namely, chili sauce, curry paste, gochujang (hot pepper paste), tandoori chicken (roasted chicken prepared with yogurt and spices), powder soup, and shrimp powder ranged from 70.2% to 102.8% and 0.8% to 8.0%, respectively. The intraday precision of the recovery test ranged from 1.7% to 4.5%, whereas the interday precision ranged from 3.7% to 7.7%. The reported method has been successfully applied to basic colorant determination in various processed foods such as fat-based food matrices (curry paste and tandoori chicken), chili products (gochujang and chili sauce), and proteinbased products

（shrimp powder and powder soup）. Thin layer chromatography and liquid chromatography/mass spectrometry methods for the determination of basic colorants in processed foods were also developed for rapid analysis and identification, respectively. These methods are very useful for monitoring unauthorized basic colorants in inspection centers or quarantine laboratories in many countries.

Keywords: Auramine O, pararosaniline, rhodamine B

Ohtsuki T, Sato K, Abe Y, Sugimoto N, Akiyama H: Quantification of acesulfame potassium in processed foods by quantitative ^{1}H NMR.

Talanta 2015;131:712-8.

Acesulfame potassium（AceK）, a high-intensity and non-caloric artificial sweetener, is used in various processed foods as a food additive. In this study, we established and validated a method for determining the AceK content in various processed foods by solvent extraction and quantitative ^{1}H NMR, using a certified reference material as the internal standard. In the recovery test, the proposed method gave satisfactory recoveries（88.4%－99.6%）and repeatabilities（0.6%－5.6%）for various processed foods. The limit of quantification was confirmed as 0.13 g kg^{-1}, which was sufficiently low for the purposes of monitoring AceK levels. In the analysis of commercially processed foods containing AceK, all AceK contents determined by the proposed method were in good agreement with those obtained by a conventional method based on dialysis and HPLC. Moreover, this method can achieve rapid quantification and yields analytical data with traceability to the International System of Units（SI）without the need for an authentic analyte standard. Therefore, the proposed method is a useful and practical tool for the determination of AceK in processed foods.

Keywords: processed food, quantitative NMR, acesulfame potassium

山崎太一[*1]，大槻崇，三浦亨[*2]，末松孝子[*3]，堀之内嵩暁[*4]，村上雅代[*1]，齋藤剛[*1]，井原俊英[*1]，多田敦子，田原麻衣子，合田幸広，穐山浩，中尾慎治[*2]，山田裕子[*2]，小池亮[*4]，杉本直樹：^{1}H NMRによる精確な定量分析のための内標準液を用いる試料調製法の検討.

分析化学 2014;63:323-9.

核磁気共鳴（NMR）を用いた定量分析法（定量NMR法）は原子核を基準にできる特性から，測定対象の標準物質を必要としないで定量できるユニバーサルな分析法として急速に注目を集めている．特に内標準法は精確な値が得られることから公定法にも採用され始めている一方で，現状の内標準法では，試料調製において高分解能な天秤びんを用いた精確な秤量が必要とされるという点が課題となっている．そこで本研究では，試料調製におけるコストと時間を低減するために，内標準液を用いた定量NMR法について検討した．質量比混合法と容量法の二つの方法で試料調製を行い，測定結果に与える調製方法の影響について考察した．さらに複数機関による共同分析を実施することで，内標準液を用いた方法の妥当性を評価し，より簡便な容量法においても1%以下の精確さで定量分析ができることを明らかにした．

Keywords: 定量NMR，内標準法，内標準液

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Tada A, Ishizuki K, Yamazaki T*, Sugimoto N, Akiyama H: Method for the determination of natural ester-type gum bases used as food additives via direct analysis of their constituent wax esters using high-temperature GC/MS.

Food Science & Nutrition 2014;2:417-25.

Natural ester-type gum bases, which are used worldwide as food additives, mainly consist of wax esters composed of long chain fatty acids and long chain fatty alcohols. There are many varieties of ester-type gum bases, and thus a useful method for their discrimination is needed in order to establish official specifications and manage their quality control. Herein is reported a rapid and simple method for the analysis of different ester-type gum bases used as food additives by high-temperature gas chromatography/mass spectrometry（GC/MS）. With this method, the constituent wax esters in ester-type gum bases can be detected without hydrolysis and derivatization. The method was applied to the determination of ten types of gum bases, including beeswax, carnauba wax, lanolin, and jojoba wax, and it was demonstrated that the gum bases derived from identical origins have specific and characteristic total ion chromatogram（TIC）patterns and ester compositions. Food additive gum bases were thus distinguished from one another based on their TIC patterns and then more clearly discriminated

using simultaneous monitoring of the fragment ions corresponding to the fatty acid moieties of the individual molecular species of the wax esters. This direct high-temperature GC/MS method was shown to be very useful for the rapid and simple discrimination of varieties of ester-type gum bases used as food additives.
Keywords: food additive, gum base, wax ester

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Shimamura T*1, Sumikura Y*1, Yamazaki T*2, Tada A, Kashiwagi T*1, Ishikawa H*3, Matsui T*4, Sugimoto N, Akiyama H, Ukeda H*1: Applicability of DPPH assay for evaluation of antioxidant capacity of food additives -Inter-laboratory evaluation study-.
Analytical Sciences 2014;30:717-21.

An inter-laboratory evaluation study was conducted in order to evaluate the antioxidant capacity of food additives by using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Four antioxidants used as existing food additives (i.e., tea extract, grape seed extract, enju extract, and *d*-α-tocopherol) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were used as analytical samples, and 14 laboratories participated in this study. The repeatability relative standard deviation (RSDr) of the IC_{50} of Trolox, four antioxidants, and the Trolox equivalent antioxidant capacity (TEAC) were 1.8 – 2.2%, 2.2 – 2.9%, and 2.1 – 2.5%, respectively. Thus, the proposed DPPH assay showed good performance within the same laboratory. The reproducibility relative standard deviation (RSDR) of IC_{50} of Trolox, four antioxidants, and TEAC were 4.0 – 7.9%, 6.0 – 11%, and 3.7 – 9.3%, respectively. The RSDR/RSDr values of TEAC were lower than, or nearly equal to, those of IC_{50} of the four antioxidants, suggesting that the use of TEAC was effective for reducing the variance among the laboratories. These results showed that the proposed DPPH assay could be used as a standard method to evaluate the antioxidant capacity of food additives.
Keywords: DPPH assay, inter-laboratory study, antioxidant

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食品衛生学雑誌 2014;55:117-34.

ガラス製，陶磁器製またはホウロウ引きの器具・容器包装，ならびに金属缶のカドミウム（Cd）および鉛（Pb）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17機関が参加し，濃度非明示の8濃度16検体についてフレーム方式原子吸光光度法（AAS），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CdおよびPbの定量を行った．その結果，AAS，ICP-OESおよびICP-MS（内標法）では真度が93～105%，併行精度（RSD_r）が0.7～8.4%，室間再現精度（RSD_R）が2.6～19.3%であり，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．一方，GF-AASではいくつかの結果でRSD_rが10%を超えており，適切な精度管理が必要であった．
Keywords: カドミウム，鉛，溶出試験

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野村千枝[*15]，疋田晃典[*16]，松山重倫[*17]，村上亮[*18]，山口未来，和田岳成[*19]，渡辺一成[*20]，穐山浩：合成樹脂製器具・容器包装におけるカドミウムおよび鉛材質試験法の性能比較.

食品衛生学雑誌 2014;55:269-78.

食品衛生法における合成樹脂製器具・容器包装のカドミウム（Cd）および鉛（Pb）材質試験について，公定法と各種代替法の性能を比較した．19機関が試験室間共同試験に参加し，3種のポリ塩化ビニル製ペレット中のCdおよびPbを定量した．公定法は，試料を灰化後，塩酸に溶解した溶液を水浴上で蒸発乾固し，原子吸光光度法（AAS）または誘導結合プラズマ発光強度測定法（ICP-OES）で測定する．その真度は86～95%，併行精度（RSD_r）は3.1～9.4%，室間再現精度（RSD_R）は8.6～22.1%であり，その性能は規格試験法として十分であった．ホットプレート上で蒸発乾固しAASおよびICP-OESで測定する方法は，公定法よりも真度とRSD_rが劣っていたが，代替法として適用可能である．マイクロウェーブ分解法（MW法）による試験溶液の調製は公定法よりも性能がよく，代替法として十分に適用可能である．また，誘導結合プラズマ質量分析（ICP-MS）法は測定法の代替法として適用可能であるが，試料を完全に灰化する必要がある．

Keywords: カドミウム，鉛，材質試験

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Abe Y, Yamaguchi M, Mutsuga M, Kawamura Y, Akiyama H: Survey of volatile substances in kitchen utensils made from acrylonitrile–butadiene–styrene and acrylonitrile–styrene resin in Japan.

Food Science & Nutrition 2014;2:236-43.

Residual levels of 14 volatile substances, including 1,3-butadiene, acrylonitrile, benzene, ethylbenzene, and styrene, in 30 kitchen utensils made from acrylonitrile–butadiene–styrene resin（ABS）and acrylonitrile–styrene resin（AS）such as slicers, picks, cups, and lunch boxes in Japan were simultaneously determined using headspace gas chromatography/mass spectroscopy（HS-GC/MS）. The maximum residual levels in the ABS and AS samples were found to be 2000 and 2800 μg/g of styrene, respectively. The residual levels of 1,3-butadiene ranged from 0.06 to 1.7 μg/g in ABS, and three of 15 ABS samples exceeded the regulatory limit for this compound as established by the European Union（EU）. The residual levels of acrylonitrile ranged from 0.15 to 20 μg/g in ABS and from 19 to 180 μg/g in AS. The levels of this substance in seven ABS and six AS samples exceeded the limit set by the U.S. Food and Drug Administration（FDA）. Furthermore, the levels of acrylonitrile in three AS samples exceeded the voluntary standard established by Japanese industries. These results clearly indicate that the residual levels of some volatile compounds are still high in ABS and AS kitchen utensils and further observations are needed.

Keywords: acrylonitrile–butadiene–styrene（ABS）, acrylonitrile–styrene（AS）, volatile substances

Ohno H[*], Mutsuga M, Kawamura Y: Identification and quantitation of volatile organic compounds in poly（methyl methacrylate）kitchen utensils by headspace gas chromatography/mass spectrometry.

Journal of AOAC International 2014;97:1452-8.

A headspace GC/MS method was developed for identification and quantitation of residual volatile organic compounds in poly（methyl methacrylate）（PMMA）kitchen utensils. A sample was cut into small pieces, then *N,N*-dimethylacetamide was added in a headspace vial and sealed. After storing for more than 1 day at room temperature, the vial was incubated for 1 h at 90℃, and the headspace gas was analyzed by GC/MS. In 24 PMMA kitchen utensils, 16 volatile organic compounds including methyl methacrylate, methyl acrylate, toluene, 2-methyl-1-butene, 2-methyl-2-butene, 2-methylpropanal, methyl propionate, methyl isobutyrate, *trans*-3-heptene,

heptane, *cis*-3-heptene, *trans*-2-heptene, *cis*-2-heptene, 2,4,4-trimethyl-1-pentene, 2,4,4-trimethyl-2-pentene, and 1-octene were identified and quantitated. These 15 volatile compounds except methyl methacrylate were found for the first time in PMMA kitchen utensils. Recovery rates from spiked samples were 97.4–104.0% with CV values of 2.8–9.6%. Samples contained 190–7900 μg/g of methyl methacrylate, 26–810 μg/g of methyl acrylate, and 2–1300 μg/g of toluene; other compounds were at levels less than 100 μg/g. Methyl methacrylate was the main monomer of PMMA and methyl acrylate was a comonomer; toluene should be used as a solvent.

Keywords: poly（methyl methacrylate）, volatile substances, kitchen utensils

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Kyoui D*, Takahashi H*, Miya S*, Kuda T*, Igimi S, Kimura B*: Genetic distance in the whole-genome perspective on *Listeria monocytogenes* strains F2-382 and NIHS-28 that show similar subtyping results.
BMC Microbiol. 2014;14:309.

Genome subtyping approaches could provide useful epidemiological information regarding food pathogens. However, the full genomic diversity of strains that show similar subtyping results has not yet been completely explored. Most subtyping methods are based on the differences of only a portion of the genome. We investigated two draft genome sequences of Listeria monocytogenes strain F2-382 and NIHS-28, which have been identified as closely related strains by subtyping（identical multi-virulence-locus sequence typing and multiple-locus variable number tandem repeat analysis sequence types and very similar pulsed-field gel electrophoresis patterns）, despite their different sources.

Keywords: Genome subtyping, *Listeria monocytogenes*, genome sequence

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Ogihara H*, Kiribe N*, Fukuda N*, Furukawa S*. Morinaga Y*, Igimi S: *Cronobacter* spp. In commercially available dried food in Japan.
Biocontrol Sciences 2014;19:209-13.

A total of 140 samples of dried food sold in Japan were surveyed and tested for the presence of viable bacteria, distributing of coliform bacteria, and contamination with *Cronobacter* spp. The samples were purchased from retail stores in Tokyo, and Kanagawa Prefecture. Out of the 140 samples tested, viable bacteria were found in 135 samples and coliform bacteria were found in 23 samples. Qualitative and quantitative testing revealed the presence of *Cronobacter* spp. In 35（25 %）and 11 samples（7.9%）, respectively. The most commonly found *Cronobacter* species were *C. sakazakii*, with the next most common, in order, being *C. muytjensii* and *C. turicensis*. The actual numbers of *Cronobacter* species in the tested dried foods were low, but the widespread contamination particularly in dried herbs and vegetables was confirmed.

Keywords: *Cronobacter* spp., dried food, *Cronobacter sakazakii*,

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Miya S*, Takahashi H*, Nakagawa M*, Kuda T*, Igimi S, Kimura B*: Genetic characteristics of Japanese clinical *Listeria monocytogenes* isolates.
PLoS One 2015;10(3):e0122902.

Listeria monocytogenes causes foodborne illnesses through consumption of ready-to-eat foods. Although 135-201annual listeriosis cases have been estimated in Japan, the details regarding the clinical isolates such as infection source, virulence level, and other genetic characteristics, are not known. In order to uncover the trends of listeriosis in Japan and use the knowledge for prevention measures to be taken, the genetic characteristics of the past human clinical isolates needs to be elucidated. For this purpose, multilocus tandem-repeat sequence analysis（MLTSA）and multi-virulence-locus sequence typing（MVLST）were used in this study. The clinical isolates showed a variety of genetically distant genotypes, indicating they were from sporadic cases. However, the MVLST profiles of 7 clinical isolates were identical to those of epidemic clone（EC）I isolates, which have caused several serious outbreaks in other countries, suggesting the possibility that they have strong virulence potential and originated from a single outbreak. Moreover, 6 Japanese food isolates shared their genotypes with ECI isolates, indicating that there may be risks for listeriosis outbreak in Japan. This is the first investigational study on genetic characteristics of Japanese listeriosis isolates. The listeriosis cases happened in the past are presumably sporadic, but it is still possible that some

isolates with strong virulence potential have caused listeriosis outbreaks, and future listeriosis risks also exist.

Keywords: *Listeria monocytogenes*, MLTSA, MVLST

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Suzuki H: Influence of body weight of mice on the susceptibility to okadaic acid, a diarrhetic shellfish poisoning toxin.
Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2014;31:719-22.

The mouse bioassay（MBA）for diarrhetic shellfish poisoning（DSP）toxins has been widely used in many countries of the world. However, different body weight ranges of mice are designated to be used in the Japanese official method and European Union procedure. In this study we investigated whether and to what extent the body weights of the mice affect the susceptibility to DSP toxins. A lethal dose of okadaic acid, one of the representative DSP toxins, was injected intraperitoneally into mice of five different body weight range groups, from 14 to 24 g. The mice were observed until 24 h after injection. The lethality was 100% in the 14-15 and 16-17 g groups, 80% in the 19-20 g group, 50% in the 21-22 g group, and 40% in the 23-24 g group, with significant differences. Survival analysis indicated a relationship between body weights of mice and susceptibility to okadaic acid. These results would be quite useful not only for the MBA, but also to improve understanding of the biological responses to DSP toxins.

Keywords: mouse bioassay, diarrhetic shellfish poisoning toxin, okadaic acid

Suzuki H, Machii K: Comparison of toxicity between saxitoxin and decarbamoyl saxitoxin in the mouse bioassay for paralytic shellfish poisoning toxins.
J Vet Med Sci. 2014;76:1523-5.

The mouse bioassay（MBA）for paralytic shellfish poisoning（PSP）toxins has been used in the AOAC Official Method and the official Japanese method. In the AOAC Official Method, the saxitoxin（STX）standard provided by the U.S. Food and Drug Administration（FDA）is used, but no standard is used in the official Japanese method. The objective of this study was to compare the toxicity of decarbamoyl STX（dcSTX）, one of the derivatives of STX and a candidate standard for the MBA for PSP toxins in Japan, to that of FDA STX in the MBA platform. In this study, the toxicity of dcSTX was 918.0 ± 44.9 mouse units/μmol, and the relative toxicity ratio of dcSTX to FDA STX based on moles was 0.478.

Keywords: mouse bioassay, saxitoxin（STX）, decarbamoyl STX（dcSTX）

Yogi K*1, Sakugawa S*2, Oshiro N, Ikehara T*3, Sugiyama K*4, Yasumoto T*5: Determination of toxins involved in ciguatera fish poisoning in the pacific by LC/MS.
J AOAC Int. 2014;97:398-402.

Ciguatera fish poisoning is the most extensive and difficult to control of the seafood poisonings. To facilitate monitoring of fish toxicity, toxin profiles were investigated by an LC/MS/MS method using 14 reference toxins on eight representative species of fish collected in four different areas of the Pacific. Snappers and groupers from Okinawa contained ciguatoxin-1B（CTX1B）and two deoxy congeners at variable but species-specific ratios, while red snapper, *Lutjanus bohar*, from Minami-Torishima, and amberjack, *Seriola dumerili*, from Hawaii, contained both CTX1B-type and CTX3C-type toxins. Spotted knifejaw, *Oplegnathus punctatus*, from Okinawan waters, contained mainly CTX4A and CTX4B, but the same species caught at Miyazaki was contaminated primarily with the CTX3C-type toxins. Otherwise, the toxin profiles were consistently species-specific in fish collected from various locations around Okinawa over 20 years. The LC/MS/MS and mouse bioassay results agreed well, indicating the LC/MS/MS method is a promising alternative to the mouse bioassay. Pure CTX1B and CTX3C were prepared for use in future LC/MS/MS analysis.

Keywords: ciguatera, ciguatoxin, LC-MS/MS

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辰野竜平*1，反町太樹*1，谷山茂人*1，大城直雅，久保弘文*2，高谷智裕*1，荒川修*1：腐肉食性小型巻貝2種に対するフグ毒給餌実験.

食品衛生学雑誌 2014;55:152-6.

腐肉食性小型巻貝のテトロドトキシン（TTX）蓄積能・蓄積機構解明に資するため，ムシロガイ科のコブムシロとアラムシロを用いて毒化モデル実験を行った．両種にTTX含有餌料を投与すると，ともに内臓と筋肉が僅かに毒化した．組織中のTTX量の最高値は，コブムシロ内臓で2.85 MU/g，筋肉0.86 MU/g，アラムシロ内臓0.80 MU/g，筋肉0.81 MU/gで，コブムシロ内臓で毒が最も多く残存する傾向が見られた．TTX残存率（推定TTX摂取量に対する総残存TTX量の割合）は，おおむねコブムシロで4%未満，アラムシロで2%未満と非常に低く，これら2種が食品衛生上問題となるほど高毒化する可能性は低いものと推察された．

Keywords: 腐肉食性巻貝，フグ毒，テトロドトキシン

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Momose Y, Asakura H, Kitamura M, Okada Y, Ueda Y*1, Hanabara Y*1, Sakamoto T*2, Matsumura T*3, Iwaki M*4, Kato H*4, Shibayama K*4, Igimi S: Food-borne botulism in Japan in March 2012.

Int J Infect Dis. 2014;24:20-2.

In March 2012, two patients were transported urgently to the hospital in Tottori Prefecture, Japan, because of symptoms suggestive of botulism. Botulinum neurotoxin type A was detected in the clinical specimens and the food consumed by the two patients (vacuum packed adzuki-batto, a sweet adzuki bean soup containing noodles). We were able to make a prompt diagnosis of food botulism associated with the consumption of adzuki-batto, from which the causative pathogen *Clostridium botulinum* Ab was cultured.

Keywords: *Clostridium botulinum*, food-borne botulism, Japan

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Asahata S*, Hirai Y* Ainoda Y*, Fujita T*, Okada Y, Kikuchi K*: Fournier's gangrene caused by *Listeria monocytogenes* as the primary organism.

Can J Infect Dis Med Microbiol. 2015;26:44-6.

A 70-year-old man with a history of tongue cancer presented with Fournier's gangrene caused by *Listeria monocytogenes* serotype 4b. Surgical debridement revealed undiagnosed rectal adenocarcinoma. The patient did not have an apparent dietary or travel history but reported daily consumption of sashimi (raw fish). Old age and immunodeficiency due to rectal adenocarcinoma may have supported the direct invasion of *L. monocytogenes* from the tumour. The present article describes the first reported case of Fournier's gangrene caused by *L. monocytogenes*. The authors suggest that raw ready-to-eat seafood consumption be recognized as a risk factor for listeriosis, especially in cases of skin and soft tissue infection.

Keywords: Fournier's gangrene, *Listeria monocytogenes*, Raw ready-to-eat food

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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

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

Food Environ Virol. 2015;Mar 22:DOI 10.1007/s12560-

015-9191-7.

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, Real-time PCR

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三好龍也*1，内野清子*1，岡山文香*1，芝田有理*1，吉田永祥*1，小林和夫*1，左近直美*2，土生川洋*3，田中智之*4，野田衛：臨床検体および下水検体を用いた堺市内のA型肝炎の流行解析.

病原微生物検出情報 2010;36:6-7.

2014年当初から国内でA型肝炎患者報告数が過去4年間に比し，大きく増加し，堺市内でも2013年10月～2014年5月までに4例の報告があり，過去3年間（2011年0例，2012年1例，2013年9月まで0例）より多い報告数であった．そこで，下水流入水およびA型肝炎患者等からHAV遺伝子検出を試みた．その結果，2013年12月にC処理場にて，2014年2月，3月にB処理場にて採水した流入水からHAV遺伝子を検出した．遺伝子型は，2013年12月：III A型，2014年2月：IA型，2014年3月：IIIA型であった．一方，家族内感染事例からはIA型が検出された．下水中のウイルス遺伝子検出や遺伝子型解析は，流入地域における感染や浸淫状況，さらに感染源や経路を把握する上で有用な情報を提供すると考えられた.

