

Namekata, I.^{*}, Shimada, H.^{*}, Kawanishi, T., Tanaka, H.^{*} and Shigenobu, K.^{*}: **Reduction by SEA0400 of myocardial ischemia-induced cytoplasmic and mitochondrial Ca²⁺ overload.**

Eur J Pharmacol., **543**, 108-115 (2006)

The cardioprotective effects of SEA0400, a novel Na⁺-Ca²⁺ exchanger inhibitor, were examined in isolated guinea pig myocardial tissue and ventricular myocytes. In a coronary-perfused right ventricular tissue preparation, SEA0400 had no cardiosuppressive effect during normoxia and experimental ischemia, but enhanced the recovery of contractile force during reperfusion. SEA0400 had no effect on tissue ATP content during normoxia, but attenuated its decrease during ischemia. Treatment of ventricular myocytes with an ischemia mimetic solution (high K⁺, glucose free, pH 6.0, gassed with N₂) resulted in the depolarization of the mitochondrial membrane potential and an increase in cytoplasmic and mitochondrial Ca²⁺ concentration, which had a similar time course. SEA0400 significantly delayed these changes. These results suggest that SEA0400 maintains mitochondrial function and tissue ATP content during ischemia through the inhibition of cytoplasmic and mitochondrial Ca²⁺ overload.

Keywords: Na⁺-Ca²⁺ exchange, Calcium, cardiomyocyte

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Namekata, I.^{*1}, Kawanishi, T., Iida-Tanaka, N.^{*2}, Tanaka, H.^{*1} and Shigenobu, K.^{*1}: **Quantitative fluorescence measurement of cardiac Na⁺/Ca²⁺ exchanger inhibition by kinetic analysis in stably transfected HEK293 cells.**

J Pharmacol Sci., **101**, 356-360 (2006)

We developed a method to quantitatively evaluate the potency of Na⁺/Ca²⁺ exchanger (NCX) inhibitors with fluorescence microscopy in NCX1-transfected HEK 293 cells. The reverse mode and forward mode NCX activities were measured as the ascending slope of the early phase increase in cytoplasmic Ca²⁺ concentration after change to low Na⁺ extracellular solution and the descending rate (inverse of the exponential time constant) on return to normal solution, respectively. Both modes of NCX were inhibited by SEA0400 (2-[4-(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline) and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea methanesulfonate), and the concentration-inhibition relationships for both inhibitors

were in good agreement with those previously reported in voltage clamped cardiomyocytes.

Keywords: Na⁺-Ca²⁺ exchange, cardiomyocyte, fluorescence microscopy

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Tanaka, H.^{*1}, Shimada, H.^{*1}, Namekata, I.^{*1}, Kawanishi, T., Iida-Tanaka, N.^{*2}, Shigenobu, K.^{*1}: **Involvement of the Na⁺/Ca²⁺ exchanger in ouabain-induced inotropy and arrhythmogenesis in guinea-pig myocardium as revealed by SEA0400.**

J Pharmacol Sci., **103**, 241-246 (2007)

Involvement of the Na⁺/Ca²⁺ exchanger in ouabain-induced inotropy and arrhythmogenesis was examined with a specific inhibitor, SEA0400. In right ventricular papillary muscle isolated from guinea-pig ventricle, 1 microM SEA0400, which specifically inhibits the Na⁺/Ca²⁺ exchanger by 80%, reduced the ouabain (1 μM) -induced positive inotropy by 40%, but had no effect on the inotropy induced by 100 μM isobutyl methylxanthine. SEA0400 significantly inhibited the contracture induced by low Na⁺ solution. In HEK293 cells expressing the Na⁺/Ca²⁺ exchanger, 1 microM ouabain induced an increase in intracellular Ca²⁺, which was inhibited by SEA0400. The arrhythmic contractions induced by 3 μM ouabain were significantly reduced by SEA0400. These results provide pharmacological evidence that the Na⁺/Ca²⁺ exchanger is involved in ouabain-induced inotropy and arrhythmogenesis.