Keywords: Hepatitis A virus, detection, Epidemiology

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入谷展弘*1，山元誠司*1，改田厚*1，阿部仁一郎*1，久保英幸*1，西尾孝之*2，伯井紀隆*2，大平真由*2，安井典子*2，桝田晴美*2，細井舞子*2，松本珠実*2，坂本徳裕*2，廣川秀徹*2，半羽宏之*2，野田衛：2014年9～11月に発生したノロウイルスによる胃腸炎集団事例について―大阪市.

病原微生物検出情報 2014;36:26-7.

2014年9～11月に大阪市内の保育所を中心ノロウイルスによる胃腸炎集団事例が多発した．30事例から検出されたノロウイルスはすべてGII.3型に分類され，互いに非常に近縁であった．さらに，3事例から検出された本株のRNA-dependent RNA polymerase（RdRp）領域（ORF1の3'末端側約800塩基）について遺伝子型別したところ，3株すべてが互いに近縁なGII.P12型に分類された.

Keywords: Norovirus, outbreak, phylogenetic analysis

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工藤由起子，磯部順子*1，古川一郎*2，権平文夫*3，寺嶋淳，齊藤志保子*4：腸管出血性大腸菌O26，O103，O111，O121，O145およびO157の食品からの検出における選択増菌培地および酵素基質培地の検討.

日本食品微生物学会雑誌 2015;32:60-6.

EHEC血清群O103，O121およびO145はO26，O111およびO157と同一の増菌培養法（mEC培地での42℃培養）によって十分に増殖することが確認された．また，多種類の酵素基質培地について，多数の菌株を供試してコロニーの形成および発色を検討した結果，複数または単独の対象血清群を単色または複数色で鑑別・分離されることが示された．さらに，新規に開発された血清群O103，O121およびO145に対する免疫磁気ビーズによって食品培養液中の菌を十分に濃縮する性能を示した．以上のことから，上記6血清群の食品からの分離にはmEC培地で

の増菌培養，免疫磁気ビーズ濃縮法，酵素基質培地での分離培養を効果的に組み合わせることで確立できることが示された．

Keywords: Enterohemorrhagic Escherichia coli, enrichment, chromogenic agar

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Watanabe M, Ohnishi T, Araki E[*1], Kanda T[*2], Tomita A[*3], Ozawa K[*4], Goto K[*5], Sugiyama K[*2], Konuma H[*1], Hara-Kudo Y: Characteristics of bacterial and fungal growth in plastic bottled beverages under a consuming condition model.
J Environ Sci Health Part A. 2014;49:819-26.

Microbial contamination in unfinished beverages can occur when drinking directly from the bottle. Various microorganisms, including foodborne pathogens, are able to grow in these beverages at room temperature or in a refrigerator. In this study, we elucidated the characteristics of microorganism growth in bottled beverages under consuming condition models. Furthermore, we provide insight into the safety of partially consumed bottled beverages with respect to food hygiene.

We inoculated microorganisms, including foodborne pathogens, into various plastic bottled beverages and analysed the dynamic growth of microorganisms as well as bacterial toxin production in the beverages. Eight bottled beverage types were tested in this study, namely green tea, apple juice drink, tomato juice, carbonated drink, sport drink, coffee with milk, isotonic water and mineral water, and in these beverages several microorganism types were used: nine bacteria including three toxin producers, three yeasts, and five moulds. Following inoculation, the bottles were incubated at 35℃ for 48 hrs for bacteria, 25℃ for 48 hrs for yeasts, and 25℃ for 28 days for moulds. During the incubation period, the number of bacteria and yeasts and visible changes in mould-growth were determined over time. Our results indicated that combinations of the beverage types and microorganism species correlated with the degree of growth. Regarding factors that affect the growth and toxin-productivity of microorganisms in beverages, it is speculated that the pH, static/shaking culture, temperature, additives, or ingredients, such as carbon dioxide or organic matter (especially of plant origin), may be important for microorganism growth in beverages. Our results suggest that various types of unfinished beverages have microorganism growth and can include food borne pathogens and bacterial toxins. Therefore, our results indicate that in terms of food hygiene it is necessary to consume beverages immediately after opening the bottle.

Keywords: Beverage, Bacteria, Fungi

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大塚佳代子[*1]，小林直樹，森田幸雄[*2]，宮坂次郎[*3]，和栗敦[*4]，楠原一[*5]，工藤由起子：焼肉調理における腸管出血性大腸菌の生残の解析．
日本食品衛生学雑誌 2014;55:79-87.

日本における焼肉調理過程を想定し，牛内臓肉を含む牛肉での腸管出血性大腸菌の挙動を明らかにすることを目的に，各過程での本菌の生残性を検討した．その結果，牛肉の低温保存および焼肉調味料への漬け込みにおいて，菌数の増減はほとんど認められなかった．また，ホットプレートおよび直火ガスコンロでの焼肉調理において十分に加熱した場合，菌数の著しい減少（1/7,100から1/31,000）が認められた．しかし，牛肉の種類による菌数の減少程度の違いや，加熱むらがあることに注意が必要であると考えられた．また，同一の調理器具を焼成前の汚染牛肉および焼成後の牛肉に共通して使用することによって，1/500から1/300,000の菌数の二次汚染が起こることが示された．

Keywords: 焼肉，腸管出血性大腸菌，生残

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Lee K[*1], Kobayashi N, Watanabe M, Sugita-Konishi Y[*2], Tsubone H[*1], Kumagai S[*1], Hara-Kudo Y: Spread and change in stress resistance of Shiga toxin-producing *Escherichia coli* O157 on fungal colonies.
Microbial Biotechnology 2014;7:621-9.

To elucidate the effect of mould hyphae on the behaviour of Shiga toxin-producing *Escherichia coli*（STEC）O157, the spread and change in stress resistance of the bacterium were evaluated after coculture with 11 species of food-related moulds including a fermentation starter. Spread distances of STEC O157 varied depending on the cocultured mould species, and the motile bacterial strain spread for longer distances than the non-motile strain. The population of STEC O157 increased when cocultured on colonies of nine mould species but decreased on colonies of Emericella nidulans and Aspergillus ochraceus. Confocal scanning microscopy visualization of green fluorescent protein-tagged STEC O157 on mould hyphae revealed that the bacterium colonized in the water film that existed on and between hyphae. To investigate the physiological changes in STEC O157 caused by coculturing with moulds, the bacterium was harvested after seven days of coculturing and tested for acid resistance. After coculture with eight mould species, STEC O157 showed greater acid resistance compared to those cultured without moulds. Our results indicate that mould hyphae can spread the contamination of STEC O157 and can also enhance the stress resistance of the bacteria.

Keywords: Shiga toxin-producing *Escherichia coli* O157, mould, stress resistance

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Saito S*1, Iwade Y*2, Tokuoka E*3, Nishio T*4, Otomo Y*5, Araki E*6, Konuma H*6, Nakagawa H*7, Tanaka H*8, Sugiyama K*4, Hasegawa A*9, Sugita-Konishi Y*10, Hara-Kudo Y: Epidemiological Evidence of lesser role of thermostable direct hemolysin（TDH）-related hemolysin than TDH on *Vibrio parahaemolyticus* pathogenicity.

Foodborne Pathogens and Disease 2015;12:131-8.

Vibrio parahaemolyticus carrying the *tdh* gene, encoding the thermostable direct hemolysin（TDH）, or the *trh* gene, encoding the TDH-related hemolysin（TRH）are both considered virulent strains. There are, however, disproportionally fewer reports of infections caused by seafood contaminated with *trh*-positive strains than by seafood contaminated with *tdh*-positive strains. Bivalves such as clams and oysters are the major seafood varieties associated with the infections. In this study, the prevalence of strains possessing of the *tdh* and *trh* genes was investigated in Japan in 74 samples collected in 2007-2008 and in 177 samples collected in 2010 of domestic bivalves, bloody clams, hen clams, short-neck clams and rock oysters. The *tdh* positive and *trh* negative, *tdh* negative and *trh* positive, and *tdh* positive and *trh* positive samples represented 5.4%, 12.2% and 4.1% of all samples collected in 2007-2008, and 5.1%, 18.6% and 5.6% of all samples collected in 2010, respectively. As determined by PCR, the prevalence of *tdh* negative and *trh* positive in all samples was 2-4 times higher than that of *tdh* positive and *trh* negative. In the samples collected in 2010, the *tdh* negative and *trh* positive *V. parahaemolyticus*（20 samples）was more often isolated than *tdh* positive and *trh* negative *V. parahaemolyticus*（7 samples）. The most common serotype of *tdh* positive isolates（22 of 24 strains）was pandemic O3:K6. The *trh* positive isolates（61 strains）were various serotypes including OUT:KUT. In 330 *V. parahaemolyticus* outbreaks and sporadic infections in Japan, most outbreaks and sporadic infections were caused by *tdh* positive and *trh* negative strains（89.4%）. The frequencies of infections caused by *tdh* negative and *trh* positive, and both *tdh* and *trh* positive strains were 1.2% and 3.0%, respectively. This finding suggests that the virulence of *trh* might be less than that of *tdh*, although *trh*-positive *V. parahaemolyticus* frequently contaminated bivalves.

Keywords: *Vibrio parahaemolyticus*, thermostable direct hemolysin-related hemolysin, virulence

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Kamata Y*1, Saito M*2, Irikura D*3, Yahata Y*4, Ohnishi T, Bessho T*5, Inui T*5, Watanabe M, Sugita-Konishi Y*6: A Toxin Isolated from *Sarcocystis fayeri* in raw

horsemeat maybe responsible for food poisoning.
J Food Prot. 2014;77:814-9.

Food poisoning has been reported after the consumption of raw horsemeat in Japan. Diarrhea with a short incubation period is a common symptom in such cases of food poisoning. Cysts found in horsemeat ingested by patients have been identified as *Sarcocystis fayeri* based on morphological and genetic evaluation and findings from experimental feeding of cysts to dogs, which resulted in the excretion of sporocysts. The extracts of the horsemeat containing the cysts produced a positive enterotoxic response in the rabbit ileal loop test. Intravenous injection of a 15-kDa protein isolated from the cysts induced diarrhea and lethal toxicity in rabbits, and the protein produced enterotoxicity in the ileal loop test as did the extracts of the horsemeat containing the cysts. The partial amino acid sequence of the 15-kDa protein was homologous to the actin-depolymerizing factor of *Toxoplasma gondii* and *Eimeria tenella*. These findings indicate that the 15-kDa protein of S. *fayeri* is a toxin that causes food poisoning after consumption of parasitized horsemeat.
Keywords: Food poisoning, Sarcocystis fayeri, horsemeat

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Wu W*1,2, Zhou H*2, He K*2, Pan X*2, Sugita-Konishi Y*3, Watanabe M, Zhang H*1, Pestka JJ*2: Role of cholecystokinin in anorexia induction following oral exposure to the 8-Ketotrichothecenes Deoxynivalenol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, fusarenon X and nivalenol.
Toxicol Sci. 2014;138:278-89.

Cereal grain contamination by trichothecene mycotoxins is known to negatively impact human and animal health with adverse effects on food intake and growth being of particular concern. The head blight fungus *Fusarium graminearum* elaborates five closely related 8-ketotrichothecene congeners: (1) deoxynivalenol (DON), (2) 3-acetyldeoxynivalenol (3-ADON), (3) 15-acetyldeoxynivalenol (15-ADON), (4) fusarenon X (FX), and (5) nivalenol (NIV). While anorexia induction in mice exposed intraperitoneally to DON has been linked to plasma elevation of the satiety hormones cholecystokinin (CCK) and peptide $YY_{3\text{-}36}$ ($PYY_{3\text{-}36}$), the effects of oral gavage of DON or of other 8-keotrichothecenes on release of these gut peptides have not been established. The purpose of this study was to (1) compare the anorectic responses to the aforementioned 8-ketotrichothecenes following oral gavage at a common dose (2.5 mg/kg bw) and (2) relate these effects to changes plasma CCK and $PYY_{3\text{-}36}$ concentrations. Elevation of plasma CCK markedly corresponded to anorexia induction by DON and all other 8-ketotrichothecenes tested. Furthermore, the CCK1 receptor antagonist SR 27897 and the CCK2 receptor antagonist L-365,260 dose-dependently attenuated both CCK- and DON-induced anorexia, which was consistent with this gut satiety hormone being an important mediator of 8-ketotrichothecene-induced food refusal. In contrast to CCK, $PYY_{3\text{-}36}$ was moderately elevated by oral gavage with DON and NIV but not by 3-ADON, 15-ADON, or FX. Taken together, the results suggest that CCK plays a major role in anorexia induction following oral exposure to 8-ketotrichothecenes, whereas $PYY_{3\text{-}36}$ might play a lesser, congener-dependent role in this response.
Keywords: 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, fusarenon X

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Wu W*1, He K*2, Zhou H*2, Berthiller F*3, Adam G*3, Sugita-Konishi Y*4, Watanabe M, Krantis A*5, Durst T*5, Zhang H*1, Pestka JJ*2: Effects of oral exposure to naturally-occurring and synthetic deoxynivalenol congeners on proinflammatory cytokine and chemokine mRNA expression in the mouse.
Toxicol Appl Pharmacol. 2014;278:107-15.

The foodborne mycotoxin deoxynivalenol (DON) induces a ribotoxic stress response in mononuclear phagocytes that mediate aberrant multi-organ upregulation of TNF-α, interleukins and chemokines in experimental animals. While other DON congeners also exist as food contaminants or pharmacologically-active derivatives, it is not known how these compounds affect expression of these cytokine genes in vivo. To address this gap, we compared in mice the acute effects of oral DON exposure to that of seven relevant congeners on splenic

expression of representative cytokine mRNAs after 2 and 6h. Congeners included the 8-ketotrichothecenes 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FX), nivalenol (NIV), the plant metabolite DON-3-glucoside (D3G) and two synthetic DON derivatives with novel satiety-inducing properties (EN139528 and EN139544). DON markedly induced transient upregulation of TNF-α IL-1β, IL-6, CXCL-2, CCL-2 and CCL-7 mRNA expressions. The two ADONs also evoked mRNA expression of these genes but to a relatively lesser extent. FX induced more persistent responses than the other DON congeners and, compared to DON, was: 1) more potent in inducing IL-1β mRNA, 2) approximately equipotent in the induction of TNF-α and CCL-2 mRNAs, and 3) less potent at upregulating IL-6, CXCL-2, and CCL-2 mRNAs. EN139528's effects were similar to NIV, the least potent 8-ketotrichothecene, while D3G and EN139544 were largely incapable of eliciting cytokine or chemokine mRNA responses. Taken together, the results presented herein provide important new insights into the potential of naturally-occurring and synthetic DON congeners to elicit aberrant mRNA upregulation of cytokines associated with acute and chronic trichothecene toxicity.

Keywords: 8-Ketotrichothecenes, Chemokine, Deoxynivalenol-3-glucoside

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Ohnishi T, Akuzawa S*1, Furusawa H, Yoshinari T, Kamata Y*2, Sugita-Konishi Y*3: Inactivation of *Kudoa septempunctata* in olive flounder.
Biocontrol Sci. 2014;19:135-8.

Kudoa septempunctata in olive flounder meat was inactivated using 3 distinct freezing methods : liquid freezing for 5 min, air blast freezing at −30℃ for 5 h, and −80℃ for 1 h. The fracture curve of olive flounder meat subjected to liquid freezing resembled that of meat stored at 4℃ , indicating that the structure of olive flounder muscle was well preserved. In contrast, air blast freezing induced the disappearance of the fracture point in the fracture curve, indicating that there was deterioration in the meat quality. Liquid freezing preserved the transparency of olive flounder meat to the same degree as that of meat stored at 4℃ . However, air blast freezing induced meat cloudiness. These results indicate that liquid freezing can be used for *K. septempunctata* inactivation without affecting the meat quality.

Keywords: Kudoa, Parasite, food-borne disease

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Sugita-Konishi Y*1, Fukuda Y*2, Mori K*3, Mekata T*3, Namba T*4, Kuroda M*5, Yamazaki A, Ohnishi T: New validated rapid screening methods for identifying *Kudoa septempunctata* in olive flounder (paralichthys olivaceus).
JJID. 2015;68:145-7.

Kudoa septempunctata is a newly identified causative agent of foodborne diseases associated with consuming raw olive flounder. Qualitative PCR and quantitative real-time PCR have been used as notification methods to identify *K. septempunctata* in Japan. However, these methods require expensive equipment and are time-consuming (2–3 h for screening). To address these problems, in this study, we developed new rapid and simple methods using real-time loop-mediated isothermal amplification (LAMP) and nucleic acid sequence based amplification-nucleic acid chromatography (NASBA-NAC). Using these methods, the total procedure required approximately 45 min and did not require any expensive equipment. With regard to validating these new methods in comparison with the notification methods used in Japan, we performed an inter-laboratory study of 5 laboratories using samples that included olive flounders infected with 4 different amounts of *K. septempunctata*. These results demonstrated that the sensitivity of NASBA-NAC was equivalent to that of qualitative PCR, and that the sensitivity of real-time LAMP was equivalent to that of quantitative real-time PCR, which indicated that these new methods were acceptable screening methods for identifying *K. septempunctata*.

Keywords: Kudoa, Parasite, Food-borne disease

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Yahata Y*1, Sugita-Konishi Y*2, Ohnishi T, Toyokawa T*3, Nakamura N*4, Taniguchi K*5, Okabe N*6: *Kudoa septempunctata* induced gastroenteritis in humans after flounder consumption in Japan: a case-control study.

JJID. 2015;68:119-23.

Raw fish consumption is increasing worldwide. Since around the year 2000, western regions of Japan have reported a foodborne disease of unknown cause that occurred after the consumption of flounder. In October 2010, a particularly large outbreak was reported in these regions among individuals who consumed flounder fish that had been raised in aquaculture systems. The median incubation period was 5 h (range, 4–19 h), and the most frequently reported symptom was diarrhea (80%). The risk estimate of the consumption of flounder was significantly higher than that of the development of symptoms (odds ratio = 9.50; 95% confidence interval, 1.59–∞). According to a trace-back investigation, all of the flounder responsible for the outbreak were raised in aquaculture systems. Microscopic examination revealed that the median amount of *Kudoa septempunctata* present in the muscle of flounder fish from the aquaculture farm was 4.5×10^3 spores/g (range, $1.0 \times 10^3 - 9.6 \times 10^6$ spores/g). The number of *K. septempunctata* spores required for the development of illness, as estimated using the Monte Carlo simulation, was 7.2×10^7 spores/g; therefore, thus this might be the minimum ingestion threshold for the development of gastrointestinal symptoms. As a public health measure, the current study results should be referred to for the prevention of the gastrointestinal symptoms related to the consumption of flounder; the national public health authority has disseminated these results. We concluded that *K. septempunctata*-contaminated flounder fish were associated with the gastrointestinal symptoms of this recent outbreak.

Keywords: Kudoa, Parasite, Epidemiology

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Yoshinari T, Takeuchi H*1, Aoyama K*2, Taniguchi M*3, Hashiguchi S*4, Kai S*5, Ogiso M*6, Sato T*7, Akiyama Y*8, Nakajima M*3, Tabata S*9, Tanaka T*10, Ishikuro E*6, Sugita-Konishi Y*11: Occurrence of four fusarium mycotoxins, deoxynivalenol, zearalenone, T-2 Toxin, and HT-2 Toxin, in wheat, barley, and Japanese retail food.

J Food Prot. 2014;77:1940-6.

A survey of the contamination of wheat, barley, and Japanese retail food by four Fusarium mycotoxins, deoxynivalenol (DON), zearalenone (ZEN), T-2 toxin (T-2), and HT-2 toxin (HT-2), was performed between 2010 and 2012.

Keywords: deoxynivalenol, T-2 toxin, zearalenone,

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Parker CG*1, Dahlgren MK*1, Li DT*1, Douglass EF*1, Shoda T, Jawanda N*1, Spasov KA*1, Lee S*2, Zhou N*2, Domaoal RA*1, Sutton R*1, Anderson KS*1, Krystal M*2, Jorgensen WL*1, Spiegel DA*1: Illuminating HIV-1 gp120–Ligand recognition through computationally-driven optimization of antibody-recruiting molecules.

Chem Sci. 2014;5:2311-7.

Here we report on the structure-based optimization of antibody-recruiting molecules targeting HIV gp120 (ARM-H). These studies have leveraged a combination of medicinal chemistry, biochemical and cellular assay analysis, and computation. Our findings have afforded an optimized analog of ARM-H, which is ~1000 fold more potent in gp120-binding and MT-2 antiviral assays than our previously reported derivative. Furthermore, computational analysis, taken together with experimental data, provides evidence that azaindole- and indole-based attachment inhibitors bind gp120 at an accessory hydrophobic pocket beneath the CD4-binding site and

can also adopt multiple unique binding modes in interacting with gp120. These results are likely to prove highly enabling in the development of novel HIV attachment inhibitors, and more broadly, they suggest novel applications for ARMs as probes of conformationally flexible systems.
Keywords: antibody, antibody-recruiting molecules, HIV

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Oba M*1, Takazaki H*2, Kawabe N*2, Doi M*3, Demizu Y, Kurihara M, Kawakubo H*4, Nagano M*2, Suemune H*2, Tanaka M*1: Helical peptide-foldamers having chiral five-membered ring amino acid with two azido functional groups.
J Org Chem. 2014;79:9125-40.

A chiral five-membered ring α,α-disubstituted α-amino acid (*R*,*R*)-Ac_5c^{dN3} having two azido functional groups has been designed and synthesized. The cyclic amino acid (*R*,*R*)-Ac_5c^{dN3} could be efficiently converted into several cyclic amino acids with various two 1,2,3-triazole functional groups. (*R*,*R*)-Ac_5c^{dN3} homochiral peptides (up to heptapeptide) and (*R*,*R*)-Ac_5c^{dN3}-containing L-Leu-based peptides were prepared, and their conversion of azido functional groups into triazole groups was completed. The preferred conformation of oligomers, before and after the "click reaction", together with the azido gauche effect of amino acid residues were studied using FT-IR absorption, CD, ^{1}H NMR, and X-ray crystallographic analysis. The cyclic amino acid (*R*,*R*)-Ac_5c^{dN3} could be used as a helical conformation controlling residue and also has a versatile functionalizing site in its oligopeptides.
Keywords: foldamer, peptide, azido functional group

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Oba M*1, Kawabe N*2, Takazaki H*2, Demizu Y, Doi M*3, Kurihara M, Suemune H*2, Tanaka M*1: Conformational studies on peptides having chiral five-membered ring amino acid with two azido or triazole functional groups within the sequence of Aib residues.
Tetrahedron 2014;70:8900-7.

The chiral cyclic α,α-disubstituted α-amino acid, (3*R*,4*R*)-1-amino-3,4-diazido-1-cyclopentanecarboxylic acid [(*R*,*R*)-Ac_5c^{dN3}], was introduced into achiral α-aminoisobutyric acid (Aib) peptides. The azido groups of (*R*,*R*)-Ac_5c^{dN3} in the peptides were efficiently converted into 1,2,3-triazole functional groups. FT-IR, ^{1}H NMR, and CD spectra revealed that the dominant conformations of all peptides in solution were 3_{10}- helical structures without controlling the helical-screw sense. X-ray crystallographic analyses of peptides containing (*R*,*R*)-Ac_5c^{dN3} showed that both the right-handed (P) and left-handed (M) 3_{10}-helical structures were present in the crystal state.
Keywords: amino acids, chirality, conformation analysis

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Nagakubo T, Demizu Y, Kanda Y, Misawa T, Shoda T, Okuhira K, Sekino Y, Naito M, Kurihara M: Development of cell-penetrating R7 fragment-conjugated helical peptides as inhibitors of estrogen receptor-mediated transcription.
Bioconjugate Chem. 2014;25:1921-4.

The heptaarginine (R7)-conjugated peptide 5 was designed and synthesized as an inhibitor of ER-coactivator interactions and ER-mediated transcription at the cellular level. The R7-conjugated peptide 5 was able to enter ER-positive T47D cells efficiently, and treatment with 3 μM of 5 downregulated the mRNA expression of pS2 (an ER-mediated gene) by 87%.
Keywords: estrogen receptor, peptide, transcriptional inhibitor

Sakakibara N*1, Baba M*2, Okamoto M*2, Toyama M*1, Demizu Y, Misawa T, Kurihara M, Irie K*1, Kato Y*1, Maruyama T*1: Design, synthesis and anti-HIV-1 activity of 1-aromatic methyl-substituted 3-(3,5-dimethylbenzyl) uracil and *N*-3,5-dimethylbenzyl-substituted urea derivatives.
Antiviral Chem Chemother. 2015;24:3-18.

A new series of 1-aromatic methyl-substituted 3-(3,5-dimethylbenzyl) uracil and N-3,5-dimethylbenzyl- substituted urea derivatives were synthesized and evaluated as non-nucleoside HIV-1 reverse transcriptase inhibitors. A series of new 6-azido and 6-amino derivatives of 1-substituted-3-(3,5-dimethylbenzyl) uracils were synthe-

sized using our previously reported method, and three acyclic derivatives were synthesized from urea. The anti-HIV-1 activities of these compounds were determined based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. The cytotoxicities of the compounds were evaluated using the viability of mock-infected cells. Some of these compounds showed good-to-moderate activities against HIV-1 with half maximal effective concentration (EC_{50}) values in the submicromolar or subnanomolar range. Compared with emivirine, compound 6-amino-3-(3,5-dimethylbenzyl)-1-(4-aminobenzyl) uracil showed significant anti-HIV-1 activity with an EC_{50} value of 10 nM and a high selectivity index of 1923. Preliminary structure–activity relationship studies and molecular modeling analyses were carried out to explore the major interactions between HIV-1 reverse transcriptase and the potent inhibitor 6-amino-3-(3,5-dimethylbenzyl) -1-(4-aminobenzyl) uracil; these results may be important for further development of this class of compounds as anti-HIV-1 agents. The excellent activity of 6-amino-3-(3,5-dimethylbenzyl)-1-(4-aminobenzyl) uracil (EC_{50}: 0.01 μM, SI: >1923) may serve as the basis for conducting further investigations on the behavior of this class of compounds against drug-resistant mutants.
Keywords: anti-HIV-I agents, uracil analogs, HIV-I reverse transcriptase

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Demizu Y, Yamashita H, Misawa T, Doi M*1, Tanaka M*2, Kurihara M: Effects of D-Leu residues on the helical secondary structures of L-Leu-based nonapeptides.
Chem Pharm Bull. 2015;63:218-24.

The influence of D-Leu residues on the helical structures of L-Leu-based-nonapeptides was investigated. Specifically, the preferred conformations of four diastereomeric nonapeptides, Boc-(L-Leu-L-Leu-Aib)$_3$-OMe (1); Boc-(L-Leu-L-Leu-Aib)$_2$-L-Leu-D-Leu-Aib-OMe (2), which contained one D-Leu residue; Boc-L-Leu- D-Leu-Aib-L-Leu-L-Leu-Aib-L-Leu-D-Leu-Aib-OMe (3), which contained two D-Leu residues; and Boc- (L-Leu-D-Leu-Aib)$_3$-OMe (4), were analyzed in solution and in the crystalline state. Peptide 1 formed a right- handed (*P*) 3_{10}-helix in solution. Peptides 2 and 3 both formed (*P*) 3_{10}-helices in solution and (*P*)α-helices in the crystalline state. Peptide 4 formed a (*P*)α-helix both in solution and in the crystalline state.
Keywords: amino acid, peptide, conformation

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Misawa T, Demizu Y, Kawamura M, Yamagata N, Kurihara M: Structural development of stapled short helical peptides as vitamin D receptor (VDR)-coactivator interaction inhibitors.
Bioorg Med Chem. 2015;23:1055-61.

We developed several stabilized helical heptapeptides (DPI-01–10) composed of L-leucine residues, an α,α-disubstituted α-amino acid (α-aminoisobutyric acid [Aib] or hydroxymethylserine [Hms]), and a stapled side chain as inhibitors of vitamin D receptor (VDR)–coactivator interactions. The inhibitory activity of these peptides against VDR–coactivator interactions was evaluated using a receptor cofactor assay system, and DPI-08 demonstrated strong activity (IC_{50}: 3.2 μM).
Keywords: vitamin D receptor, protein-protein interaction, stapled peptide

Yamashita H, Demizu Y, Misawa T, Shoda T, Kurihara M: Synthesis of a bis-cationic α,α-disubstituted amino acid (9-amino-bispidine-9-carboxylic acid) and its effects on the conformational properties of peptides.
Tetrahedron 2015;71:2241-5.

A new bis-cationic cyclic amino acid, 9-amino-3,7-diazabicyclo [3.3.1] nonane-9-carboxylic acid (9-amino-bispidine-9-carboxylic acid; Abp), which is available for both solution phase and solid phase peptide synthesis, was designed and synthesized. Furthermore, a heterotripeptide Cbz-Leu-Abp-Ala-OMe (9) containing Abp was prepared, and its dominant conformation was analyzed by examining its nuclear magnetic resonance and infrared spectra and performing molecular modeling. The tripeptide 9 formed a β-turn structure as its preferred conformation in solution.
Keywords: cationic amino acid, peptide, conformation analysis

Hirata T*1, Ueda A*2, Oba M*2, Doi M*3, Demizu Y, Kurihara M, Nagano M*1, Suemune H*1, Tanaka M*2: Amino equatorial effect of a six-membered ring amino acid on its peptide 3_{10}- and α-helices.
Tetrahedron 2015;71:2409-20.

Two diastereomeric six-membered ring α,α-disubstituted

α-amino acids (1*R*,3*R*)- and (1*S*,3*R*)-1-amino-3-methylcyclohexanecarboxylic acids (Ac_6c^{3M}); side-chain restricted leucine analogs, were stereoselectively synthesized from (3*R*)-3-methylcyclohexanone by a BuchereBergs or Strecker reaction. Two series of homo-chiral homopeptides Cbz-[(1*R*,3*R*)- and (1*S*,3*R*)-Ac_6c^{3M}]$_n$-OMe, up to hexapeptides, were prepared, respectively, and the preferred conformations of cyclohexane rings of amino acid residues and the peptide-backbones were studied. In solution, these peptides formed helical structures, but the helical-screw control to one-handedness was not possible for the hexapeptide length. In the crystal state, all (1*R*,3*R*)-Ac_6c^{3M} residues formed cyclohexane chair form conformations with a 3-methyl substituent at equatorial orientation and an amino group at the axial position, whereas all (1*S*,3*R*)-Ac_6c^{3M} residues assumed cyclohexane chair forms with the 3-methyl and amino groups at equatorial orientations. The preferred peptide-backbone structure of (1*R*,3*R*)-Ac_6c^{3M} hexapeptide had (*P*) and (*M*) 3_{10}-helices, and that of (1*S*,3*R*)-Ac_6c^{3M} hexapeptide had (*P*) and (*M*) α-helices in the crystal state.

Keywords: amino acids, chirality, peptide

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Demizu Y, Misawa T, Yamagata N, Doi M*, Kurihara M: Methyl 2-[(2-aminophenyl)ethynyl]benzoate and 2-[(2-acetamidophenyl)ethynyl] benzoic acid.
Molbank 2015;M854:doi:10.3390/M854.

The title compound was prepared by inducing amide bond formation between methyl 2-[(2-aminophenyl) ethynyl] benzoate and 2-[(2-acetamidophenyl) ethynyl] benzoic acid in the presence of dichlorotriphenylphosphorane. The structure of the synthesized compound was determined on the basis of its ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and mass spectral data. Furthermore, the compound's crystal structure is also reported.

Keywords: foldamer, aromatic amide, X-ray crystallographic analysis

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Misawa T, Dodo K*1, Ishikawa M*2, Hashimoto Y*2, Sagawa M*3, Kizaki M*3, Aoyama H*4: Structure-activity relationships of benzhydrol derivatives based on 1'-acetoxychavicol acetate (ACA) against Multiple Myeloma cell-growth inhibition via inactivation of NF-κB pathway.
Bioorg Med Chem. 2015;23;2241-6.

1'-Acetoxychavicol acetate (ACA), which was isolated from the rhizomes of Zingiberaceae, showed several biological activity such as anti-inflammatory activity, anti-humanimmunodeficiency virus (HIV) activity, and anti-cancer activity. Especially, it has been expected that ACA could be an attractive candidate for the treatment of broad cancers. Here, we describe the structure-activity relationship of ACA derivatives based on benzhydrol skeleton against Human leukemia cells (HL-60). Moreover, we revealed that the synthesized ACA derivatives (ACA, **1**, and **18**) showed the cell-growth-inhibitory activity against multiple myeloma cells (IM-9 cells) via inactivation of NF-κB pathway.

Keywords: 1'-Acetoxychavicol acetate (ACA), NF-κB, Multiple Myeloma

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Cui H, Wu W, Okuhira K, Miyazawa K*1, Hattori T, Sai K, Naito M, Suzuki K, Nishimura T, Sakamoto Y*2, Ogata A*2, Maeno T*2, Inomata A*2, Nakae D*2, Hirose A, Nishimaki-Mogami T: High-temperature calcined fullerene nanowhiskers as well as long needle-like multi-wall carbon nanotubes have abilities to induce NLRP3-mediated IL-1β secretion.
Biochem Biophys Res Commun. 2014;452:593-9.

Because multi-wall carbon nanotubes (MWCNTs) have asbestos-like shape and size, concerns about their pathogenicity have been raised. Contaminated metals of MWCNTs may also be responsible for their toxicity. In this study, we employed high-temperature calcined fullerene nanowhiskers (HTCFNWs), which are needle-like nanofibers composed of amorphous carbon having similar sizes to MWCNTs but neither metal impurities nor tubular structures, and investigated their ability to induce production a major proinflammatory cytokine IL-1β via the Nod-like receptor pyrin domain containing 3 (NLRP3)-containing flammasome-mediated mechanism.

When exposed to THP-1 macrophages, long-HTCFNW exhibited robust IL-1β production as long and needle-like MWCNTs did, but short-HTCFNW caused very small effect. IL-1β release induced by long-HTCFNW as well as by long, needle-like MWCNTs was abolished by a caspase-1 inhibitor or siRNA-knockdown of NLRP3, indicating that NLRP3-inflammasome-mediated IL-1β production by these carbon nanofibers. Our findings indicate that the needle-like shape and length, but neither metal impurities nor tubular structures of MWCNTs were critical to robust NLRP3 activation.

Keywords: carbon nanotubes, fullerene nanowhiskers, IL-1β

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Kitagawa M*, Nakamura K, Kondo K, Ubukata S*, Akiyama H: Examination of processed vegetable foods for the presence of common DNA sequences of genetically modified tomatoes.

Shokuhin Eiseigaku Zasshi. 2014;55:247-53.

The contamination of processed vegetable foods with genetically modified (GM) tomatoes was investigated by the use of qualitative PCR methods to detect the *Cauliflower mosaic virus* 35S promoter (P35S) and the kanamycin resistance gene (*NPTII*). DNA fragments of P35S and *NPTII* were detected in vegetable juice samples, possibly due to contamination with the genomes of *Cauliflower mosaic virus* infecting juice ingredients of *Brassica* species and soil bacteria, respectively. Therefore, to detect the transformation construct sequences of GM tomatoes, primer pairs were designed for qualitative PCR to specifically detect the border region between P35S and *NPTII*, and the border region between nopaline synthase gene promoter and *NPTII*. No amplification of the targeted sequences was observed using genomic DNA purified from the juice ingredients. The developed qualitative PCR method is considered to be a reliable tool to check contamination of products with GM tomatoes.

Keywords: processed vegetable food, genetically modified, tomato

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田中秀典*，北﨑康生*，中村公亮，穐山浩，明石良*：遺伝子組換えパパイヤ（PRSV-YK）の簡易検出法の確立.

育種学研究 2014;16:158-61.

様々な遺伝子組換え（GM）作物が各国で開発され流通量が拡大することに伴い，意図せずに未承認のGM作物が混入し流通する恐れが高まっている．そのような中でパパイヤリングスポットウイルス台湾株（YK）の外被タンパク質の遺伝子が導入されたGM パパイヤ（PRSV-YK）のパパイヤ加工食品への混入が報告された．本研究では，PRSV-YKの葉を用いて，簡易かつ低コストで多検体処理が可能な検知法を検討したので報告する．

Keywords: 遺伝子組換え体検出，PRSV-YK，FTAカード

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Takabatake R*1, Onishi M*1, Futo S*2, Minegishi Y*3, Noguchi A, Nakamura K, Kondo K, Teshima R, Mano J*1, Kitta K*1: Comparison of the specificity, stability, and PCR efficiency of six rice endogenous sequences for detection analyses of genetically modified rice.

Food Control 2014;50:949-55.

Species-specific endogenous reference sequences are indispensable in the development of methods to detect genetically modified (GM) crops and food/feed. We evaluated and compared the applicability of 6 rice (*Oryza sativa*) endogenous sequences, including 5 previously reported sequences; SPS1 derived from the *sucrose phosphate synthase* (SPS) gene, PLD1 and PLD2 derived from the *phospholipase D* (PLD) gene, GOS9 derived from the root-specific gene *gos*9, and ppi-PPF derived from the *ppi-phosphofructokinase* (ppi-PPF) gene, as well as a newly designed sequence, SPS2 in the rice *SPS* gene promoter region. PCR efficiency and stability were evaluated with 28 rice cultivars, and species specificity was evaluated using gDNAs isolated from major crops and rice-related species. SPS1 and GOS9 were less easy to be amplified and showed lower PCR amplification stabilities than the other sequences among rice cultivars. On the other hand, PLD1 showed high PCR efficiency and stability but low specificity against rice. Meanwhile, ppi-PPF was moderate in all evaluated characteristics. SPS2 and PLD2 showed higher PCR efficiencies and stabilities than those of other sequences, and also had acceptable species specificities. We conclude that SPS2 and PLD2 are ideal endogenous sequences for use in the development of methods to detect and quantify GM rice.

Keywords: Rice, Endogenous, genetically modified

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Noguchi A, Akiyama H, Nakamura K, Sakata K, Minegishi Y*1, Mano J*2, Takabatake R*2, Futo S*3, Kitta K*2, Teshima R, Kondo K, Nishimaki-Mogami T: A novel trait-specific real-time PCR method enables quantification of genetically modified（GM）maize content in ground grain samples containing stacked GM maize.

Eur Food Res Technol. 2014;240:413-22.

Stacked genetically modified（GM）maize is increasingly produced; thereby, current event-specific quantitative real-time polymerase chain reaction（qPCR）methods have led to the overestimation of GM organism（GMO）content compared with the actual weight/weight percentage of GM organism in maize samples. We developed a feasible qPCR method in which the GMO content is calculated based on the quantification of two herbicidetolerant trait genes, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4（*cp4epsps*）and phosphinothricin *N*-acetyl-transferase from *Streptomyces viridochromogenes*（*pat*）to quantify the GMO content in ground grain samples containing stacked GM maize. The GMO contents of two genes were quantified using a plasmid calibrant and summed for quantification of total GMO content. The trait-specific method revealed lower biases for examination of test samples containing stacked GM maize compared with the event-specific method. Our results clearly show that the trait-specific method is not only simple and cost-effective, but also useful in quantifying the GMO content in ground grain samples containing stacked GM maize, which are expected to be major events in the near future. The developed method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize for the verification of the labeling regulation.

Keywords: genetically modified maize, qPCR, trait-specific method

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Morita T, Miyajima A, Hatano A, Honma M: Effects of lowering the proposed top-concentration limit in an in vitro chromosomal aberration test on assay sensitivity and on the reduction of the number of false positives.

Mutat Res. 2014;769:34-49.

The effect of a reduction in the top-concentration limit on sensitivity and specificity was investigated by use of a dataset on 435 chemicals obtained from the CGX database（267 CA-positives and 168 CA-negatives; 317 carcinogens and 118 non-carcinogens）where three TGs（i.e., 1997-OECD, revised OECD and ICH）were applied. The results suggest that the revised OECD TG will not affect the sensitivity or specificity for the detection of rodent carcinogens, indicating the usefulness of the guideline. However, nearly no improvement with respect to a reduction in the number of false positives should be expected.

Keywords: Top-concentration limit, In vitro chromosomal aberration test, Sensitivity

Kirkland D*1, Zeiger E*2, Madia F*3, Gooderham N*4, Kasper P*5, Lynch A*6, Morita T, Ouedraogo G*7, Parra Morte JM*8, Pfuhler S*9, Rogiers V*10, Schulz M*11, Thybaud V*12, van Benthem J*13, Vanparys P*14, Worth A*3, Corvi R*3: Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predictcarcinogenic or in vivo genotoxic activity? I. Reports of individualdatabases presented at an EURL ECVAM Workshop.

Mutat Res. 2014;755-6:55-68.

Positive results in the Ames test correlate well with carcinogenic potential in rodents. This correlation is not perfect because mutations are only one of many stages in tumour development. Since most chemicals are also tested for genotoxicity in mammalian cells, the pattern of mammalian cell results may help identify whether Ames-positive results predict carcinogenic or in vivo mutagenic activity. A workshop was therefore organised and sponsored by the EU Reference Laboratory for Alternatives to Animal Testing（EURL ECVAM）to investigate this further. Possible reasons why a positive Ames test may not be associated with in vivo activity and what additional investigations/tests might contribute to a more robust evaluation were discussed.

Keywords: Positive Ames tests, Database, Carcinogenicity

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Hanatani T, Sai K, Tohkin M*1, Segawa K, Kimura M*2, Hori K*2, Kawakami J*2, Saito Y: A detection algorithm for drug-induced liver injury in medical information databases using the diagnostic scale in Japan as compared to the CIOMS/RUCAM scale.
Pharmacoepidemiol Drug Saf. 2014;23:984-8.

Drug-induced liver injury (DILI) is one of the primary targets for pharmacovigilance using medical information databases (MIDs). Using an MID from the Hamamatsu University Hospital, we constructed a DILI detection algorithm on the basis of the Digestive Disease Week Japan 2004 (DDW-J) scale, and compared it with the Council for International Organizations of Medical Sciences/the Roussel Uclaf Causality Assessment Method (CIOMS/RUCAM) scale. We examined the characteristics of DILI after antibiotic treatment using a Hamamatsu database and a commercial database including data from 124 hospitals. The DDW-J and CIOMS/RUCAM algorithms were equivalent for identifying the DILI cases (Spearman rank correlation: 0.952 P<0.0001). Men showed a significantly higher risk for DILI after antibiotic treatments in both MIDs. This study provides evidence supporting the utility of MID analyses to improve pharmacovigilance.
Keywords: Drug-induced liver injury, Medical information database, Drug safety

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Hanatani T, Sai K, Tohkin M*1, Segawa K, Antoku Y*2, Nakashima N*2, Yokoi H*3, Ohe K*4, Kimura M*5, Hori K*5, Kawakami J*5, Saito Y: Evaluation of two Japanese regulatory actions using medical information databases: a "Dear Doctor" letter to restrict oseltamivir use in teenagers, and label change caution against co-administration of omeprazole with clopidogrel.
J Clin Pharm Ther. 2014;39:361-7.

We conducted quantitative assessment of the impact of the two regulatory actions by the Japanese government: (1) restriction of oseltamivir use in teenagers, and (2) caution against the co-administration of omeprazole (OPZ) with clopidogrel (CPG). To estimate the impact of the actions, we conducted segmented regression analysis using interrupted time series data from four hub hospitals in Japan. The use of oseltamivir in the teenagers was significantly declined (63.16%) just after the intervention (P = 0.0008). Although no change was observed in the co-administration of OPZ and CPG (OPZ+CPG), when restricted to new users of CPG, concurrent OPZ+CPG use dropped significantly (3.87%, P = 0.0003) and remained at the lower level. The current analysis reveals the effectiveness of two regulatory actions and the results support the benefit of MID research for improving pharmacovigilance.
Keywords: Medical information database, Drug safety, Label change

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Takahashi H*1, Sai K, Saito Y, Kaniwa N, Matsumura Y*2, Hamaguchi T*2, Shimada Y*2, Ohtsu A*2, Yoshino T*2 Doi T*2, Okuda H, Ichinohe R*2, Takahashi A*3, Doi A*2, Odaka Y*2, Okuyama M*2, Saijo N*2, Sawada J, Sakamoto H*2, Yoshida T*2: Application of a combination of a knowledge-based algorithm and 2-stage screening to hypothesis-free genomic data on irinotecan-treated patients for identification of a candidate single nucleotide polymorphism related to an adverse effect.
PLoS One 2014;9:e105160.

We applied a combined method consisting of a knowledge-based algorithm, 2-stages of screening, and a permutation test for identifying SNPs associated with irinotecan-

induced diarrhea. Among 109,365 SNPs in 168 cancer patients treated with irinotecan, we identified the SNP rs9351963 in potassium voltage-gated channel subfamily KQT member 5（KCNQ5）as a candidate factor. The p value for rs9351963 was 3.3161025 in Fisher's exact test and 0.0289 in the permutation test. The model involving rs9351963 showed sensitivity of 77.8% and specificity of 57.6% in the evaluation by means of logistic regression. This finding suggests that rs9351963 in KCNQ5 is a possible predictive factor of incidence of diarrhea in cancer patients treated with irinotecan. Clinical importance of rs9351963 should be further elucidated.

Keywords: Irinotecan, Single nucleotide polymorphism, Knowledge-based algorithm

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Saito K, Maekawa K, Ishikawa M, Senoo Y, Urata M, Murayama M, Nakatsu N*, Yamada H*, Saito Y: Glucosylceramide and lysophosphatidylcholines as potential blood biomarkers for drug-induced hepatic phospholipidosis.
Toxicol Sci. 2014;141:377-86.