Keywords: Na⁺/Ca²⁺ exchange, cardiomyocyte, fluorescence microscopy

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Izutsu, K., Yomota, C., and Aoyagi, N.: **Inhibition of mannitol crystallization in frozen solutions by sodium phosphates and citrates.**

Chem. Pharm. Bull., **55**, 565-570 (2007)

Effects of co-solutes on the physical property of mannitol and sorbitol in frozen solutions and freeze-dried solids were studied as a model of controlling component crystallinity in pharmaceutical formulations. A frozen mannitol solution (500 mM) showed a eutectic crystallization exotherm at -22.8 degrees C, whereas sorbitol remained amorphous in the freeze-concentrated fraction in the thermal scan. Various

inorganic salts reduced the eutectic mannitol crystallization peak. Trisodium and tripotassium phosphates or citrates prevented the mannitol crystallization at much lower concentrations than other salts. They also raised transition temperatures of the frozen mannitol and sorbitol solutions (T_g : glass transition temperature of maximally freeze-concentrated amorphous phase). Crystallization of some salts (e.g., NaCl) induced crystallization of mannitol at above certain salt concentration ratios. Thermal and near-infrared analyses of cooled-melt amorphous sorbitol solids indicated increased intermolecular hydrogen-bonding in the presence of trisodium phosphate. The sodium phosphates and citrates should prevent crystallization of mannitol in frozen solutions and freeze-dried solids by the intense hydrogen-bonding and reduced molecular mobility in the amorphous phase.

Keywords: amorphous, crystallization, formulation, freeze-drying, thermal analysis

Yonezawa, Y. ^{*1}, Izutsu, K., Tokunaga, H. ^{*1}, Maeda, H. ^{*1}, Arakawa, T. ^{*2} and Tokunaga, M. ^{*1}: **Dimeric structure of nucleoside diphosphate kinase from moderately halophilic bacterium: contrast to tetrameric *Pseudomonas* counterpart.**

FEMS Microbiol Lett., **268**, 52-58 (2007)

Light scattering and chemical cross-linking analyses of nucleoside diphosphate kinase (NDK) from moderate halophile, *Halomonas* sp. 593 (HaNDK), unambiguously demonstrated that this enzyme formed a dimeric structure, in contrast to the *Pseudomonas* NDK (PaNDK), a non-halophilic counterpart, and other NDKs from Gram-negative bacteria, which all formed a tetrameric structure. Comparison of HaNDK and PaNDK showed that the HaNDK was less thermally stable than the PaNDK: the optimum temperature of PaNDK enzyme activity was 20 degrees C higher than that of HaNDK. However, the HaNDK readily refolded and reassembled back to the active dimeric structure, upon heat denaturation at 0.2 M NaCl, as soon as the temperature was lowered. On the contrary, the thermally more stable PaNDK was irreversibly denatured at its melting temperature.

Keywords: protein structure, stability, halophilic, nucleoside diphosphate kinase

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Yomota, C., Ohnishi, Y.: **Determination of biotin fol-**

lowing derivatization with 2-nitrophenylhydrazine by high-performance liquid chromatography-UV detection-electrospray ionization mass spectrometry.

J.Chromatogr. A, **1142**, 231-235 (2007)

Currently, biotin is typically determined in Japan using a microbiological method. Such microbiological assays are sensitive, but they are not always highly specific and are also rather tedious and time-consuming. In the present study, RP-HPLC and LC-MS methods for the determination of biotin have been developed by coupling the carboxyl group with 2-nitrophenylhydrazine hydrochloride. 2-Nitrophenylhydrazine is used for the derivatization of carboxylic acids, and these derivatives are known to be applicable to LC-MS detection. Good recovery rates of over 80% were obtained for the addition of 0.20~0.41 μ mol of biotin per formulation. The detection limit in HPLC at 400 nm was 5 ng per injection, with good linearity being obtained over the concentration range 0.01-1.5 μ g per injection. Further, derivatives were determined by LC-MS with electrospray ionization, where the spectra indicated the molecular-related ions $[M+H]^+$. The detection limit was 0.2 ng per injection in SIMS analysis, and linearity was observed in the range of 5~50 ng per injection. The proposed method could be used to specifically determine the presence of biotin for relatively clean samples with almost pharmacologic amounts of biotin.