Drug-induced phospholipidosis is one of the major concerns in drug development and clinical treatment. The present study involved the use of a nontargeting lipidomic analysis with liquid chromatography-mass spectrometry to explore noninvasive blood biomarkers for hepatic phospholipidosis from rat plasma. We used three tricyclic antidepressants（clomipramine［CPM］, imipramine［IMI］, and amitriptyline［AMT］）for the model of phospholipidosis in hepatocytes and ketoconazole（KC）for the model of phospholipidosis in cholangiocytes and administered treatment for 3 and 28 days each. Total plasma lipids were extracted and measured. Lipid molecules contributing to the separation of control and drug-treated rat plasma in a multivariate orthogonal partial least squares discriminant analysis were identified. Four lysophosphatidylcholines（LPCs）（16:1, 18:1, 18:2, and 20:4）and 42:1 hexosylceramide（HexCer）were identified as molecules separating control and drug-treated rats in all models of phospholipidosis in hepatocytes. In addition, 16:1, 18:2, and 20:4 LPCs and 42:1 HexCer were identified in a model of hepatic phospholipidosis in cholangiocytes, although LPCs were identified only in the case of 3-day treatment with KC. The levels of LPCs were decreased by drug-induced phospholipidosis, whereas those of 42:1 HexCer were increased. The increase in 42:1 HexCer was much higher in the case of IMI and AMT than in the case of CPM; moreover, the increase induced by IMI was dose-dependent. Structural characterization determining long-chain base and hexose delineated that 42:1 HexCer was d18:1/24:0 glucosylceramide（GluCer）. In summary, our study demonstrated that d18:1/24:0 GluCer and LPCs are potential novel biomarkers for drug-induced hepatic phospholipidosis.

Keywords: Phospholipidosis biomarker, Lipidomics

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Saito K, Maekawa K, Pappan KL*[1], Urata M, Ishikawa M, Kumagai Y*[2], Saito Y: Differences in metabolite profiles between blood matrices, ages, and sexes among Caucasian individuals and their inter-individual variations.
Metabolomics 2014;10:402-13.

Endobiotic metabolites are associated with biological processes in the body and therefore may serve as biomarkers for disease states or therapeutic efficacy and toxicity. However, information is limited regarding how differences between blood matrices, patient backgrounds, and sample handling affect human metabolite profiles. Our objective was to obtain metabolite profiles from Caucasian individuals, based on different matrices（plasma and serum）, subject backgrounds（male/female and young/old）, and storage conditions（2 or 10 freeze–thaw cycles）. In total, 297 metabolites were detected by LC/MS and GC/MS, and more than 75 % of them were highly represented in all sample groups. The multivariate discriminant analysis（OPLS-DA as a model）singled out the matrix type as the most important variable influencing global metabolic profiles; that is, more than 100 metabolites were significantly different based on the matrix type. The influence of subject backgrounds on global metabolic profiles was consistent between plasma and serum. Age-associated differences were more predominant in females than males, whereas gender-associated differences were more prevalent in young subjects than old individuals were. The relative standard deviation of metabolite levels in subjects with the same background ranked from 0.1 to 1.5. Moreover,

the changes of metabolite levels caused by freeze–thaw cycles were limited, and the effect was more prominent in plasma than serum. These data demonstrate the impact of matrix, age, gender, and freeze–thaw cycles on the metabolite profiles and reveal metabolites affected by these factors. Thus, our results provide would useful fundamental information for exploring and qualifying biomarkers for clinical applications.

Keywords: Metabolomics, Plasma and serum

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Saito K, Ishikawa M, Murayama M, Urata M, Senoo Y, Toyoshima K, Kumagai Y*, Maekawa K, Saito Y: Effects of sex, age, and fasting conditions on plasma lipidomic profiles of fasted Sprague-Dawley rats.

PLoS One 2014;9:e112266.

Circulating lipid molecules reflect biological processes in the body and, thus, are useful tools for preclinical estimation of the efficacy and safety of newly developed drugs. However, background information on profiles of circulating lipid molecules in preclinical animal models is limited. Therefore, we examined the effects of multiple factors such as sex (fasted male vs. female), age (fasted 10 vs. 30 weeks old), and feeding conditions (feeding vs. fasting, 16 vs. 22 hr fasting, 10 AM vs. 4 PM blood collection), on the global profiles of lipid molecules in plasma from Sprague-Dawley rats by using a lipidomic approach. Our assay platform determined 262 lipid molecules (68 phospholipids, 20 sphingolipids, 138 neutral lipids, and 36 polyunsaturated fatty acids and their metabolites) in rat plasma. Multivariate discriminant analysis (orthogonal partial least squares discriminant analysis) and heat maps of statistically significant lipid molecules revealed that the plasma lipid profiles in rats are predominantly influenced by feeding conditions, followed by sex and age. In addition, the fasting duration (16 vs. 22 hr fasting) or the time of blood collection (10 AM vs. 4 PM blood collection) has limited or no contribution on the profiles of lipid molecules in rat plasma. Our results provide useful, fundamental information for exploring and validating biomarkers in future preclinical studies and may help to establish regulatory standards for such studies.

Keywords: Lipidomics, Circulating lipids

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Furihata T*, Kawamatsu S*, Ito R*, Saito K, Suzuki S*, Kishida S*, Saito Y, Kamiichi A*, Chiba K*: Hydrocortisone enhances the barrier properties of HBMEC/ciβ, a brain microvascular endothelial cell line, through mesenchymal-to-endothelial transition-like effects.

Fluids Barriers CNS. 2015;12:7.

Because in vitro blood-brain barrier (BBB) models are important tools for studying brain diseases and drug development, we recently established a new line of conditionally immortalized human brain microvascular endothelial cells (HBMEC/ciβ) for use in such models. Since one of the most important functional features of the BBB is its strong intercellular adhesion, in this study, we aimed at improving HBMEC/ciβ barrier properties by means of culture media modifications, thus enhancing their use for future BBB studies. In addition, we simultaneously attempted to obtain insights on related mechanistic properties. Several types of culture media were prepared in an effort to identify the medium most suitable for culturing HBMEC/ciβ. The barrier properties of HBMEC/ciβ were examined by determining Na(+)-fluorescein permeability and transendothelial electric resistance (TEER). Endothelial marker mRNA expression levels were determined by quantitative real-time polymerase chain reaction. Adherens junction (AJ) formation was examined by immunocytochemistry. Cell migration ability was analyzed by scratch assay. Furthermore, cellular lipid composition was examined by liquid chromatography-time-of-flight mass spectrometry. Our initial screening tests showed that addition of hydrocortisone (HC) to the basal medium significantly reduced the Na(+)-fluorescein permeability and increased the TEER of HBMEC/ciβ monolayers. It was also found that, while AJ proteins were diffused in the cytoplasm of HBMEC/ciβ cultured without HC, those expressed in cells cultured with HC were primarily localized at the cell border. Furthermore, this facilitation of AJ formation by HC was in concert with increased endothelial marker mRNA levels and increased ether-type phosphatidylethanolamine levels, while cell migration was retarded in the presence of HC. Our results show

that HC supplementation to the basal medium significantly enhances the barrier properties of HBMEC/ciβ. This was associated with a marked phenotypic alteration in HBMEC/ciβ through orchestration of various signaling pathways. Taken together, it appears that overall effects of HC on HBMEC/ciβ could be summarized as facilitating endothelial differentiation characteristics while concurrently retarding mesenchymal characteristics.

Keywords: Brain microvascular endothelial cells, In vitro BBB model, Mesenchymal-to-endothelial transition

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Wan D[*1], Ludolf F[*2], Alanine DG[*1], Stretton O[*1], Ali Ali E[*1], Al-Barwary N[*1], Wang X[*1], Doenhoff MJ[*1], Mari A[*3], Fitzsimmons CM[*4], Dunne DW[*4], Nakamura R, Oliveira GC[*2], Alcocer MJ[*1], Falcone FH[*1]: Use of humanised rat basophilic leukaemia cell line RS-ATL8 for the assessment of allergenicity of schistosoma mansoni proteins.

PLoS Negl Trop Dis. 2014;8:e3124.

Parasite-specific IgE is thought to correlate with protection against Schistosoma mansoni infection or re-infection. Only a few molecular targets of the IgE response in S. mansoni infection have been characterised. A better insight into the basic mechanisms of anti-parasite immunity could be gained from a genome-wide characterisation of such S. mansoni allergens. This would have repercussions on our understanding of allergy and the development of safe and efficacious vaccinations against helminthic parasites.

Keywords: allergen, RS-ATL8, Schistosoma mansoni

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Iwamoto S[*1], Yonekawa T[*1], Azuma E[*1], Fujisawa T[*2], Nagao M[*2], Shimada E[*3], Nakamura R, Teshima R, Ohishi K[*4], Toyoda H[*1], Komada Y[*1]: Anaphylactic transfusion reaction in homozygous haptoglobin deficiency detected by CD203c expression on basophils.

Pediatr Blood Cancer. 2014;61:1160-1.

Some patients with anhaptoglobinemia develop anaphylactic or allergic transfusion reactions related to antihaptoglobin（Hp）antibodies. However, if anti-Hp IgE antibody is negative, it is difficult to elucidate an anaphylactic reaction. The IgE antibody against Hp was not detected by by the ELISA or EXiLE methods, however, it is possible that the serum IgE concentration was too low to be detected, whereas the amount of cell-bound IgE was sufficient to induce anaphylaxis.

Keywords: haptoglobin, IgE, transfusion reaction

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Fukuzawa K[*1,2], Watanabe C[*2], Kurisaki I[*3], Taguchi N[*4], Mochizuki Y[*2,4], Nakano T, Tanaka S[*5], Komeiji K[*6]: Accuracy of the fragment molecular orbital（FMO）calculations for DNA: Total energy, molecular orbital, and inter-fragment interaction energy.

Comput Theor Chem. 2014;1034:7-16.

The fragment molecular orbital（FMO）method can calculate the electronic structure of macromolecules such as DNA by dividing them into several fragments and introducing suitable approximations. To establish guiding principles for FMO calculation of DNA, benchmark tests were performed for several small DNA models consisting of one or two bases or two base pairs.

Keywords: FMO, DNA, benchmark

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Ueta M[*1], Kaniwa N, Sotozono C[*1], Tokunaga K[*2], Saito Y, Sawai H[*2], Miyadera H[*2], Sugiyama E, Maekawa K, Nakamura R, Nagato M[*3], Aihara M[*4], Matsunaga K[*5], Takahashi Y[*6], Furuya H[*7], Muramatsu M[*8], Ikezawa Z[*9], Kinoshita S[*1]: Independent strong association of HLA-A*02:06 and HLA-B*44:03 with cold medicine-related Stevens-Johnson syndrome with severe mucosal involvement.

Sci Rep. 2014;4:4862.

Stevens-Johnson syndrome (SJS) and its severe variant, toxic epidermal necrolysis (TEN), are acute inflammatory vesiculobullous reactions of the skin and mucous membranes. Cold medicines including non-steroidal anti-inflammatory drugs (NSAIDs) and multi-ingredient cold medications are reported to be important inciting drugs. We used two sample sets of Japanese patients to investigate the association between HLA genotypes and cold medicine-related SJS/TEN (CM-SJS/TEN), including acetaminophen-related SJS/TEN (AR-SJS/TEN) with severe mucosal involvement such as severe ocular surface complications (SOC). *HLA-A*02:06* was strongly associated with CM-SJS/TEN with SOC and AR-SJS/TEN with SOC. *HLA-B*44:03* was also detected as an independent risk allele for CM-, including AR-SJS/TEN with SOC. Analyses using data obtained from CM-SJS/TEN patients without SOC and patients with CM-unrelated SJS/TEN with SOC suggested that these two susceptibility alleles are involved in the development of only CM-SJS/TEN with SOC patients.

Keywords: FMO, DNA, benchmark

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Chung WH*1, Chang WC*2, Lee YS*3, Wu YY*2, Yang CH*1, Ho HC*1, Chen MJ*1, Lin JY*1, Hui RC*1, Ho JC*1, Wu WM*1, Chen TJ*2, Wu T*1, Wu YR*1, Hsih MS*1, Tu PH*1, Chang CN*1, Hsu CN*1, Wu TL*1, Choon SE*4, Hsu CK*5, Chen DY*6, Liu CS*7, Lin CY*8, Kaniwa N, Saito Y, Takahashi Y*9, Nakamura R, Azukizawa H*10, Shi Y*1, Wang TH*1, Chuang SS*1: Tsai SF*11, Chang CJ*1, Chang YS*12, Hung SI*2, Taiwan Severe Cutaneous Adverse Reaction Consortium, Japan Pharmacogenomics Data Science Consortium: Genetic variants associated with phenytoin-related severe cutaneous adverse reactions.
JAMA. 2014;312:525-34.

To investigate the genetic factors associated with phenytoin-related severe cutaneous adverse reactions. Case-control study conducted in 2002-2014 among 105 cases with phenytoin-related severe cutaneous adverse reactions (n=61 Stevens-Johnson syndrome/toxic epidermal necrolysis and n=44 drug reactions with eosinophilia and systemic symptoms), 78 cases with maculopapular exanthema, 130 phenytoin-tolerant control participants, and 3655 population controls from Taiwan, Japan, and Malaysia. A genome-wide association study (GWAS), direct sequencing of the associated loci, and replication analysis were conducted using the samples from Taiwan. The initial GWAS included samples of 60 cases with phenytoin-related severe cutaneous adverse reactions and 412 population controls from Taiwan. The results were validated in (1) 30 cases with severe cutaneous adverse reactions and 130 phenytoin-tolerant controls from Taiwan, (2) 9 patients with Stevens-Johnson syndrome/toxic epidermal necrolysis and 2869 population controls from Japan, and (3) 6 cases and 374 population controls from Malaysia. The GWAS discovered a cluster of 16 single-nucleotide polymorphisms in *CYP2C* genes at 10q23.33 that reached genome-wide significance. Direct sequencing of *CYP2C* identified missense variant rs1057910 (*CYP2C9*3*) that showed significant association with phenytoin-related severe cutaneous adverse reactions (odds ratio, 12; 95% CI, 6.6-20; P=1.1 × 10(-17)). The statistically significant association between *CYP2C9*3* and phenytoin-related severe cutaneous adverse reactions was observed in additional samples from Taiwan, Japan, and Malaysia. A meta-analysis using the data from the 3 populations showed an overall odds ratio of 11 (95% CI, 6.2-18; z=8.58; P<.00001) for *CYP2C9*3* association with phenytoin-related severe cutaneous adverse reactions. Delayed clearance of plasma phenytoin was detected in patients with severe cutaneous adverse reactions, especially *CYP2C9*3* carriers, providing a functional link of the associated variants to the disease. This study identified *CYP2C* variants, including *CYP2C9*3*, known to reduce drug clearance, as important genetic factors associated with phenytoin-related severe cutaneous adverse reactions.

Keywords: Severe cutaneous adverse reaction, phenytoin, CYP2C9

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相﨑健一，北嶋聡，菅野純：シリーズ：日本毒性学会との連携（1）～遺伝子の発現からみた毒性学.
中毒研究 2014;27:358-63.

数万種に及ぶと言われる身の回りの化学物質の毒性評価は，実験動物の所見を人に外挿する事によって実施され，種差や個体差は「安全係数（不確実係数）」により，量的な安全マージンをとる事で勘案されてきた．しかし，サリドマイドに代表されるが如く，これには科学的な限界があり，人の安全性確保をより確実にするためには「毒性学の近代化」が必要である．それには従来法に加え，ブラックボックスであった毒性発現機序の分子レベルでの把握が重要であり，そのための研究手法としては網羅的に遺伝子発現変動をプロファイリングする（精緻に記述する）ことによるトキシコゲノミクス研究法が特に有効である．本稿では，我々が進めているトキシコゲノミクス研究と，その応用によって得た知見例を紹介した．
Keywords: Percellome Toxicogenomics, quantitative toxicology, translational toxicology

Ohtake F, Saeki Y*1, Sakamoto K*2, Ohtake K*2, Nishikawa H*3, Tsuchiya H*1, Ohta T*3, Tanaka K*1, Kanno J: Ubiquitin acetylation inhibits polyubiquitin chain elongation.
EMBO Rep. 2015;16:192-201.

Ubiquitylation is a versatile post-translational modification (PTM). The diversity of ubiquitylation topologies, which encompasses different chain lengths and linkages, underlies its widespread cellular roles. Here, we show that endogenous ubiquitin is acetylated at lysine (K)-6 (AcK6) or K48. Acetylated ubiquitin does not affect substrate monoubiquitylation, but inhibits K11-, K48-, and K63-linked polyubiquitin chain elongation by several E2 enzymes in vitro. In cells, AcK6-mimetic ubiquitin stabilizes the monoubiquitylation of histone H2B-which we identify as an endogenous substrate of acetylated ubiquitin-and of artificial ubiquitin fusion degradation substrates. These results characterize a mechanism whereby ubiquitin, itself a PTM, is subject to another PTM to modulate mono- and polyubiquitylation, thus adding a new regulatory layer to ubiquitin biology.
Keywords: ubiquitin, acetylation, post-translational modification

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Tanaka M*, Aisaki K, Kitajima S, Igarashi K, Kanno J, Nakamura T*: Gene expression response to EWS–FLI1 in mouse embryonic cartilage.
Genomics Data 2014;2:296-8.

Ewing's sarcoma is a rare bone tumor that affects children and adolescents. We have recently succeeded to induce Ewing's sarcoma-like small round cell tumor in mice by expression of EWS–ETS fusion genes in murine embryonic osteochondrogenic progenitors. The Ewing's sarcoma precursors are enriched in embryonic superficial zone (eSZ) cells of long bone. To get insights into the mechanisms of Ewing's sarcoma development, gene expression profiles between EWS–FLI1-sensitive eSZ cells and EWS–FLI1-resistant embryonic growth plate (eGP) cells were compared using DNA microarrays. Gene expression of eSZ and eGP cells (total, 30 samples) was evaluated with or without EWS–FLI1 expression 0, 8 or 48 h after gene transduction. Our data provide useful information for gene expression responses to fusion oncogenes in human sarcoma.
Keywords: Percellome analysis, EWS–FLI1, Ewing's sarcoma

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Tanaka M*, Yamazaki Y*, Kanno Y*, Igarashi K, Aisaki K, Kanno J, Nakamura T*1: Ewing's sarcoma precursors are highly enriched in embryonic osteochondrogenic progenitors.
J Clin Invest. 2014;124:3061-74.

Ewing's sarcoma is a highly malignant bone tumor

found in children and adolescents, and the origin of this malignancy is not well understood. Here, we introduced a Ewing's sarcoma-associated genetic fusion of the genes encoding the RNA-binding protein EWS and the transcription factor ETS（EWS-ETS）into a fraction of cells enriched for osteochondrogenic progenitors derived from the embryonic superficial zone（eSZ）of long bones collected from late gestational murine embryos. EWS-ETS fusions efficiently induced Ewing's sarcoma-like small round cell sarcoma formation by these cells. Analysis of the eSZ revealed a fraction of a precursor cells that express growth/differentiation factor 5（Gdf5), the transcription factor Erg, and parathyroid hormone-like hormone（Pthlh), and selection of the Pthlh-positive fraction alone further enhanced EWS-ETS-dependent tumor induction. Genes downstream of the EWS-ETS fusion protein were quite transcriptionally active in eSZ cells, especially in regions in which the chromatin structure of the ETS-responsive locus was open. Inhibition of β-catenin, poly（ADP-ribose）polymerase 1（PARP1), or enhancer of zeste homolog 2（EZH2）suppressed cell growth in a murine model of Ewing's sarcoma, suggesting the utility of the current system as a preclinical model. These results indicate that eSZ cells are highly enriched in precursors to Ewing's sarcoma and provide clues to the histogenesis of Ewing's sarcoma in bone.

Keywords: Ewing's sarcoma, mouse model, Percellome analysis

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Yokoyama A*[1,2], Igarashi K*[3], Sato T*[4], Takagi K*[5], Otsuka M*[3], Shishido Y*[6], Baba T*[6], Ito R*[1,2], Kanno J, Ohkawa Y[7], Morohashi KI*[7], Sugawara A*[1]: Identification of myelin transcription factor 1(MyT1) as a Subunit of the Neural Cell Type-specific Lysine-specific demethylase 1（LSD1）Complex.

J Biol Chem. 2014;289(26):18152-62.

Regulation of spatiotemporal gene expression in higher eukaryotic cells is critical for the precise and orderly development of undifferentiated progenitors into committed cell types of the adult. It is well known that dynamic epigenomic regulation（including chromatin remodeling and histone modifications by transcriptional coregulator complexes）is involved in transcriptional regulation. Precisely how these coregulator complexes exert their cell type and developing stage-specific activity is largely unknown. In this study we aimed to isolate the histone demethylase lysine-specific demethylase 1（LSD1）complex from neural cells by biochemical purification. In so doing, we identified myelin transcription factor 1（MyT1）as a novel LSD1 complex component. MyT1 is a neural cell-specific zinc finger factor, and it forms a stable multiprotein complex with LSD1 through direct interaction. Target gene analysis using microarray and ChIP assays revealed that the Pten gene was directly regulated by the LSD1-MyT1 complex. Knockdown of either LSD1 or MyT1 derepressed the expression of endogenous target genes and inhibited cell proliferation of a neuroblastoma cell line, Neuro2a. We propose that formation of tissue-specific combinations of coregulator complexes is a critical mechanism for tissue-specific transcriptional regulation.

Keywords: LSD1 Complex, MyT1, Percellome analysis

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Xu J*[1,2], Alexander DB*[1], Futakuchi M*[3], Numano T*[3], Fukamachi K*[3], Suzui M*[3], Omori T*[4], Kanno J, Hirose A, Tsuda*[1]: H. Size- and shape-dependent pleural translocation, deposition, fibrogenesis and mesothelial proliferation by multi-walled carbon nanotubes.

Cancer Sci. 2014;105(7):763-9.

Multiwalled carbon nanotubes（MWCNT）have a fibrous structure similar to asbestos, raising concern that MWCNT exposure may lead to asbestos-like diseases. Previously we showed that MWCNT translocated from

the lung alveoli into the pleural cavity and caused mesothelial proliferation and fibrosis in the visceral pleura. Multiwalled carbon nanotubes were not found in the parietal pleura, the initial site of development of asbestos-caused pleural diseases in humans, probably due to the short exposure period of the study. In the present study, we extended the exposure period to 24 weeks to determine whether the size and shape of MWCNT impact on deposition and lesion development in the pleura and lung. Two different MWCNTs were chosen for this study: a larger sized needle-like MWCNT（MWCNT-L; l = 8 μm, d = 150 nm), and a smaller sized MWCNT（MWCNT-S; l = 3 μm, d = 15 nm), which forms cotton candy-like aggregates. Both MWCNT-L and MWCNT-S suspensions were administered to the rat lung once every 2 weeks for 24 weeks by transtracheal intrapulmonary spraying. It was found that MWCNT-L, but not MWCNT-S, translocated into the pleural cavity, deposited in the parietal pleura, and induced fibrosis and patchy parietal mesothelial proliferation lesions. In addition, MWCNT-L induced stronger inflammatory reactions including increased inflammatory cell number and cytokine/chemokine levels in the pleural cavity lavage than MWCNT-S. In contrast, MWCNT-S induced stronger inflammation and higher 8-hydroxydeoxyguanosine level in the lung tissue than MWCNT-L. These results suggest that MWCNT-L has higher risk of causing asbestos-like pleural lesions relevant to mesothelioma development.

Keywords: multiwalled carbon nanotubes, pleural translocation, mesothelial proliferation

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Hirabayashi Y, Tsuboi I*1, Nakachi K*2, Kusunoki Y*2, Inoue T*1: Experimentally induced, synergistic late effects of a single dose of radiation and aging: Significance in LKS fraction as compared with mature blood cells.

J Appl Toxicol. 2015;35:230-40.

The number of murine mature blood cells recovered within 6 weeks after 2-Gy whole-body irradiation at 6 weeks of age, whereas in the case of the undifferentiated hematopoietic stem/progenitor cell（HSC/HPC）compartment［cells in the lineage-negative, c-kit-positive and stem-cell-antigen-1-positive（LKS）fraction］, the numerical differences between mice with and without irradiation remained more than a year, but conclusively the cells showed numerical recovery. When mice were exposed to radiation at 6 months of age, acute damages of mature blood cells were rather milder probably because of their maturation with age; but again, cells in the LKS fraction were specifically damaged, and their numerical recovery was significantly delayed probably as a result of LKS-specific cellular damages. Interestingly, in contrast to the recovery of the number of cells in the LKS fraction, their quality was not recovered, which was quantitatively assessed on the basis of oxidative-stress-related fluorescence intensity. To investigate why the recovery in the number of cells in the LKS fraction was delayed, expression levels of genes related to cellular proliferation and apoptosis of cells in the bone marrow and LKS fraction were analyzed by real-time polymerase chain reaction（RT-PCR）. In the case of 21-month-old mice after radiation exposure, Ccnd1, PiK3r1 and Fyn were overexpressed solely in cells in the LKS fraction. Because Ccnd1and PiK3r1 upregulated by aging were further upregulated by radiation, single-dose radiation seemed to induce the acceleration of aging, which is related to the essential biological responses during aging based on a lifetime-dependent relationship between a living creature and xenobiotic materials.

Keywords: Radiation late effects, Hematopoietic stem cells, Gene expression profile

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Naruse M*1,2, Ono R, Irie M*1,2, Nakamura K*3,4, Furuse T*5, Hino T*3,6, Oda K*3,7, Kashimura M*5, Yamada I*5, Wakana S*5, Yokoyama M*3,7, Ishino F*1,8, Kaneko-Ishino T*2 : Sirh7/Ldoc1 knockout mice exhibit placental P4 overproduction and delayed parturition.

Development 2014;141(24):4763-71.

Sirh7/Ldoc1［sushi-ichi retrotransposon homolog 7/

leucine zipper, downregulated in cancer 1, also called mammalian retrotransposon-derived 7 (Mart7)] is one of the newly acquired genes from LTR retrotransposons in eutherian mammals. Interestingly, Sirh7/Ldoc1 knockout (KO) mice exhibited abnormal placental cell differentiation/maturation, leading to an overproduction of placental progesterone (P4) and placental lactogen 1 (PL1) from trophoblast giant cells (TGCs). The placenta is an organ that is essential for mammalian viviparity and plays a major endocrinological role during pregnancy in addition to providing nutrients and oxygen to the fetus. P4 is an essential hormone in the preparation and maintenance of pregnancy and the determination of the timing of parturition in mammals; however, the biological significance of placental P4 in rodents is not properly recognized. Here, we demonstrate that mouse placentas do produce P4 in mid-gestation, coincident with a temporal reduction in ovarian P4, suggesting that it plays a role in the protection of the conceptuses specifically in this period. Pregnant Sirh7/Ldoc1 knockout females also displayed delayed parturition associated with a low pup weaning rate. All these results suggest that Sirh7/Ldoc1 has undergone positive selection during eutherian evolution as a eutherian-specific acquired gene because it impacts reproductive fitness via the regulation of placental endocrine function.

Keywords: Placenta, Progesterone, Retrotransposon

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Fujieda T*, Koganezawa N*, Ide Y*, Sekino Y: An inhibitory pathway controlling the gating mechanism of the mouse lateral amygdala revealed by voltage-sensitive dye imaging.
Neurosci Lett. 2015;590:126-31.

The lateral amygdala nucleus (La) is known as a gateway for emotional learning that interfaces sensory inputs from the cortex and the thalamus. In the La, inhibitory GABAergic inputs control the strength of sensory inputs and interfere with the initial step of the acquisition of fear memory. In the present study, we investigated the spatial and temporal patterns of the inhibitory responses in mouse La using voltage-sensitive dye imaging. Stimulating the external capsule (EC) induced large and long-lasting hyperpolarizing signals in the La. We focused on these hyperpolarizing signals, revealing the origins of the inhibitory inputs by means of surgical cuts on the possible afferent pathways with four patterns. Isolating the medial branch of EC (ECmed), but not the lateral branch of EC (EClat), from the La strongly suppressed the induction of the hyperpolarization. Interestingly, isolating the ECmed from the caudate putamen did not suppress the hyperpolarization, while the surgical cut of the ECmed fiber tract moderately suppressed it. Glutamatergic antagonists completely suppressed the hyperpolarizing signals induced by the stimulation of EC. When directly stimulating the dorsal, middle or ventral part of the ECmed fiber tract in the presence of glutamatergic antagonists, only the stimulation in the middle part of the ECmed caused hyperpolarization. These data indicate that the GABAergic neurons in the medial intercalated cluster (m-ITC), which receive glutamatergic excitatory input from the ECmed fiber tract, send inhibitory afferents to the La. This pathway might have inhibitory effects on the acquisition of fear memory.

Keywords: GABA, external capsule, voltage-sensitive dye imaging

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Ishikawa M*1, Shiota J*1, Ishibashi Y*1, Hakamata T*1, Shoji S*1, Fukuchi M*1, Tsuda M*1, Shirao T*2, Sekino Y, Baraban JM*3, Tabuchi A*1: Cellular localization and dendritic function of rat isoforms of the SRF coactivator MKL1 in cortical neurons.
Neuroreport 2014;25(8):585-92.

The ability of megakaryoblastic leukemia 1 (MKL1) to function as a serum response factor (SRF) coactivator

is regulated through its association with G-actin. In the cytoplasm, MKL1 binds to G-actin through RPXXXEL（RPEL）motifs. However, dissociation of MKL1 from G-actin triggers its translocation into the nucleus where it stimulates SRF-mediated gene expression. Previous characterization of rat MKL1 gene products has identified several isoforms: full-length MKL1, basic, SAP, and coiled-coil domain（BSAC）, MKL1-elongated derivative of yield（MELODY）, and MKL1met. In this study, we have investigated whether these MKL1 isoforms, which contain different numbers of RPEL motifs, differ in their subcellular localization, transcriptional activity, and effect on dendritic number and axonal length. Immunofluorescent staining of cultured cortical neurons expressing individual FLAG-tagged MKL1 isoforms indicated that all MKL1 isoforms are present in both the cytoplasm and the nucleus. However, MKL1met, which contains two RPEL motifs, shows enhanced nuclear staining compared with the other three isoforms, full-length MKL1, basic, SAP, and coiled-coil domain, and MKL1-elongated derivative of yield, which contain three RPEL motifs. Consistent with its preferential nuclear localization, overexpression of MKL1met, but not other isoforms, increases SRF-mediated transcriptional responses and reduces the number of dendrites. In contrast to the inhibitory effect of MKL1met on dendritic number, axonal length is not affected by overexpression of any of the MKL1 isoforms. These findings suggest that the subcellular localization of MKL1 isoforms, which is mediated by the number of actin-binding RPEL motifs, regulates their effect on SRF-mediated gene expression and dendritic morphology.

Keywords: megakaryoblastic leukemia 1（MKL1）, serum response factor（SRF）, dendritic morphology

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Shigemoto-Mogami Y, Fujimori K, Ikarashi Y, Hirose A, Sekino Y, Sato K: Residual metals in carbon nanotubes suppress the proliferation of neural stem cells.
Fundam Toxcol Sci. 2014;1(3):87-94.

Carbon nanotubes（CNTs）are used in many fields; however, little is known about the effects of CNTs on the central nervous system（CNS）. In this study, we found that extracts of sonicated CNTs suppressed the proliferation of neural stem cells（NSCs）. Single-walled CNTs（SWCNTs）and multiple-walled CNTs（MWCNTs）were suspended in PBS（1 mg/mL）and sonicated for 5 hr using a water bath sonicator. Supernatants from both types of CNTs suppressed NSC proliferation. The effects weakened in a dilution-ratio-dependent manner and strengthened in a sonication time-dependent manner. Metal concentrations extracted from SCNTs and MCNTs after 5-hr of sonication were determined using inductively coupled plasma mass spectrometry. Mn, Rb, Cs, Tl, and Fe were detected in the SWCNT supernatant, and Mn, Cs, W, and Tl were detected in the MWCNT supernatant. The concentration of Mn, Rb, and Fe eluted from the SWCNTs and Rb eluted from MWCNTs following sonication were sufficient to suppress NSC proliferation alone. N-acetyl cysteine（NAC）and ascorbic acid（AA）reversed the effects of Mn and Fe and restored NSC proliferation. The effects of Rb and Tl were not affected by the antioxidants. Both antioxidants largely restored the suppression of NSC proliferation induced by the SWCNT and MWCNT supernatants. These results suggest that metals extracted from CNTs via a strong vibration energy can suppress NSC proliferation through ROS production by the extracted metals.

Keywords: carbon nanotube, neural stem cell, reactive oxygen species

Fujimori K*, Takaki J*, Shigemoto-Mogami Y, Sekino Y, Suzuki T*, Sato K: Paroxetine prevented the down-regulation of astrocytic L-Glu transporters in neuroinflammation.
J Pharmacol Sci. 2015;127(1):145-9.

The extracellular L-glutamate（L-Glu）concentration is elevated in neuroinflammation, thereby causing excitotoxicity. One of the mechanisms is down-regulation of astrocyte L-Glu transporters. Some antidepressants have anti-inflammatory effects. We therefore investigated effects of various antidepressants on the down-regulation of astrocyte L-Glu transporters in the in vitro neuroinflammation model. Among these antidepressants, only paroxetine was effective. We previously demonstrated that the downregulation of astrocyte L-Glu transporters was caused by L-Glu released from activated microglia. We here clarified that only paroxetine inhibited L-Glu release from microglia. This is the novel action of paroxetine, which may bring advantages on the therapy of neuroinflammation.

Keywords: L-glutamate transporter, astrocyte, microglia

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Hayakawa T*[1], Kunihiro T*[1], Ando T*[2], Kobayashi S*[1], Matsui E*[1], Yada H*[1], Kanda Y, Kurokawa J*[2], Furukawa T*[2]: Image-based evaluation of contraction-relaxation kinetics of human-induced pluripotent stem cell-derived cardiomyocytes: correlation and complementarity with extracellular electrophysiology.
J Mol Cell Cardiol. 2014;77:178-91.

In this study, we used high-speed video microscopy with motion vector analysis to investigate the contractile characteristics of hiPS-CM monolayer, in addition to further characterizing the motion with extracellular field potential (FP), traction force and the Ca(2+) transient. Results of our traction force microscopy demonstrated that the force development of hiPS-CMs correlated well with the cellular deformation detected by the video microscopy with motion vector analysis. In the presence of verapamil and isoproterenol, contractile motion of hiPS-CMs showed alteration in accordance with the changes in fluorescence peak of the Ca(2+) transient, i.e., upstroke, decay, amplitude and full-width at half-maximum. Simultaneously recorded hiPS-CM motion and FP showed that there was a linear correlation between changes in the motion and field potential duration in response to verapamil (30-150nM), isoproterenol (0.1-10μM) and E-4031 (10-50nM). In addition, tetrodotoxin (3-30μM)-induced delay of sodium current was corresponded with the delay of the contraction onset of hiPS-CMs. These results indicate that the electrophysiological and functional behaviors of hiPS-CMs are quantitatively reflected in the contractile motion detected by this image-based technique. In the presence of 100nM E-4031, the occurrence of early after-depolarization-like negative deflection in FP was also detected in the hiPS-CM motion as a characteristic two-step relaxation pattern. These findings offer insights into the interpretation of the motion kinetics of the hiPS-CMs, and are relevant for understanding electrical and mechanical relationship in hiPS-CMs.

Keywords: iPS cells, motion vector, contraction

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Hiyoshi H*[1], Goto N*[1], Tsuchiya M*[1], Iida K*[2], Nakajima Y*[2], Hirata N, Kanda Y, Nagasawa K*[2], Yanagisawa J*[1]: 2-(4-Hydroxy-3-methoxyphenyl)-benzothiazole suppresses tumor progression and metastatic potential of breast cancer cells by inducing ubiquitin ligase CHIP.
Scientific Reports 2014;4:7095.

Breast cancer is the most common malignancy among women and has poor survival and high recurrence rates for aggressive metastatic disease. Notably, triple-negative breast cancer (TNBC) is a highly aggressive cancer and there is no preferred agent for TNBC therapy. In this study, we show that a novel agent, 2-(4-hydroxy-3-methoxyphenyl)-benzothiazole (YL-109), has ability to inhibit breast cancer cell growth and invasiveness in vitro and in vivo. In addition, YL-109 repressed the sphere-forming ability and the expression of stem cell markers in MDA-MB-231 mammosphere cultures. YL-109 increased the expression of carboxyl terminus of Hsp70-interacting protein (CHIP), which suppresses tumorigenic and metastatic potential of breast cancer cells by inhibiting the oncogenic pathway. YL-109 induced CHIP transcription because of the recruitment of the aryl hydrocarbon receptor (AhR) to upstream of CHIP gene in MDA-MB-231 cells. Consistently, the antitumor effects of YL-109 were depressed by CHIP or AhR knockdown in MDA-MB-231 cells. Taken together, our findings indicate that a novel agent YL-109 inhibits cell growth and metastatic potential by inducing CHIP expression through AhR signaling and reduces cancer stem cell properties in MDA-MB-231 cells. It suggests that YL-109 is a potential candidate for breast cancer therapy.

Keywords: ubiquitin ligase, chemical biology, aryl hydrocarbon receptor

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Tsuchiya M*, Nakajima Y*, Hirata N, Morishita T*, Kishimoto H*, Kanda Y, Kimura K*: Ubiquitin ligase CHIP suppresses cancer stem cell properties in a population of breast cancer cells.
Biochemical and Biophysical Research Communications 2014;452:928-32.

Cancer stem cells (CSCs) have several distinctive characteristics, including high metastatic potential,

tumor-initiating potential, and properties that resemble normal stem cells such as self-renewal, differentiation, and drug efflux. Because of these characteristics, CSC is regarded to be responsible for cancer progression and patient prognosis. In our previous study, we showed that an ubiquitin E3 ligase carboxyl terminus of Hsc70-interacting protein（CHIP）suppressed breast cancer malignancy. Moreover, a recent clinical study reported that CHIP expression levels were associated with favorable prognostic parameters of patients with breast cancer. Here we show that CHIP suppresses CSC properties in a population of breast cancer cells. CHIP depletion resulted in an increased proportion of CSCs among breast cancers when using several assays to assess CSC properties. From our results, we propose that inhibition of CSC properties may be one of the functions of CHIP as a suppressor of cancer progression.

Keywords: cancer stem cells, ubiquitin ligase, breast cancer

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Hirata N, Yamada S, Shoda T, Kurihara M, Sekino Y, Kanda Y: Sphingosine-1-phosphate regulates cancer stem cell phenotype via Notch signaling.

Nature Communications 2014;5:4806.

Many tumours originate from cancer stem cells（CSCs）, which is a small population of cells that display stem cell properties. However, the molecular mechanisms that regulate CSC frequency remain poorly understood. Here, using microarray screening in aldehyde dehydrogenase（ALDH）-positive CSC model, we identify a fundamental role for a lipid mediator sphingosine-1-phosphate（S1P）in CSC expansion. Stimulation with S1P enhances ALDH-positive CSCs via S1P receptor 3（S1PR3）and subsequent Notch activation. CSCs overexpressing sphingosine kinase 1（SphK1）, an S1P-producing enzyme, show increased ability to develop tumours in nude mice, compared with parent cells or CSCs. Tumorigenicity of CSCs overexpressing SphK1 is inhibited by S1PR3 knockdown or S1PR3 antagonist. Breast cancer patient-derived mammospheres contain SphK1(+)/ALDH1(+) cells or S1PR3(+)/ALDH1(+) cells. Our findings provide new insights into the lipid-mediated regulation of CSCs via Notch signalling, and rationale for targeting S1PR3 in cancer.

Keywords: cancer stem cells, lipid mediator, notch

Yamada S, Kotake Y*, Demizu Y*, Kurihara M*, Sekino Y, Kanda Y: NAD-dependent isocitrate dehydrogenase as a novel target of tributyltin in human embryonic carcinoma cells.

Scientific Reports 2014;4:5952.

Tributyltin（TBT）is known to cause developmental defects as endocrine disruptive chemicals（EDCs）. At nanomoler concentrations, TBT actions were mediated by genomic pathways via PPAR/RXR. However, non-genomic target of TBT has not been elucidated. To investigate non-genomic TBT targets, we performed comprehensive metabolomic analyses using human embryonic carcinoma NT2/D1 cells. We found that 100 nM TBT reduced the amounts of α-ketoglutarate, succinate and malate. We further found that TBT decreased the activity of NAD-dependent isocitrate dehydrogenase（NAD-IDH）, which catalyzes the conversion of isocitrate to α-ketoglutarate in the TCA cycle. In addition, TBT inhibited cell growth and enhanced neuronal differentiation through NAD-IDH inhibition. Furthermore, studies using bacterially expressed human NAD-IDH and in silico simulations suggest that TBT inhibits NAD-IDH due to a possible interaction. These results suggest that NAD-IDH is a novel non-genomic target of TBT at nanomolar levels. Thus, a metabolomic approach may provide new insights into the mechanism of EDC action.

Keywords: Tin compound, TCA cycle, Neurotoxicity

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Nakamura Y*[1], Matsuo J*[1,2], Miyamoto N*[3], Ojima A*[3], Ando K*[1], Kanda Y, Sawada K*[3], Sugiyama A*[1], Sekino Y: Standardization of testing methods with iPS derived cardiomyocytes for evaluating drug-induced repolarization delay.

Journal of Pharmaceutical Sciences 2014;124:494-501.

A prospective comparison study across 3 independent research laboratories of a pure IKrblocker E-4031 was conducted by using the same batch of human iPS cell–derived cardiomyocytes in order to verify the utility and reliability of our original standard protocol. Field potential waveforms were recorded with a multi-electrode array system to measure the inter-spike interval and field potential duration. The effects of E-4031 at

concentrations of 1 to 100 nM were sequentially examined every 10 min. In each facility, E-4031 significantly prolonged the field potential duration corrected by Fridericia's formula and caused early afterdepolarizations occasionally resulting in triggered activities, whereas it tended to decrease the rate of spontaneous contraction. These results were qualitatively and quantitatively consistent with previous non-clinical in vitro and in vivo studies as well as clinical reports. There were inter-facility differences in some absolute values of the results, which were not observed when the values were normalized as percentage change. Information described in this paper may serve as a guide when predicting the drug-induced repolarization delay and arrhythmias with this new technology of stem cells.

Keywords: iPS cells, QT prolongation, proarrythmia

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Zeiger E*1, Gollapudi B*2, Aardema MJ*3, Auerbach S*4, Boverhof D*2, Custer L*5, Dedon P*6, Honma M, Ishida S, Kasinski AL*7, Kim JH*8, Manjanatha MG*9, Marlowe J*10, Pfuhler S*11, Pogribny I*9, Slikker W*9, Stankowski LF Jr*12, Tanir JY*8, Tice R*4, van Benthem J*13, White P*14, Witt KL*4, Thybaud V*15: Opportunities to integrate new approaches in genetic toxicology: an ILSI-HESI workshop report.
Environmental and Molecular Mutagenesis 2014;56: 277-85.

Genetic toxicity tests currently used to identify and characterize potential human mutagens and carcinogens rely on measurements of primary DNA damage, gene mutation, and chromosome damage in vitro and in rodents. ILSI-HESI Committee on the Relevance and Follow-up of Positive Results in In Vitro Genetic Toxicity Testing held an April 2012 Workshop in Washington, DC, to consider the impact of new understanding of biology and new technologies on the identification and characterization of genotoxic substances, and to identify new approaches to inform more accurate human risk assessment for genetic and carcinogenic effects. A summary of the workshop are provided.

Keywords: epigenetics, genetic toxicity, iPS cells

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Kim S-R, Kubo T, Kuroda Y, Hojyo M, Matsuo T*, Miyajima A, Usami M, Sekino Y, Matsushita T*, Ishida S: Comparative metabolome analysis of cultured fetal and adult hepatocytes in humans.
The Journal of Toxicological Sciences 2014;39:717-23.

The liver is the central organ of metabolism, but its function varies during development from fetus to adult. In this study, we comprehensively analyzed and compared metabolites in fetal and adult hepatocytes from human donors. We identified 211 metabolites by CE-TOFMS in the hepatocytes cultured in vitro. The amounts of most metabolites in the glycolysis/glyconeogenesis pathway, tricarboxylic acid cycle and urea cycle were lower in fetal hepatocytes than in adult hepatocytes. These results suggest different susceptibility of the fetal and adult liver to toxic insults affecting energy metabolism.

Keywords: Metabolome, CE-TOFMS, Human fetal hepatocytes

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Dubois-Pot-Schneider H*1,2, Fekir K*1,2, Coulouarn C*1,2,

Glaise D*[1,2], Aninat C*[1,2], Jouarnen K*[1], Le Guével L*[3], Kubo T, Ishida S, Morel F*[1,2], Corlu A*[1-3]: Inflammatory cytokines promote the retrodifferentiation of tumor-derived hepatocyte-like cells to progenitor cells.
Hepatology 2014;60:2077-90.

Human hepatocellular carcinoma（HCC）heterogeneity promotes recurrence and resistance to therapies. Recent studies have reported that HCC may be derived not only from adult hepatocytes and hepatoblasts but also hepatic stem/progenitors. In this study we report the mechanisms and molecular effectors involved in the retrodifferentiation of HepaRG cells into bipotent progenitors. HepaRG cell retrodifferentiation is mediated by crosstalk between transforming growth factor beta 1（TGFβ1）and inflammatory cytokine pathways. Interestingly, the retrodifferentiation process is blocked by the histone deacetylase inhibitor trichostatin A.
Keywords: HepaRG cells, retrodifferentiation, inflammatory cytokines

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Usami M, Mitsunaga K*[1], Irie T, Miyajima A, Doi O*[2]: Simple in vitro migration assay for neural crest cells and the opposite effects of all-trans-retinoic acid on cephalic- and trunk-derived cells.
Congenit Anom（Kyoto）. 2014;54(3):184-8.