Keywords: biotin, 2-nitrophenylhydrazine derivative, LC-MS, electrospray ionization

四方田千佳子, 保立仁美, 伊豆津健一, 青柳伸男: **皮膚適用製剤の溶出試験に関する研究.**

医薬品研究, **38**, 235-241 (2007)

皮膚適用製剤は、皮膚への作用を目的とした局所製剤の他、経口投与でバイオアベイラビリティが低い医薬品等の注射剤に変わる剤形としても広く検討されている。現在、欧州薬局方 (EP) や米国薬局方 (USP) には、皮膚適用製剤の品質評価法として溶出試験のベッセル内に製剤を固定する装置を設置する方法、パドル部分を改変した溶出試験法等が数種収載されている。しかし、日本薬局方 (日局) では、皮膚適用製剤の品質評価法を収載しておらず、日局への適切な取り込みを目指すこととした。それぞれの収載試験法を試みると共に、新たなメンブランフィルターを熱溶着する手法を開発し、有用な試験法であることを示した。

簡便な試験法として、貼付剤の他、軟膏剤等への広い応用が期待される。

Keywords: 貼付剤, 放出試験, 溶出試験器, メンブラン

フィルター

Kawamura, M.^{*1}, Shibata, H., Kamada, H.^{*2}, Okamoto, T.^{*1}, Mukai, Y.^{*1}, Sugita, T.^{*1}, Abe, Y.^{*1}, Imai, S.^{*1}, Nomura, T.^{*1}, Nagano, K.^{*1}, Mayumi, T.^{*3}, Nakagawa, S.^{*1}, Tsutsumi, Y.^{*2}, Tsunoda, S.^{*2} : **A novel method for constructing of gene fragment library to searching epitopes.**

Biochem. Biophys. Res. Commun., **346**,198-201 (2006)

Identification of the epitope sequence or the functional domain of proteins is a laborious process but a necessary one for biochemical and immunological research. To achieve intensive and effective screening of these functional peptides in various molecules, we established a novel screening method using a phage library system that displays various lengths and parts of peptides derived from target protein. Applying this library for epitope mapping, epitope peptide was more efficiently identified from gene fragment library than conventional random peptide library. Our system may be a most powerful method for identifying functional peptides.

Keywords: Phage display system, Gene fragment library, Random peptide library, Epitope mapping, TNF- α

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Imai, S.^{*1}, Mukai, Y.^{*1}, Nagano, K.^{*1}, Shibata, H., Sugita, T.^{*1}, Abe, Y.^{*1}, Nomura, T.^{*1}, Tsutsumi, Y.^{*2}, Kamada, H.^{*2}, Nakagawa, S.^{*1}, Tsunoda, S.^{*2} : **Quality enhancement of the non-immune phage scFv library to isolate effective antibodies.**

Biol. Pharm. Bull., **29**, 1325-30 (2006)

In the present study, we re-generated the library primer sets newly and constructed an improved library from non-immune mice that was far superior in terms of variety and quality. This new library contained 2.4 billion independent clones. In addition, we optimized the selection step from this library to isolate high-affinity antibodies. The optimization of an affinity panning protocol by the incorporation of an automated Microfluidics instrument led to the successful isolation of three different monoclonal antibodies for human vascular endothelial growth factor receptor 2 (KDR). These antibodies were demonstrated to exhibit high specificity and were able to detect a mere 0.6 fmol of KDR by dot blot analysis. Previously reported antibodies for luciferase were also isolated successfully from this li-

brary. Our results clearly demonstrate the importance of the improved protocol for the library preparation of antibodies and the resulting isolation of antibodies for clinical and research applications.

Keywords: non-immune antibody library, phage display system, single-chain Fv, high-throughput screening, vascular endothelial growth factor receptor 2

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Mukai, Y.^{*1}, Sugita, T.^{*1}, Yamato, T.^{*1}, Yamanada, N.^{*1}, Shibata, H., Imai, S.^{*1}, Abe, Y.^{*1}, Nagano, K.^{*1}, Nomura, T.^{*1}, Kamada, H.^{*2}, Nakagawa, S.^{*1}, Tsutsumi, Y.^{*2}, Tsunoda, S.^{*2} : **Creation of novel protein transduction domains (PTDs) under phage display system-based high-throughput screening methods.**

Biol. Pharm. Bull., **29**, 1570-4 (2006)

Significant research effort is currently focused on Protein Transduction Domains (PTDs) as potential intracellular drug delivery carriers. However, the application of this technology is limited because the transduction efficiencies are often insufficient for therapeutic purposes, even using HIV-1 Tat peptide. Here we describe a high-throughput screening method based on a phage display system for isolating novel PTDs with improved cell penetration activity. The screening method involves using protein synthesis inhibitory factor (PSIF) as cargo of PTD. Using this method, several Tat-PTD mutants of superior cell-penetrating activity were isolated. Interestingly, the amino acid sequence of the PTD mutants contained some characteristic residues, such as proline. Thus, our screening method may prove useful in determining the relationship between protein transduction and amino acid sequence.