Here, we describe a simple in vitro neural crest cell（NCC）migration assay and the effects of all-trans-retinoic acid（RA）on NCCs. Neural tubes excised from the rhombencephalic or trunk region of day 10.5 rat embryos were cultured for 48 h to allow emigration and migration of NCCs. Migration of NCCs was measured as the change in the radius（radius ratio）calculated from the circular spread of NCCs between 24 and 48 h of culture. RA was added to the culture medium after 24 h at embryotoxic concentrations determined by rat whole embryo culture. RA（10 μM）reduced the migration of cephalic NCCs, whereas it enhanced the migration of trunk NCCs, indicating that RA has opposite effects on these two types of NCCs.
Keywords: Migration assay, Neural crest cell, Rat

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Onoue S*[1], Hosoi K*[2], Toda T*[3], Takagi H*[4], Osaki N*[4], Matsumoto Y*[5], Kawakami S*[6], Wakuri S*[7], Iwase Y*[8], Yamamoto T*[8], Nakamura K*[3], Ohno Y, Kojima H: Intra-/inter-laboratory validation study on reactive oxygen species assay for chemical photosafety evaluation using two different solar simulators.
Toxicol In Vitro. 2014;28(4):515-23.

A previous multi-center validation study demonstrated high transferability and reliability of reactive oxygen species（ROS）assay for photosafety evaluation. The present validation study was undertaken to verify further the applicability of different solar simulators and assay performance. In 7 participating laboratories, 2 standards and 42 coded chemicals, including 23 phototoxins and 19 non-phototoxic drugs/chemicals, were assessed by the ROS assay using two different solar simulators（Atlas Suntest CPS series, 3 labs; and Seric SXL-2500V2, 4 labs）. Irradiation conditions could be optimized using quinine and sulisobenzone as positive and negative standards to offer consistent assay outcomes. In both solar simulators, the intra- and inter-day precisions（coefficient of variation; CV）for quinine were found to be below 10%. The inter-laboratory CV for quinine averaged 15.4%（Atlas Suntest CPS）and 13.2%（Seric SXL-2500V2）for singlet oxygen and 17.0%（Atlas Suntest CPS）and 7.1%（Seric SXL-2500V2）for superoxide, suggesting high inter-laboratory reproducibility even though different solar simulators were employed for the ROS assay. In the ROS assay on 42 coded chemicals, some chemicals（ca. 19-29%）were unevaluable because of limited solubility and spectral interference. Although several false positives appeared with positive predictivity of ca. 76-92%（Atlas Suntest CPS）and ca. 75-84%（Seric SXL-2500V2）, there were no false negative predictions in both solar simulators. A multi-center validation study on the ROS assay demonstrated satisfactory transferability, accuracy, precision, and predictivity, as well as the availability of other solar simulators.
Keywords: Phototoxicity, Reactive oxygen species, Validation

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Kojima H, Katoh M*1, Shinoda S*2, Hagiwara S*2, Suzuki T*3, Izumi R*3, Yamaguchi Y*4, Nakamura M*4, Kasahawa T*5, Shibai A*5: A catch-up validation study of an in vitro skin irritation test method using reconstructed human epidermis LabCyte EPI-MODEL24.
J Appl Toxicol. 2014;34(7):766-74.

Three validation studies were conducted by the Japanese Society for Alternatives to Animal Experiments in order to assess the performance of a skin irritation assay using reconstructed human epidermis (RhE) LabCyte EPI-MODEL24 (LabCyte EPI-MODEL24 SIT) developed by the Japan Tissue Engineering Co., Ltd.(J-TEC), and the results of these studies were submitted to the Organisation for Economic Co-operation and Development (OECD) for the creation of a Test Guideline (TG). In the summary review report from the OECD, the peer review panel indicated the need to resolve an issue regarding the misclassification of 1-bromohexane. To this end, a rinsing operation intended to remove exposed chemicals was reviewed and the standard operating procedure (SOP) revised by J-TEC. Thereafter, in order to confirm general versatility of the revised SOP, a new validation management team was organized by the Japanese Center for the Validation of Alternative Methods (JaCVAM) to undertake a catch-up validation study that would compare the revised assay with similar in vitro skin irritation assays, per OECD TG No. 439 (2010). The catch-up validation and supplementary studies for LabCyte EPI-MODEL24 SIT using the revised SOPs were conducted at three laboratories. These results showed that the revised SOP of LabCyte EPI-MODEL24 SIT conformed more accurately to the classifications for skin irritation under the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS), thereby highlighting the importance of an optimized rinsing operation for the removal of exposed chemicals in obtaining consistent results from in vitro skin irritation assays.

Keywords: reconstructed human epidermis, skin irritation, validation

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Hamada S*1, Ohyama W*2, Takashima R*1, Shimada K*3, Matsumoto K*4, Kawakami S*5, Uno F*6, Sui H*7, Shimada Y*8, Imamura T*9, Matsumura S*10, Sanada H*11, Inoue K*12, Muto S*13, Ogawa I*14, Hayashi A*15, Takayanagi T*16, Ogiwara Y*17, Maeda A*18, Okada E*2, Terashima Y*19, 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 Genet Toxicol Environ Mutagen. 2015;Mar: 780-1.

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-four testing laboratories belonging to the Mammalian Mutagenicity Study Group, a subgroup of the Japanese Environmental Mutagen Society, participated in this trial. 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. In addition to the RDLMN assay, we simultaneously conducted gastrointestinal tract MN assays using 6 of the above carcinogens as an optional trial of the collaborative study. The MN assay using the glandular stomach, which is the first contact site of the test chemical when administered by oral gavage, was able to detect chromosomal aberrations with 3 test chemicals including a stomach-targeted carcinogen. The treatment regime was the 14- and/or 28-day

repeated-dose, and the regime is sufficiently promising to incorporate these methods into repeated-dose toxicological studies. The outcomes of our collaborative study indicated that the new techniques to detect chromosomal aberrations in vivo in several tissues worked successfully.

Keywords: Liver, Micronucleus, Repeated-dose

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Onami S, Cho YM, Toyoda T, Mizuta Y, Yoshida M, Nishikawa A, Ogawa K: A 13-week repeated dose study of three 3-monochloropropane-1,2-diol fatty acid esters in F344 rats.
Arch Toxicol. 2014;88:871-80.

3-monochloropropane-1,2-diol（3-MCPD）, a rat renal and testicular carcinogen, has been reported to occur in various foods and food ingredients as free or esterified forms. Since reports about toxicity of 3-MCPD esters are limited, we conducted a 13-week rat subchronic toxicity study of 3-MCPD esters（palmitate diester: CDP, palmitate monoester: CMP, oleate diester: CDO）. We administered a carcinogenic dose（3.6×10^{-4} mol/kg B.W./day）of 3-MCPD or these esters at equimolar concentrations and two 1/4 lower doses by gavage with olive oil as a vehicle five times a week for 13 weeks to F344 male and female rats. As a result, five out of ten 3-MCPD-treated females died from acute renal tubular necrosis, but none of the ester-treated rats. Decreased HGB was observed in all high-dose 3-MCPD fatty acid ester-treated rats, except CDO-treated males. The absolute and relative kidney weights were significantly increased in the ester-treated rats at medium and high doses. Relative liver weights were significantly increased in the esters-treated rat at high dose, except for CMP females. Significant increase in apoptotic epithelial cells in the initial segment of the epididymis of high-dose ester-treated males was also observed. The results suggested that although acute renal toxicity was lower than 3-MCPD, these three 3-MCPD fatty acid esters have the potential to exert subchronic toxicity to the rat kidneys and epididymis, to a similar degree as 3-MCPD under the present conditions. NOAELs（no-observed-adverse-effect levels）of CDP, CMP and CDO were suggested to be 14, 8 and 15 mg/kg B.W./day, respectively.

Keywords: 3-MCPD fatty acid esters, F344 rats, epididymis

Onami S, Cho YM, Toyoda T, Horibata K, Ishii Y, Umemura T, Honma M, Nohmi T, Nishikawa A, Ogawa K: Absence of *in vivo* genotoxicity of 3-monochloropropane-1,2-diol and associated fatty acid esters in a 4 week comprehensive toxicity study using F344 *gpt* delta rats.
Mutagenesis 2014;29:295-302.

3-Monochloropropane-1,2-diol（3-MCPD）is regarded as a rat renal and testicular carcinogen and has been classified as a possible human carcinogen（group 2B）by International Agency for Research on Cancer. This is potentially of great importance given that esters of this compound have recently found to be generated in many foods and food ingredients as a result of food processing. There have been a few reports about their toxicity, although we have recently found that the toxicity profile of 3-MCPD esters was similar to that of 3-MCPD in a rat 13-week repeated dose study, except for the acute renal toxicity seen in 3-MCPD-treated females. In the present study, to examine *in vivo* genotoxicity we administered equimolar doses of 3-MCPD or 3-MCPD fatty acid esters（palmitate diester, palmitate monoester and oleate diester）to 6-week-old male F344 *gpt* delta rats carrying a reporter transgene for 4 weeks by intragastric administration. *In vivo* micronucleus, Pig-a mutation and *gpt* assays were performed, as well as investigations of major toxicological parameters including histopathological features. As one result, the relative

kidney weights of the 3-MCPD and all three ester groups were significantly increased compared with the vehicle control group. However, the frequency of micronucleated reticulocytes and Pig-a mutant red blood cells did not differ among groups. Moreover, no changes were observed in mutant frequencies of *gpt* and *red/gam* (Spi^-) genes in the kidney and the testis of 3-MCPD and 3-MCPD-fatty-acid-esters-treated rats. In histopathological analyses, no treatment related changes were observed, except for decrease of eosinophilic bodies in the kidneys of all treated groups. These results suggest that 3-MCPD and its fatty acid esters are not *in vivo* genotoxins, although they may exert renal toxicity.

Keywords: 3-MCPD fatty acid esters, *in vivo* genotoxicity, *gpt* delta rat

Ishii Y, Matsushita K, Kuroda K, Yokoo Y, Kijima A, Takasu S, Kodama Y, Nishikawa A, Umemura T: Acrylamide induces specific DNA adduct formation and gene mutations in a carcinogenic target site, the mouse lung.
Mutagenesis 2015;30:227-35.

Acrylamide（AA）is a contaminant in heated foods and is carcinogenic in multiple organs of rodents. There have been many reports regarding AA-induced DNA modification and genotoxicity. However, the data are insufficient to understand fully the relationship between the two events. A recent report demonstrated carcinogenicity in the mouse lung. The lung is advantageous for investigation of AA-induced genotoxicity because DNA adduct levels are relatively high in this organ. In the present study, reporter gene mutation assays and quantitative analyses of specific DNA adducts were performed in the lungs of mature gpt delta mice treated with AA at doses of 100, 200 and 400 p.p.m. in drinking water for 4 weeks. N7-GA-Gua was detected in all AA-treated mice in a dose-dependent manner. *gpt* mutant frequencies（MFs）were significantly increased in the middle- and high-dose groups. In the analysis of mutation spectra, significant increases in GC-TA transversions and single base deletion mutations were observed in the high-dose group. Spi^- MFs were significantly increased in the high-dose group. Analysis of Spi- mutants revealed significant increases in the frequencies of single base deletion mutation in runs of G/C and A/T. Analyses of immature mice under the same experimental conditions showed that there were no differences of susceptibility to AA-induced genotoxicity in the two age classes. The overall data clearly show the causal relationship between AA-induced DNA adducts and the gene mutations at carcinogenic target sites.

Keywords: acrylamide, DNA adduct, *gpt* delta mouse

Ishii Y, Takasu S, Kuroda K, Matsushita K, Kijima A, Nohmi T, Ogawa K, Umemura T: Combined application of comprehensive analysis for DNA modification and reporter gene mutation assay to evaluate kidneys of *gpt* delta rats given madder color or its constituents.
Anal Bioanal Chem. 2014;406:2467-75.

DNA adductome analysis using liquid chromatography-tandem mass spectrometry is a promising tool to exhaustively search DNA modifications. Given that the molecular weight of chemical-specific adducts is determined by the total molecular weights of the active form and nucleotide bases, we developed a new method of comprehensive analysis for chemical-specific DNA adducts based on the principle of adductome analysis. The actual analytical mass range was 50 mass units up or down from the average molecular weight of the four DNA bases plus the molecular weight of the expected active form of the chemical. Using lucidin-3-O-primeveroside（LuP）, lucidin-modified bases formed by its active form were exhaustively searched using this new method. Various DNA adducts, including Luc-N^2-dG and Luc-N^6-dA, were identified in the kidneys of rats given LuP. Together with measurement of 8-hydroxydeoxyguanosine（8-OHdG）levels, the combined application of this new method with a reporter gene mutation assay was performed to clarify renal carcinogenesis induced by madder color（MC）that includes LuP and alizarin（Alz）as constituent agents. A DNA adductome map derived from MC-treated rats was almost identical to that of LuP-treated rats, but not Alz-treated rats. Although 8-OHdG levels were elevated in MC- and Alz-treated rats, significant increases in *gpt* and Spi^- mutant frequencies were observed only in MC- and LuP-treated rats. In addition, the spectrum of *gpt* mutants in MC-treated rats showed almost the same pattern as those in LuP-treated rats. The overall data suggest that LuP may be responsible for MC-induced carcinogenicity and that the proposed methodology is appropriate for exploring and understanding mechanisms of chemical carcinogenesis.

Keywords: DNA adduct, *gpt* delta, madder color

Yamada T*, Wei M*, Toyoda T, Yamano S*, Wanibuchi H*: Inhibitory effect of *Raphanobrassica* on *Helicobacter pylori*-induced gastritis in Mongolian gerbils.

Food Chem Toxicol. 2014;70:107-13.

Helicobacter pylori（*H. pylori*）infection is well known to be associated with chronic gastritis and also development of gastric cancer. *Raphanobrassica*（RB）is an intergeneric hybrid of the genera *Raphanus*（radish）and *Brassica*（cabbages）containing appreciable amounts of glucoraphanin（GR）and glucoraphenin（GRe）, which are actively hydrolyzed by the enzyme myrosinase to sulforaphene and sulforaphene, respectively. Both of these metabolites exert antimicrobial and anti-inflammatory activity. The purpose of the present study was to investigate the effect of two freeze-dried products of RB（RB1 and RB2）on *H. pylori*-induced gastritis in Mongolian gerbils. Six-week-old male Mongolian gerbils were inoculated orally with *H. pylori*（ATCC 43504）, and two weeks later were fed diets containing no additives or diets supplemented with 2% RB1（containing both GR and GRe）or 2% RB2（containing GR only）for 10 weeks. In the RB1, but not the RB2 group, mononuclear cell infiltration, mRNA expression of IL-6, and cell proliferation in the gastric mucosa were significantly suppressed. These results indicate that RB1 containing both GR and GRe exerted significant inhibitory effects on *H. pylori*-induced gastritis in Mongolian gerbils apparently mediated via suppression of IL-6 expression and chronic inflammation.

Keywords: *Helicobacter pylori*, *Raphanobrassica*, chemoprevention

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Takahashi M, Yoshida M, Inoue K, Morikawa T, Nishikawa A, Ogawa K: Chronic toxicity and carcinogenicity of semicarbazide hydrochloride in Wistar Hannover GALAS rats.

Food Chem Toxicol. 2014;73:84-94.

We performed a combined study to determine the chronic toxicity and carcinogenicity of semicarbazide hydrochloride（SEM-HCl）. Male and female Wistar Hannover GALAS rats were fed a diet containing SEM-HCl at 0, 10, 50, and 250 ppm for 52 weeks（10 rats/sex/group）or for 104 weeks（50 rats/sex/group）. Enlargement of the knee joints was apparent in both sexes at 250 ppm. Reduced body weight was observed at 250 ppm from week 76 only in males. SEM-HCl exerted no toxic effects on hematology, serum biochemistry, or organ weights. Histopathologically, disarrangement of chondrocytes accompanied by increased connective tissues, and degeneration of articular cartilage were found in males at 50 ppm and above and in females at 250 ppm. Mild changes in the elastic laminae were observed at 250 ppm for both sexes in the chronic toxicity study. There were no significant intergroup differences in the incidences or types of any tumors. Taken together, toxicological effects of chronic exposure to SEM-HCI mainly occurred in the bone, cartilage, and aorta. Based on histopathological findings, the no-observed-adverse-effect-level was 10 ppm in males and 50 ppm in females（equal to 0.6 mg/kg/day in males and 3.9 mg/kg/day in females）. SEM-HCl was not carcinogenic in rats.

Keywords: semicarbazide hydrochloride, chronic toxicity, carcinogenicity

Toyoda T, Cho YM, Mizuta Y, Akagi J, Ogawa K: A 13-week subchronic toxicity study of ferric citrate in F344 rats.

Food Chem Toxicol. 2014;74:68-75.

Ferric citrate has been used as a food additive for supplementation of iron. We performed a 13-week subchronic toxicity study of ferric citrate in F344 rats with oral administration in the diet at concentrations of 0%, 0.25%, 1.0%, and 4.0%. Reduction of body weight gain was noted in 4.0% males and females. On hematology assessment, decreases of red blood cells and lymphocytes and increases of platelets and eosinophils were noted in 4.0% males and females. Serum biochemistry demonstrated increased iron and decreased total protein and transferrin in both sexes treated with 4.0% ferric citrate. In addition, an increase of serum inorganic phosphorus levels was noted in 4.0% females. Regarding organ weights, an increase of relative spleen weights was detected in 4.0% males and females and a decrease of absolute and relative heart weights in 4.0% females. On histopathological assessment, colitis with infiltration of eosinophils and hyperplasia of mucosal epithelium, eosinophilic infiltration in mesenteric lymph nodes, and increased hemosiderosis in spleen were observed as treatment-related toxicological changes in 4.0% males and females. Based on the results, the no-observed-adverse-effect level（NOAEL）of ferric citrate was estimated to be 1.0%（596 mg/kg bw/day

for males and 601 mg/kg bw/day for females).
Keywords: ferric citrate, subchronic toxicity, eosinophilic enteritis

Tamura K, Inoue K, Takahashi M, Matsuo S, Irie K, Kodama Y, Gamo T*, Ozawa S*, Yoshida M: Involvement of constitutive androstane receptor in liver hypertrophy and liver tumor development induced by triazole fungicides.
Food Chem Toxicol. 2015;78:86-95.

We clarified the involvement of constitutive androstane receptor (CAR) in triazole-induced liver hypertrophy and tumorigenesis using CAR-knockout (CARKO) mice. Seven-week-old male CARKO and wild-type (WT) mice were treated with 200 ppm cyproconazole (Cypro), 1500 ppm tebuconazole (Teb), or 200 ppm fluconazole (Flu) in the diet for 27 weeks after initiation by diethyl nitrosamine (DEN). At weeks 4 (without DEN) and 13 (with DEN), WT mice in all treatment groups and CARKO mice in Teb group revealed liver hypertrophy with mainly Cyp2b10 and following Cyp3a11 inductions in the liver. Teb also induced Cyp4a10 in both genotypes. Cypro induced slight and durationdependently liver hypertrophy in CARKO mice. At week 27, Cypro and Teb significantly increased eosinophilic altered foci and/or adenomas in WT mice. These proliferating lesions were clearly reduced in CARKO mice administered both compounds. The eosinophilic adenomas caused by Flu decreased in CARKO mice. The present study indicates that CAR is the main mediator of liver hypertrophy induced by Cypro and Flu, but not Teb. In contrast, CAR played a crucial role in liver tumor development induced by all three triazoles.
Keywords: triazole, constitutive androstane receptor, liver hypertrophy

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Nozawa K*, Nagaoka K*, Zhang H*, Usuda K*, Okazaki S*, Taya K*, Yoshida M, Watanabe G*: Neonatal exposure to 17α-ethynyl estradiol affects ovarian gene expression and disrupts reproductive cycles in female rats.
Reprod Toxicol. 2014;46:77-84.

Neonatal exposure to synthetic estrogen causes delayed reproductive dysfunction in female rats. Exposure to 17α-ethynyl estradiol (EE, low: 20 and high: 2000μg/kg) induced an abnormal estrous cycle during PND171-190 in low-dose and PND126-145 in high-dose group. At PND90 within normal estrous cycle, high-dose animals showed lack of LH surge and low of ovarian hormones in serum level. Gene expression analysis demonstrated that level of mRNA encoding luteinizing hormone/chorionic gonadotropin receptor (LHCGR) was higher in EE-treated ovaries than in control ovaries, and LHCGR protein colocalized with apoptosis-related proteins in the interstitial area of the ovary. At PND1, ovarian LHCGR mRNA levels were higher in EE-treated rats than in control rats, and direct induction of LHCGR expression by EE was observed in vitro. Our results indicate that neonatal exposure to EE induces irregular LHCGR expression in the immature ovary, which may influence the occurrence of delayed reproductive dysfunction in adult animals.
Keywords: 17α-ethynyl estradiol, endocrine disruptor, luteinizing hormone/chorionic gonadotropin receptor

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Ichimura R, Takahashi M, Morikawa T, Inoue K, Maeda J, Usuda K*1, Yokosuka M*2, Watanabe G*1, Yoshida M: Prior attenuation of KiSS1/GPR54 signaling in the anteroventral periventricular nucleus is a trigger for the delayed effect induced by neonatal exposure to 17alpha-ethynylestradiol in female rats.
Reprod Toxicol. 2015;51:145-56.

Neonatal exposure to 17alpha-ethynylestradiol (EE) cause delayed effect, a late-occurring irreversible damage to reproductive functions characterized by the early onset of age-matched abnormal estrous cycling. To clarify the involvement of a hypothalamic key cycling regulator KiSS1/GPR54 in the delayed effect, we investigated artificially-induced LH surges and KiSS1 mRNA expression in the anteroventral periventricular nucleus (AVPV) of cycling young adult rats neonatally exposed to EE, and compared these parameters to those in about 5 months old middle-aged rats. KiSS1 mRNA expression, the number of KiSS1-positive cells and KiSS1/ERα co-expressing cells in the AVPV decreased in both EE-exposed and middle-aged rats. The peak area and levels of LH surge dose-dependently decreased in EE-exposed rats, and reduction was more evident in middle-aged rats. These results indicate that the prior attenuation of KiSS1 and consequent depression

of LH surges plays a key role in the onset of abnormal estrous cycling in the delayed effect.

Keywords: delayed effect, neonatal exposure, kisspeptin

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Usuda K*, Nagaoka K*, Nozawa K*, Zhang H*, Taya K*, Yoshida M, Watanabe G*: Neonatal exposure to 17α-ethynyl estradiol affects kisspeptin expression and LH-surge level in female rats.

J Vet Med Sci. 2014;76:1105-10.

Contamination of estrogenic compounds disrupts endocrinological and neurological reproductive systems in animals. Neonatal exposure to 17α-ethinyl estradiol (EE) induced an abnormal estrous cycle at postnatal day (PND) 180, but not at PND90. We found that serum level of luteinizing hormone (LH) at the latter half of proestrus in EE-treated rats was lower than in the controls at PND90 when there was no significant difference on estrous cyclicity. Additionally, kiss1 mRNA levels in the anteroventral periventricular nucleus-preoptic area (AVPV/POA) were lower in EE-treated rats than in the controls. The expression of GnRH precursor (GNRH1) mRNA in the AVPV/POA and that of LH beta subunit (LHb) mRNA in the pituitary were similar in the control- and EE-treated groups. Our results indicated that neonatal exposure to EE leads to reduced expression of kiss1 mRNA in AVPV/POA and LH-surge, which is likely related to the delayed reproductive dysfunction seen in adult female rats.

Keywords: 17α-ethynyl estradiol, endocrine disruptor, kisspeptin

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Kawano M*1,2, Qin XY*1, Yoshida M, Fukuda T*3, Nansai H*1, Hayashi Y*4, Nakajima T*5, Sone H*1: Peroxisome proliferator-activated receptor α mediates di-(2-ethylhexyl) phthalate transgenerational repression of ovarian Esr1 expression in female mice.

Toxicol Lett. 2014;228:235-40.

Di-(2-ethylhexyl)-phthalate (DEHP) is a phthalate ester that binds peroxisome proliferator-activated receptor α (PPARα) to induce proliferation of peroxisomes and regulate the expression of specific target genes. The question of whether the effect of DEHP on female reproductive processes is mediated via PPARα-dependent signaling is controversial. In this study, we investigated the effect of exposure to DEHP on ovarian expression of estrogen receptor α (Esr1) and aromatase (Cyp19a1) in three generations of Sv/129 wild-type (WT,++) and PPARα (-/-) knockout mice. Compared with untreated controls, ovarian expression of Esr1 decreased in response to DEHP treatment in the F0 (0.56-fold, P=0.19), F1 (0.45-fold, P=0.023), and F2 (0.35-fold, P=0.014) generations of WT mice, but not PPARα-null mice. Our data indicate that transgenerational repression by DEHP of ovarian Esr1 gene expression is mediated by PPARα-dependent pathways. Further studies are required to elucidate the mechanisms underlying crosstalk between PPARα and Esr1 signaling in reproductive processes.

Keywords: DEHP, PPARα, transgenerational

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Matsushita K, Kuroda K, Ishii Y, Takasu S, Kijima A, Kawaguchi H*, Miyoshi N*, Nohmi T, Ogawa K, Nishikawa A, Umemura T: Improvement and validation of a medium-term *gpt* delta rat model for predicting chemical carcinogenicity and underlying mode of action.

Exp Toxicol Pathol. 2014;66:313-21.

We have developed a new medium-term animal model, "GPG", in which an *in vivo* mutation assay in partially hepatectomized tissue and a tumor-promoting assay were performed. The tumor-promoting assay measures glutathione S-transferase placental form positive foci induced by diethylnitrosamine (DEN) in the residual tissue. Given that a limitation of the original protocol is the potential interaction between the test chemical and DEN, the present study establishes a modified protocol that includes a test chemical washout period. Using CYP2E1 inhibitor and CYP1A or CYP2B inducers, a period of 2 weeks after cessation of exposure to the chemicals was confirmed to be sufficient to return their enzymatic activities to normal levels. Additionally, to avoid the effects of DEN on the pharmacokinetics of the test chemical, re-exposure to the test chemical started

1 week after DEN injection, in which tumor-promoting activities were clearly detected. Consequently, a modified protocol has been established with 2- and 1-week washout periods before and after DEN injection, respectively. The applicability of the modified protocol was demonstrated using the genotoxic hepatocarcinogen, estragole (ES), the genotoxic renal carcinogen, aristolochic acid (AA), and the non-genotoxic hepatocarcinogens, β-naphthoflavone and barbital. Furthermore, the increase of cell cycle-related parameters in ES-treated livers, but not in AA-treated livers, may indicate that the liver is not the carcinogenic target site of AA despite its genotoxic role. Thus, since various parameters related to carcinogenesis can be evaluated concurrently, the GPG model could be a rapid and reliable assay for the assessment of human cancer hazards.

Keywords: *in vivo* mutagenicity, medium-term animal model, *gpt* delta rat

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Matsushita K, Ishii Y, Takasu S, Kuroda K, Kijima A, Tsuchiya T, Kawaguchi H*, Miyoshi N*, Nohmi T, Ogawa K, Nishikawa A, Umemura T: A medium-term *gpt* delta rat model as an *in vivo* system for analysis of renal carcinogenesis and the underlying mode of action.

Exp Toxicol Pathol. 2015;67:31-9.

The kidney is a major target site of chemical carcinogenesis. However, a reliable *in vivo* assay for rapid identification of renal carcinogens has not been established. The purpose of this study was to develop a new medium-term *gpt* delta rat model (the GNP model) to facilitate identification of renal carcinogens. In this model, we carried out an *in vivo* mutation assay using unilaterally nephrectomized kidney tissue and a tumor-promoting assay using residual kidney tissue, with diethylnitrosamine (DEN) as the renal tumor initiator. To clarify the optimal time of DEN injection after nephrectomy, time-dependent changes in bromodeoxyuridine-labeling indices in the tubular epithelium of nephrectomized rats were examined. The optimal dose of DEN injection and sufficient duration of subsequent nitrilotriacetic acid treatment were determined for detection of renal preneoplastic lesions. The standard protocol for the GNP model was determined as follows. Six-week-old female *gpt* delta rats were treated with test chemicals for 4 weeks, followed by a 2-week washout period, and 40mg/kg DEN was administered intraperitoneally to initiate renal carcinogenesis. Unilateral nephrectomy was performed 48h before DEN injection, followed by *gpt* assays using excised kidney tissues. One week after DEN injection, rats were further exposed to test chemicals for 12 weeks, and histopathological analysis of renal preneoplastic lesions was performed as an indicator of tumor-promoting activity in residual kidney tissue. Validation studies using aristolochic acid, potassium dibasic phosphate, phenylbutazone, and d-limonene indicated the reliability of the GNP model for predicting renal carcinogens and the underlying mode of action.

Keywords: medium-term animal model, *gpt* delta rat, *in vivo* mutagenicity

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Takasu S, Ishii Y, Matsushita K, Kuroda K, Kijima A, Kodama Y, Ogawa K, Umemura T: No effect of high fat diet-induced obesity on spontaneous reporter gene mutations in *gpt* delta mice.

Asian Pac J Cancer Prev. 2014;15:7149-52.

A large number of epidemiological studies have demonstrated that obesity is a risk factor for several human cancers. Several animal studies using rodents with diet-induced or genetic obesity have also demonstrated that obesity can promote tumor development. However, the effects of obesity on the early stages of carcinogenesis, and especially on the spontaneous occurrence of somatic gene mutations, remain unclear. To investigate the effects of obesity on the rate of spontaneous gene mutations, we performed reporter gene mutation assays in liver, kidney, and colon, organs in which obesity appears to be associated with cancer development on the basis of epidemiological or animal studies, in mice with high fat diet (HFD)-induced obesity. Six-week-old male and female C57BL/6 *gpt* delta mice were fed HFD or standard diet (STD) for 13 or 26 weeks. At the end of the experiments, reporter gene mutation assays of liver, kidney, and colon were performed. Final body weights and serum leptin levels of male and female mice fed HFD for 13 or 26 weeks were significantly increased compared with corresponding STD-fed groups. Reporter gene mutation assays of liver, kidney, and colon revealed that there were no significant differences in *gpt* or Spi$^-$ mutant frequencies between

STD- and HFD-fed mice in either the 13-week or 26-week groups. These results indicate that HFD treatment and consequent obesity does not appear to influence the spontaneous occurrence of somatic gene mutations.
Keywords: obesity, *in vivo* mutagenicity, *gpt* delta mouse

Saelee P*, Chaiwerawattana A*, Ogawa K, Cho YM, Tiwawech D*, Suktangman V*: Clinicopathological significance of BRCA1 promoter hypermethylation in Thai breast cancer patients.
Asian Pac J Cancer Prev. 2014;15:10585-9.

Breast cancer susceptibility gene 1 (BRCA1), mapped on chromosome 17q21, is implicated in the mechanisms of cellular DNA repair. Inactivation of this gene is involved in the development of many human cancers, including breast cancer. This study aimed to investigate the prognostic value of BRCA1 promoter hypermethylation and expression in breast cancer cases. Sixty-one breast cancers were examined for BRCA1 hypermethylation by methylation-specific polymerase chain reaction (PCR), and 45 paired normal breast tissues were analyzed for altered BRCA1 mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). Aberrant methylation status in BRCA1 was detected in 15 of 61 cases (24.6%), while reduced expression was found in 7 of 45 (15.6%). BRCA1 hypermethylation was statistically associated with tumor grade III (p=0.04), a high frequency of stage IIB (p=0.02), and triple-negative phenotype (OR= 3.64, 95%CI =1.1-12.3, p=0.03). Our findings indicated that BRCA1 promoter hypermethylation is a useful prognostic marker for breast cancer.
Keywords: breast cancer, BRCA1, DNA methylation

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Naiki-Ito A*, Chewonarin T*, Tang M*, Pitchakarn P*, Kuno T*, Ogawa K, Asamoto M*, Shirai T*, Takahashi S*: Ellagic acid, a component of pomegranate fruit juice, suppresses androgen-dependent prostate carcinogenesis via induction of apoptosis.
Prostate. 2015;75:151-60.

Ellagic acid (EA), a component of pomegranate fruit juice (PFJ), is a plant-derived polyphenol and has antioxidant properties. PFJ and EA have been reported to suppress various cancers, including prostate cancer. However, their chemopreventive effects on development and progression of prostate cancer using in vivo models have not been established yet. The transgenic rat for adenocarcinoma of prostate (TRAP) model was used to investigate the modulating effects of PFJ and EA on prostate carcinogenesis. Three-week-old male transgenic rats were treated with EA or PFJ for 10 weeks. In vitro assays for cell growth, apoptosis, and Western blot were performed using the human prostate cancer cell lines, LNCaP (androgen-dependent), PC-3 and DU145 (androgen-independent). PFJ decreased the incidence of adenocarcinoma in lateral prostate, and both EA and PFJ suppressed the progression of prostate carcinogenesis and induced apoptosis by caspase 3 activation in the TRAP model. In addition, the level of lipid peroxidation in ventral prostate was significantly decreased by EA treatment. EA was able to inhibit cell proliferation of LNCaP, whereas this effect was not observed in PC-3 and DU145. As with the in vivo data, EA induced apoptosis in LNCaP by increasing Bax/Bcl-2 ratio and caspase 3 activation. Cell-cycle related proteins, p21WAF , p27Kip, cdk2, and cyclin E, were increased while cyclin D1 and cdk1 were decreased by EA treatment. The results indicate that PFJ and EA are potential chemopreventive agents for prostate cancer, and EA may be the active component of PFJ that exerts these anti-cancer effects.
Keywords: ellagic acid, prostate cancer, apoptosis

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Tokudome S*[1], Kuriki K*[1], Yokoyama Y*[1], Sasaki M*[1], Joh T*[1], Kamiya T*[1], Cheng J*[1], Ogawa K, Shirai T*[1], Imaeda N*[1], Goto C*[1], Tokudome Y*[1], Ichikawa H*[1], Okuyama H*[2]: Dietary n-3/long-chain n-3 polyunsaturated fatty acids for prevention of sporadic colorectal tumors: A randomized controlled trial in polypectomized participants.
Prostaglandins Leukot Essent Fatty Acids. 2015;94:1-11.

To address preventive effects of n-3 PUFAs/LC n-3 PUFAs on CRTs, a randomized controlled trial was conducted. One-hundred four experimental group participants were advised to increase intake of n-3 PUFAs, including fish/shell fish, fish oil supplements and perilla oils, and to decrease consumption of n-6 PUFAs and fats/oils as a whole for 24 months. One-hundred one control group participants were only cautioned to reduce consumption of fats/oils as a whole. Random

allocation was satisfactorily attained, and participants sufficiently complied with our regimen. Intakes, plasma concentrations, and compositions of the RBC and sigmoid colon membranes of n-3 PUFAs, LC n-3 PUFAs, EPA and DHA increased, and the ratios of n-6 PUFAs/n-3 PUFAs and AA/LC n-3 PUFAs decreased without any adverse response. Twenty-four months after the intervention, the multivariate-adjusted hazard ratio (95% confidence intervals) was estimated to be 0.805 (0.536-1.209) with a signal towards the reduced CRT incidence.

Keywords: colorectal tumor, randomized controlled trial, n-3 polyunsaturated fatty acids

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Maeda J*, Kijima A, Inoue K, Ishii Y, Ichimura R, Takasu S, Kuroda K, Matsushita K, Kodama Y, Saito N*, Umemura T, Yoshida M: *In vivo* genotoxicity of *Ginkgo biloba* extract in *gpt* delta mice and constitutive androstane receptor knockout mice.
Toxicol Sci. 2014;140:298-306.

The National Toxicology Program (NTP) study of Ginkgo biloba extract (GBE), an herbal supplement, reported concerns regarding genotoxicity and clear evidence of hepatocarcinogenicity and liver hypertrophy in mice. To clarify the genotoxicity of GBE in vivo, we performed reporter gene mutation assay using gpt delta mice. We also used a combined liver comet assay and bone marrow micronucleus assay using C3H-derived constitutive androstane receptor knockout (CARKO) and wild-type mice. No remarkable increases in gpt or spi$^-$ mutation frequencies were observed in DNA extracted from the livers of gpt delta mice that had been exposed to GBE up to 2000 mg/kg bw/day. In the comet and micronucleus assays, no statistically significant increases in positive cells were observed at doses up to 2000 mg/kg bw/day of GBE in either mouse genotype. The present study provides clear evidence that GBE is not genotoxic in vivo. Our results indicate that GBE-induced hepatocarcinogenesis in mice occurs through a nongenotoxic mode of action.

Keywords: constitutive androstane receptor, genotoxicity, ginkgo biloba extract

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Kuroda K, Hibi D, Ishii Y, Yokoo Y, Takasu S, Kijima A, Matsushita K, Masumura K, Kodama Y, Yanai T*, Sakai H*, Nohmi T, Ogawa K, Umemura T: Role of *p53* in the progression from ochratoxin A-induced DNA damage to gene mutations in the kidneys of mice.
Toxicol Sci. 2015;144:65-76.

Carcinogenic doses of ochratoxin A (OTA) cause increases of mutant frequencies (MFs) of the *red/gam* gene (Spi$^-$) in the kidneys of *p53*-deficient *gpt* delta mice, but not in *p53*-proficient mice. Here, we investigated the role of *p53* in the progression from OTA-induced DNA damage to gene mutations. To this end, *p53*-proficient and -deficient mice were administered 5 mg/kg OTA for 3 days or 4 weeks by gavage. After 3 days of administration, comet assays were performed and there were no differences in the degrees of OTA induced DNA damage between *p53*-proficient and -deficient mice. However, the frequencies of γ-H2AX-positive tubular epithelial cells in *p53*-deficient mice were significantly higher than those in *p53*-proficient mice, implying that *p53* inhibited the progression from DNA damage to DNA double strand breaks (DSBs). Evaluation of global gene expression and relevant mRNA/protein expression levels demonstrated that OTA increased the expression of *Cdkn1a*, which encodes the *p21* protein, in *p53*-proficient mice, but not in *p53*-deficient mice. Moreover, in *p53*-deficient mice, mRNA levels of cell cycle progression and DSB repair (homologous recombination repair [HR])-related genes were significantly increased. Thus, G_1/S arrest due to activation of the *p53/p21* pathway may contribute to the prevention of DSBs in *p53*-proficient mice. In addition, single base deletions/insertions/substitutions were predominant, possibly due to HR. Overall, these results suggested that OTA induced DSBs at the carcinogenic target site in mice and that *p53/p21*-mediated cell cycle control prevented an increase in the formation of DSBs, leading to gene mutations.

Keywords: ochratoxin A, p53, DNA damage

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Inoue K, Morikawa T, Matsuo S, Tamura K, Takahashi M, Yoshida M: Adaptive parotid gland hypertrophy induced by dietary treatment of GSE in rats.
Toxicol Pathol. 2014;42:1016-23.

In a 13-week feeding toxicity study of grape skin

extract（GSE）performed previously, 5.0% GSE showed diffuse hypertrophy and basophilia in rat parotid glands. To clarify whether the change in the parotid glands was an adverse effect of GSE, 6-week-old male F344 rats were fed a diet containing 5.0% GSE or were administered a dose corresponding to the dietary concentration via gavage for 4 weeks, and the treatment was stopped for 2 weeks. To ascertain the effect of astringency, other animals were fed a diet containing 5.0% tannic acid（TA）using the same protocol as the GSE feed group. Control groups were fed a basal diet or were administered sterilized distilled water by gavage. In the GSE and TA feed groups, diffuse severe hypertrophy and basophilia in the parotid glandular epithelial cells were observed. Macroscopic, microscopic, and ultrastructural characteristics consistent with cellular hypertrophy was less apparent after the recovery period in both feed groups. In contrast, no changes were observed in the parotid glands of the gavage GSE and control groups at week 4. Based on these findings of parotid hypertrophy without cytotoxicity, the data from this and previous studies suggest that hypertrophy of the parotid glands induced by feeding treatment with GSE is an adaptive non-adverse effect that is reversible upon removal of the sialotrophic agent.

Keywords: grape skin extract, adaptive, rats

Matsuo S, Takahashi M, Inoue K, Tamura K, Irie K, Kodama Y, Nishikawa A, Yoshida M: Inhibitory potential of postnatal treatment with cyclopamine, a hedgehog signaling inhibitor, on medulloblastoma development in Ptch1 heterozygous mice.

Toxicol Pathol. 2014;42:1174-87.

Medulloblastomas（MBs）are thought to be derived from granular cell precursors in the external granular layer（EGL）of the developing cerebellum. Heterozygous patched1（Ptch1）knockout mice develop MBs that resemble those in humans when the sonic hedgehog（Shh）signaling pathway is activated. The present study was conducted to evaluate postnatal effects of a Shh signaling inhibitor, cyclopamine, on the development of MBs in Ptch1 mice. Ptch1 and wild-type mice were treated daily with subcutaneous cyclopamine at 40 mg/kg or vehicle from postnatal day（PND）1 to PND14, and the subsequent development of MBs and preneoplastic lesions was examined up to week 12（W12）. Proliferative lesions in the cerebellum, MBs, and preneoplastic lesions were only detected in Ptch1 mice. Cyclopamine treatment resulted in a statistically significant reduction in the incidence and/or area of proliferative lesions at PND14 and 21. The trend of decreasing preneoplastic lesions persisted up to W12. At PND7, cyclopamine treatment reduced the width and proliferation of the EGL regardless of genotype. These results indicate that inhibition of Shh signaling during cerebellar development has prolonged inhibitory potential on MB development in Ptch1 mice. This inhibitory potential might be related to inhibition of EGL proliferation, including preneoplastic MB cells.

Keywords: cyclopamine, medulloblastoma, cerebellum

Inoue K, Takahashi M, Kodama Y, Nishikawa A, Sugita-Konishi Y*, Yoshida M: The kidneys of infant mice are not sensitive to the food mycotoxin contaminant nivalenol.

J Toxicol Pathol. 2014;27:57-66.

Nivalenol（NIV）is a trichothecene mycotoxin produced by *Fusarium* fungi that frequently contaminates agricultural commodities. Dietary administration of NIV to adult mice affects the renal glomeruli, but data about NIV toxicity in human infants are limited. To evaluate the effects of NIV on infant kidneys, 3-week-old male ICR-derived glomerulonephritis（ICGN）and ICR mice were administered 0, 4, 8 or 16 ppm NIV in diet for 4 weeks, and their renal status was compared with age-matched or adult ICR mice. In ICGN mice, the number of glomeruli showing mesangial expansion and α-smooth muscle actin（SMA）-positive mesangial cells was higher with 16 ppm NIV compared with controls. No other significant differences were observed in ICGN mice. In infant ICR mice, the IgA serum concentrations were significantly elevated without glomerular morphological changes in the 16 ppm NIV group. There was no difference in NIV sensitivity in the kidneys of infant ICGN and ICR mice. These data suggest that the kidneys in infant mice are not sensitive to nivalenol under the present conditions.

Keywords: nivalenol, renal glomeruli, infants

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Takahashi M, Inoue K, Morikawa T, Matsuo S, Hayashi S, Tamura K, Watanabe G*, Taya K*, Yoshida M: Early

indicators of delayed adverse effects in female reproductive organs in rats receiving neonatal exposure to 17alpha-ethynylestradiol.

J Toxicol Sci. 2014;39:775-84.

We previously reported that neonatal exposure to 17α-ethynylestradiol (EE) led to delayed adverse effects in which age-related anovulation after sexual maturation was accelerated. To identify early indicators of these adverse effects, female Wistar Hannover GALAS rats received a single EE injection (0, 0.02, 0.2, 2, 20, or 200 μg/kg) within 24 hr of birth. Histopathological changes in ovarian and uterine development were investigated from postnatal day (PND) 14 to 10 weeks of age. Immunohistochemical expression of estrogen receptor alpha (ERα) in the uterus, serum levels of sex-related hormones and gene expression in the hypothalamus were examined. Although neonatal exposure to EE did not affect body growth or ovarian development, serum FSH tended to decrease at doses ≥ 2 μg/kg, and Kiss1 mRNA level in the whole hypothalamus was significantly decreased in all EE-treated groups at PND14.The number of uterine glands at PND21 was suppressed at doses ≥ 20 μg/kg, and ERα expression in the uterine epithelium at estrus stage decreased in a dose-dependent manner at 10 weeks of age. These results demonstrated that the various identified changes that occurred before the appearance of delayed adverse effects could be candidate early indicators.

Keywords: 17α-ethynylestradiol, neonatal exposure, delayed effects

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Ihara K[*1], Asanuma K[*2], Fukuda T[*1], Ohwada S[*1], Yoshida M, Nishimori K[*1]: MAGI-2 is critical for the formation and maintenance of the glomerular filtration barrier in mouse kidney.

Am J Pathol. 2014;184:2699-708.

Membrane-associated guanylate kinase inverted 2 (MAGI-2) is a tight junction protein in epithelial tissues. We previously reported the detailed expression patterns of MAGI-2 in mouse tissues, including kidney podocytes, based on results obtained from Venus knock-in mice for Magi2 locus. In the present study, homozygous deletion of the Magi2 gene in mice caused neonatal lethality, which was explained by podocyte morphological abnormalities and anuria. Immunohistological analysis showed that loss of MAGI-2 function induced a significant decrease in nephrin and dendrin at the slit diaphragm of the kidney, although other components of the slit diaphragm were unchanged. Furthermore, nuclear translocation of dendrin was observed in the podocytes of the MAGI-2-null mutants, along with enhanced expression of cathepsin L, which is reported to be critical for rearrangement of the actin cytoskeleton in podocytes. Expression analysis of the null mutants showed that loss of MAGI-2 function induces abnormal expression of various types of adhesion-related molecules. The present study is the first to demonstrate that MAGI-2 has a critical role in maintaining the functional structure of the slit diaphragm and that this molecule has an essential role in the functioning of the kidney filtration barrier.

Keywords: MAGI-2, kidney, glomerular filtration barrier

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Wakasugi M[*1], Sasaki T[*1], Matsumoto M[*1], Nagaoka M[*1], Inoue K[*1], Inobe M[*1], Horibata K, Tanaka K[*2], Matsunaga T[*1]: Nucleotide excision Repair-dependent DNA double-strand break formation and ATM signaling activation in mammalian quiescent cells.

J Biol Chem. 2014;289:28730-7.

Histone H2A variant H2AX is phosphorylated at Ser(139) in response to DNA double-strand break (DSB) and single-stranded DNA (ssDNA) formation. UV light dominantly induces pyrimidine photodimers, which are removed from the mammalian genome by nucleotide excision repair (NER). We previously reported that in quiescent G0 phase cells, UV induces ATR-mediated H2AX phosphorylation plausibly caused by persistent ssDNA gap intermediates during NER. In this study, we have found that DSB is also generated following UV irradiation in an NER-dependent manner and contributes to an earlier fraction of UV-induced H2AX phosphorylation. The NER-dependent DSB formation activates ATM kinase and triggers the accumulation of its downstream factors, MRE11, NBS1, and MDC1, at UV-damaged sites. Importantly, ATM-deficient cells exhibited enhanced UV sensitivity under quiescent conditions compared with asynchronously growing conditions. Finally, we show that the NER-dependent H2AX phosphorylation is also observed in murine

peripheral T lymphocytes, typical nonproliferating quiescent cells *in vivo*. These results suggest that *in vivo* quiescent cells may suffer from NER-mediated secondary DNA damage including ssDNA and DSB.
Keywords: Histone, DNA damage, DNA double-strand break

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Kawamura Y*1, Hayashi H*1, Masumura K, Numazawa S*2, Nohmi T: Genotoxicity of phenacetin in the kidney and liver of Sprague-Dawley *gpt* delta transgenic rats in 26-week and 52-week repeated-dose studies.
Toxicology 2014;324:10-7.

Transgenic rat mutation assays can be used to assess genotoxic properties of chemicals in target organs for carcinogenicity. Mutations in transgenes are genetically neutral and accumulate during a treatment period; thus, assays are suitable for assessing the genotoxic risk of chemicals using a repeated-dose treatment paradigm. However, only a limited number of such studies have been conducted. To examine the utility of transgenic rat assays in repeated-dose studies, we fed male and female Sprague-Dawley *gpt* delta rats with a 0.5% phenacetin-containing diet for 26 and 52 weeks. A long-term feeding of phenacetin is known to induce renal cancer in rats. Phenacetin administration for 52 weeks in males significantly increased *gpt* (point mutations) mutant frequency (MF) in the kidney, the target organ of carcinogenesis. In the liver, the nontarget organ of carcinogenesis, *gpt* MFs were significantly elevated in phenacetin treatment groups of both genders during 26- and 52-week treatments. Furthermore, sensitive to P2 interference (Spi(-) deletions) MF increased in the liver of both genders following 52-week treatment. MFs were higher after treatment for 52 weeks than after treatment for 26 weeks. Frequencies of phenacetin-induced mutations were higher in the liver than in the kidney, suggesting that the intensity of genotoxicity does not necessarily correlate with the induction of tumor formation. Results from *gpt* delta rat assays of repeated-dose treatments are extremely useful to elucidate the relationship between gene mutations and carcinogenesis in the target organ induced by cancer-causing agents.
Keywords: phenacetin, *gpt* delta rat, mutant frequency

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Takeiri A*1, Wada NA*1, Motoyama S*1, Matsuzaki K*1, Tateishi H*2, Matsumoto K*2, Niimi N, Sassa A, Grúz P, Masumura K, Yamada M, Mishima M*1, Jishage KI*1, Nohmi T: *In vivo* evidence that DNA polymerase kappa is responsible for error-free bypass across DNA cross-links induced by mitomycin C.
DNA Repair 2014;24:113-21.

Translesion DNA synthesis (TLS) is an important pathway that avoids genotoxicity induced by endogenous and exogenous agents. DNA polymerase kappa (Polk) is a specialized DNA polymerase involved in TLS but its protective roles against DNA damage *in vivo* are still unclear. To better understand these roles, we have established knock-in mice that express catalytically-inactive Polk and crossbred them with *gpt* delta mice, which possess reporter genes for mutations. The resulting mice (inactivated Polk KI mice) were exposed to mitomycin C (MMC), and the frequency of point mutations, micronucleus formation in peripheral erythrocytes, and γH2AX induction in the bone marrow was determined. The results suggest that Polk mediates TLS, which suppresses point mutations and DNA double-strand breaks caused by intra- and interstrand cross-links induced by MMC treatment. The established knock-in mice are extremely useful to elucidate the *in vivo* roles of the catalytic activity of Polk in suppressing DNA damage that was induced by a variety of genotoxic stresses.
Keywords: inactivated Polk knock-in mice, mitomycin C, interstrand cross-links

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Horibata K, Ukai A, Honma M: Evaluation of rats' *in vivo* genotoxicity induced by *N*-ethyl-*N*-nitrosourea in the RBC *Pig-a*, PIGRET, and *gpt* assays.
Genes and Environ. 2014;36:199-202.

The emerging *Pig-a* gene mutation assay, a powerful and promising tool for evaluating *in vivo* genotoxicity, is based on flow cytometric enumeration of red blood cells

(RBCs), which are deficient in glycosylphosphatidylinositol anchored protein. Various approaches for measuring *Pig-a* mutant cells have been developed, particularly those focused on peripheral RBCs and reticulocytes (RETs). Previously, it had been reported that *Pig-a* and *gpt* mutant frequencies were relatively increased in *N*-ethyl-*N*-nitrosourea (ENU)- and benzo[*a*]pyrene (BP)-treated mice. The capacity and characteristics of the *Pig-a* assay relative to transgenic rodent (TGR) mutation assays, however, are unclear in rats. Here, using transgenic *gpt* delta rats, we compared the *in vivo* genotoxicity of single oral doses of ENU (40 mg/kg) in the *gpt* gene mutation assay in bone marrow and liver, and *Pig-a* gene mutation assays on RBCs and RETs in the same animals. The *Pig-a* gene mutation assays were conducted at 1, 2, and 4 weeks after treatment, whereas *gpt* assays were conducted on tissues collected at the 4-week terminal sacrifice. Consequently, we detected that *Pig-a* and *gpt* mutant frequencies were clearly increased in ENU-treated rats, indicating that both the *Pig-a* and TGR gene mutation assays can detect *in vivo* ENU genotoxicity equally.

Keywords: genotoxicity, *Pig-a* gene mutation assay, transgenic rodent mutation assays

Sugiyama K, Takamune M, Furusawa H, Honma M: Human DNA methyltransferase gene-transformed yeasts display an inducible flocculation inhibited by 5-aza-2'-deoxycytidine.
Biochem Biophys Res Commun. 2015;456:689-94.

Mammalian DNA methyltransferases (DNMTs) play an important role in establishing and maintaining the proper regulation of epigenetic information. However, it remains unclear whether mammalian DNMTs can be functionally expressed in yeasts, which probably lack endogenous DNMTs. We cotransformed the budding yeast *Saccharomyces cerevisiae* with the human *DNMT1* gene, which encodes a methylation maintenance enzyme, and the *DNMT3A/3B* genes, which encode *de novo* methylation enzymes, in an expression vector also containing the *GAL1* promoter, which is induced by galactose, and examined the effects of the DNMT inhibitor 5-aza-2'-deoxycytidine (5AZ) on cell growth. Transformed yeast strains grown in galactose- and glucose-containing media showed growth inhibition, and their growth rate was unaffected by 5AZ. Conversely, 5AZ, but not 2'-deoxycytidine, dose-dependently interfered with the flocculation exhibited by *DNMT*-gene transformants grown in glucose-containing medium. Further investigation of the properties of this flocculation indicated that it may be dependent on the expression of a Flocculin-encoding gene, *FLO1*. Taken together, these findings suggest that DNMT-gene transformed yeast strains functionally express these enzymes and represent a useful tool for *in vivo* screening for DNMT inhibitors.

Keywords: DNA methyltransferase inhibitor, yeast, flocculation

Matsumoto M, Masumori S[*1], Hirata-Koizumi M, Ono A, Honma M, Yokoyama K[*2], Hirose A: Evaluation of in vivo mutagenicity of hydroquinone in Muta™ mice.
Mutat Res Genet Toxicol Environ Mutagen. 2014;775-6: 94-8.

Hydroquinone (HQ) is used in skin bleaching agents, hair dyes, and finger nail treatments. Many skin-lightening cosmetics that contain HQ are currently marketed in Japan. Concerns have been expressed regarding health risks to the general population because the carcinogenicity of HQ was previously suggested in animal studies. HQ induced hepatocellular adenomas and forestomach hyperplasias in mice and renal tubular cell adenomas in male rats. In the present study, the lacZ transgenic mutation assay was conducted according to OECD test guideline 488 to determine whether mutagenic mechanisms were involved in HQ-induced carcinogenesis. Male Muta™ mice were repeatedly administered HQ orally at dosages of 0, 25, 50, 100, or 200mg/kg bw/day for 28 days. Body weight gain was decreased in all treatment groups. No significant differences were observed in mutant frequencies in the liver, stomach, lung, or kidney between HQ-treated mice and the concurrent negative controls, whereas the significant induction of mutations was noted in the positive control, N-ethyl-N-nitrosourea. These results suggest that a mutagenic mechanism is not responsible for HQ-induced carcinogenesis.

Keywords: Hydroquinone, Transgenic mutation assay, Mouse

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Igarashi Y*[1], Nakatsu N*[1], Yamashita T*[1,2], Ono A, Ohno Y, Urushidani T*[1,3], Yamada H*[1]: Open TG-GATEs: A large-scale toxicogenomics database.
Nucleic Acids Res. 2015;43:D921-7.

Toxicogenomics focuses on assessing the safety of compounds using gene expression profiles. Gene expression signatures from large toxicogenomics databases are expected to perform better than small databases in identifying biomarkers for the prediction and evaluation of drug safety based on a compound's toxicological mechanisms in animal target organs. Over the past 10 years, the Japanese Toxicogenomics Project consortium（TGP）has been developing a large-scale toxicogenomics database consisting of data from 170 compounds（mostly drugs）with the aim of improving and enhancing drug safety assessment. Most of the data generated by the project（e.g. gene expression, pathology, lot number）are freely available to the public via Open TG-GATEs（Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System）. Here, we provide a comprehensive overview of the database, including both gene expression data and metadata, with a description of experimental conditions and procedures used to generate the database. Open TG-GATEs is available from http://toxico.nibio.go.jp/english/index.html.
Keywords: database, toxicogenomics, toxicity

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Kobayashi K, Pillai K S*, Michael M*, Cherian K M*, Ono A: Transition of Japan's statistical tools by decision tree for quantitative data obtained from the general repeated dose administration toxicity studies in rodents.
International Journal of Basic and Applied Sciences. 2014;3:507-20.

Statistical significance is one of important criteria on judgment of regulatory toxicological testing. The decision tree for analysing quantitative data obtained from repeated dose administration studies in rodents has been in use in Japan around 1981. Since then, several authors proposed improved versions of the decision tree incorporating all possible situations of statistical analysis normally encountered in such studies. Recently, a decision tree, which traces a simple route, unlike the previously proposed ones which trace complex routes has been proposed by a few researchers in Japan. While tracing to the most appropriate statistical tool using a decision tree, we propose to consider following points which also play a significant role in selecting the most appropriate statistical tool: (1) statistical tools that fails to detect a significant difference in the low dose group, (2) use of the one-sided test with high power to detect a significant difference compared with two-sided, (3) as far as possible avoid carrying out statistical analysis on the transformed data, since the analytical result of such data is difficult to interpret, (4) it is important to mention what statistical tools of the decision tree are used for the analysis, (5) examine the data for both normality and homogeneity and (6) for testing homogeneity, use Levene's test. Selection of widely accepted statistical tools is usually preferred to less popular and complex statistical analysis. It has been observed that in recent years the preferred statistical tools for analyzing quantitative data obtained from toxicity studied are of simple in nature but with high power to detect a significant difference.
Keywords: Decision Tree, Repeated Dose Administration Study, Statistical Method.

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Omura K*[1-3], Uehara T*[3,4], Morikawa Y*[3,4], Hayashi H*[5,6], Mitsumori K*[5], Minami K*[3,7], Kanki M*[1-3], Yamada H*[3], Ono A, Urushidani T*[3,8]: Detection of initiating potential of non-genotoxic carcinogens in a two-stage hepatocarcinogenesis study in rats.
J Toxicol Sci. 2014;39:785-94.

We previously reported a toxicogenomics-based prediction model for hepatocarcinogens in which the expression patterns of signature genes following repeated doses of either genotoxic or non genotoxic compounds were similar. Based on the results of our prediction model, we hypothesized that repeated doses of non-genotoxic carcinogens might have initiating potential. Here, we conducted a two stage hepatocarcinogenesis study in rats exposed to the initiating agent nitrosodiethylamine（DEN）, and hepatotoxic compounds thioacetamide（TAA）, methapyrilene（MP）and acetaminophen（APAP）for 1-2weeks followed by the

liver tumor promoter phenobarbital (PB). The duration of initial treatment was determined based on positive results from our prediction model. Combined treatment of 3 or 30 mg/kg of genotoxic DEN and PB induced marked increases in altered hepatocellular foci and a DEN dose-dependent increase in the number and area of glutathione S-transferase-placental form (GST-P)-positive foci. A low number of altered hepatocellular foci were also observed in rats treated with TAA at a dose of 45 mg/kg.MP at a dose of 100 mg/kg induced a very low number of foci, but APAP did not. Hierarchical clustering analysis using gene expression data revealed that 2-week treatment with TAA at a dose of 30 mg/kg and MP at 45 mg/kg induced specific expression of DNA damage-related genes, similar to 1-week treatment with DEN at a dose of 30 mg/kg. These results suggest that TAA and MP induce DNA damage, which partially supports our hypothesis. Although this study does not indicate whether tumor growth in response to these compounds can be assessed in this model, our results suggest that cumulative treatment with non genotoxic TAA might have initiating potential in the liver.

Keywords: Altered hepatocellular foci, GST-P carcinogenesis, Liver

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J Toxicol Sci. 2014;39:837-48.

Recent studies have shown that epigenetic alterations correlate with carcinogenesis in various tissues. Identification of these alterations might help characterize the early stages of carcinogenesis. We comprehensively analyzed DNA methylation and gene expression in livers obtained from rats exposed to nitrosodiethylamine (DEN) followed by a promoter of hepatic carcinogenesis, phenobarbital (PB). The combination of DEN and PB induced marked increases in number and area of glutathione S-transferase-placental form (GST-P)-positive foci in the liver. In the liver of rats that received 30 mg/kg of DEN, pathway analysis revealed alterations of common genes in terms of gene expression and DNA methylation, and that these alterations were related to immune responses. Hierarchical clustering analysis of the expression of common genes from public data obtained through the Toxicogenomics Project-Genomics Assisted Toxicity Evaluation system (TG-GATEs) showed that carcinogenic compounds clustered together. MBD-seq and GeneChip analysis indicated that major histocompatibility complex class Ib gene RT1-CE5, which has an important role in antigen presentation, was hypomethylated around the promoter region and specifically induced in the livers of DEN-treated rats. Further, immunohistochemical analysis indicated that the co-localization of GST-P and protein homologous to RT1-CE5 was present at the foci of some regions. These results suggest that common genes were altered in terms of both DNA methylation and expression in livers, with preneoplastic foci indicating carcinogenic potential, and that immune responses are involved in early carcinogenesis. In conclusion, the present study identified a specific profile of DNA methylation and gene expression in livers with preneoplastic foci. Early epigenetic perturbations of immune responses might correlate with the early stages of hepatocarcinogenesis.

Keywords: Altered hepatocellular foci, GST-P, DNA methylation

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Hanafusa H*1, Morikawa Y*1,2, Uehara T*1,2, Kaneto M*1, Ono A, Yamada H*2, Ohno Y, Urushidani T*2,3: Comparative gene and protein expression analyses of a panel of cytokines in acute and chronic drug-

induced liver injury in rats.

Toxicology 2014;324:43-54.

Drug-induced liver injury (DILI) is a significant safety issue associated with medication use, and is the major cause of failures in drug development and withdrawal in post marketing. Cytokines are signaling molecules produced and secreted by immune cells and play crucial roles in the progression of DILI. Although there are numerous reports of cytokine changes in several DILI models, a comprehensive analysis of cytokine expression changes in rat liver injury induced by various compounds has, to the best of our knowledge, not been performed. In the past several years, we have built a public, free, large-scale toxicogenomics database, called Open TG-GATEs, containing microarray data and toxicity data of the liver of rats treated with various hepatotoxic compounds. In this study, we measured the protein expression levels of a panel of 24 cytokines in frozen liver of rats treated with a total of 20 compounds, obtained in the original study that formed the basis of the Open TG-GATEs database and analyzed protein expression profiles combined with mRNA expression profiles to investigate the correlation between mRNA and protein expression levels. As a result, we demonstrated significant correlations between mRNA and protein expression changes for interleukin (IL)-1β, IL-1α, monocyte chemo-attractant protein (MCP)-1/CC-chemokine ligand (Ccl)2, vascular endothelial growth factor A (VEGF-A), and regulated upon activation normal T cell expressed and secreted (RANTES)/Ccl5 in several different types of DILI. We also demonstrated that IL-1β protein and MCP-1/Ccl2 mRNA were commonly up-regulated in the liver of rats treated with different classes of hepatotoxicants and exhibited the highest accuracy in the detection of hepatotoxicity. The results also demonstrate that hepatic mRNA changes do not always correlate with protein changes of cytokines in the liver. This is the first study to provide a comprehensive analysis of mRNA-protein correlations of factors involved in various types of DILI, as well as additional insights into the importance of understanding complex cytokine expression changes in assessing DILI.

Keywords: Biomarkers, Hepatotoxicity, Toxicogenomics

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Yamada T[*1], Tanaka Y[*1], Hasegawa R[*1], Sakuratani Y[*1], Yamazoe Y[*2], Ono A, Hirose A, Hayashi M[*3]: Development of a category approach to predict the testicular toxicity of chemical substances structurally related to ethylene glycol methyl ether.

Regul Toxicol Pharmacol. 2014;70:711-9.

We propose a category approach to assessing the testicular toxicity of chemicals with a similar structure to ethylene glycol methyl ether (EGME). Based on toxicity information for EGME and related chemicals and accompanied by adverse outcome pathway information on the testicular toxicity of EGME, this category was defined as chemicals that are metabolized to methoxy- or ethoxyacetic acid, a substance responsible for testicular toxicity. A Japanese chemical inventory was screened using the Hazard Evaluation Support System, which we have developed to support a category approach for predicting the repeated-dose toxicity of chemical substances. Quantitative metabolic information on the related chemicals was then considered, and seventeen chemicals were finally obtained from the inventory as a shortlist for the category. Available data in the literature shows that chemicals for which information is available on the metabolic formation of EGME, ethylene glycol ethyl ether, methoxy- or ethoxyacetic acid do in fact possess testicular toxicity, suggesting that testicular toxicity is a concern, due to metabolic activation, for the remaining chemicals. Our results clearly demonstrate practical utility of AOP-based category approach for predicting repeated-dose toxicity of chemicals.

Keywords: Category approach, Testicular toxicity, Adverse outcome pathway

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Ema M, Endoh K, Fukushima R, Fujii S, Hara H, Hirata-Koizumi M, Hirose A, Hojo H, Horimoto M, Hoshino N, Hosokawa Y, Imai Y, Inada H, Inawaka K, Itoh K, Katsumata Y, Izumi H, Kato H, Maeda M, Matsumoto K, Matsuo S, Matsuoka T, Matsuura I, Mineshima H, Miwa Y, Nakano N, Naya M, Noyori H, Ohta T, Oku H, Ono A, Shimizu T, Shimomura K,

Takakura I, Tanaka R, Tateishi T, Tominaga Y, Uesugi T, Urakawa C, Yabe K, Yamashita A, Yamauchi T, Yokoi R, A Study Group for Historical Control Data on Prenatal Developmental Toxicity Studies in Rodents: Historical control data on developmental toxicity studies in rodents.

Congenit Anom (Kyoto). 2014;54:150-61.

Historical control data on rodent developmental toxicity studies, performed between 1994 and 2010, were obtained from 19 laboratories in Japan, including 10 pharmaceutical and chemical companies and 9 contract research organizations. Rats, mice, and hamsters were used for developmental toxicity studies. Data included maternal reproductive findings at terminal cesarean sections and fetal findings including the spontaneous incidences of external, visceral, and skeletal anomalies. No noticeable differences were observed in maternal reproductive data between laboratories. Inter-laboratory variations in the incidences of fetuses with anomalies appeared to be due to differences in the selection of observation parameters, observation criteria, classification of the findings, and terminology of fetal alterations. Historical control data are useful for the appropriate interpretation of experimental results and evaluation of the effects of chemical on reproductive and developmental toxicities.

Keywords: reproductive and developmental toxicity, historical control data, rodent

Fujitani T[*1], Hojo M[*1], Inomata A[*1], Ogata A[*1], Hirose A, Nishimura T[*2], Nakae D[*1]: Teratogenicity of asbestos in mice.

J Toxicol Sci. 2014;39:363-70.

Possible teratogenicity of 3 different asbestos (crocidolite, chrysotile and amosite) was assessed in CD1(ICR) mice. Dams on day 9 of gestation were given a single intraperitoneal administration at dose of 40 mg/kg body weight of asbestos suspended in 2% sodium carboxymethyl cellulose solution in phosphate buffered saline, while dams in the control group were given vehicle (10 ml/kg body weight). Dams and fetuses were examined on day 18 of gestation. To compare with the control group, the mean percentage of live fetuses in implantations in the group given crocidolite and the incidence of dams with early dead fetuses in the groups given chrysotile or amosite were increased. While no external or skeletal malformation was observed in the control group, the incidence of external malformation (mainly reduction deformity of limb) in the group given amosite, and the incidences of skeletal malformation (mainly fusion of vertebrae) in the all dosed groups were significantly increased. The result indicated that asbestos (crocidolite, chrysotile and amosite) have fetotoxicity and teratogenicity in mice.

Keywords: teratogenicity, asbestos, mice

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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: titanium dioxide, respiratory syncytial virus, pneumonia

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