Keywords: phage display system; protein transduction domain; high-throughput screening; HIV-1 Tat; intracellular drug delivery

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Mukai, Y.^{*1}, Okamoto, T.^{*1}, Kawamura, M.^{*1}, Shibata, H.^{*1}, Sugita, T.^{*1}, Imai, S.^{*1}, Abe, Y.^{*1}, Nagano, K.^{*1}, Nomura, T.^{*1}, Kamada, H.^{*2}, Tsutsumi, Y.^{*2}, Mayumi, T.^{*3}, Nakagawa, S.^{*1}, Tsunoda, S.^{*2} : **Optimization of anti-tumor necrosis factor- α single chain Fv displayed on phages for creation of functional antibodies.**

Pharmazie. **61**, 889-90, (2006)

In this study, we converted the immunoglobulin-type anti-human tumor necrosis factor- α (TNF- α) monoclonal antibody (mAb) to a scFv-type antibody in order to assess its basic properties. The immunoglobulin VH and VL genes were isolated from the hybridoma that produced an anti-TNF- α neutralizing Mab, and they were then linked together to create scFvs of the VL-VH or VH-VL-form. The binding affinity to TNF- α was retained in both scFvs. Interestingly, the VL-VH-type scFv effectively inhibited the TNF- α -mediated cytotoxicity, while this neutralization activity was dramatically decreased in the VH-VL-type scFv. These results suggest that the VL-VH-type scFv is a suitable template to create improved versions of the anti-TNF- α antibody using a phage display system, and they also show that the structural format must be taken into account in manufacturing scFvs.

Keywords: VL, VH, anti-TNF- α antibody

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: **Creation of novel cell-penetrating peptides for intracellular drug delivery using systematic phage display technology originated from Tat transduction domain.** *Biol. Pharm. Bull.*, **30**, 218-23 (2007)

Cell penetrating peptides (CPPs) have been developed to efficiently deliver a wide variety of cargo in a fully biological active form into a range of cell types for the treatment of multiple preclinical disease models. To further develop this methodology, we established a systematic approach to identify novel CPPs using phage display technology. Firstly, we screened a phage peptide library for peptides that bound to the cell membrane. Secondly, to assess functionality as intracellular carriers, we recombined cDNAs of binding peptides with protein synthesis inhibitory factor (PSIF) to create fusion proteins. Using this systematic approach, novel and effective CPPs were rapidly identified. We suggest that these novel cell-penetrating peptides can be utilized as drug delivery tools for protein therapy or an analytical tool to study mechanisms of protein transduction into the cytoplasm.

Keywords: cell penetrating peptide, phage display, Tat

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Yoshioka, S., Miyazaki, T., Aso, Y., Kawanishi, T.: **Significance of local mobility in aggregation of β -galactosidase lyophilized with trehalose, sucrose or stachyose.**

Pharm. Res., **24**, 1660-1667 (2007)

The effect of global mobility, as reflected by glass transition temperature (T_g), on aggregation during storage of β -galactosidase (β -GA) lyophilized with sucrose, trehalose or stachyose was compared with that of local mobility, as reflected by rotating-frame spin-lattice relaxation time ($T_{1\rho}$). The aggregation rate of β -GA in lyophilized formulations exhibited a temperature dependence with a change in slope at around T_g , indicating the effect of molecular mobility on the aggregation rate. Although the T_g rank order of β -GA formulations was sucrose < trehalose < stachyose, the rank order of β -GA aggregation rate at temperatures below and above T_g was also sucrose < trehalose < stachyose, thus suggesting that β -GA aggregation rate is not related to ($T-T_g$). The local mobility of β -GA, as determined by the $T_{1\rho}$ of the β -GA carbonyl carbon, was more markedly decreased by the addition of sucrose than by the addition of stachyose. The effect of trehalose on $T_{1\rho}$ was intermediate when compared to those for sucrose and stachyose. These findings suggest that β -GA aggregation rate is primarily related to local mobility. Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

Key words: solid-state stability, local mobility, β -Galactosidase.

Yoshioka, S., Miyazaki, T., Aso, Y.: **Degradation Rate of Lyophilized Insulin, Exhibiting an Apparent Arrhenius Behavior around Glass Transition Temperature Regardless of Significant Contribution of Molecular Mobility.**

J. Pharm. Sci., **95**, 2684-2691 (2006)

The relative influences of chemical activation energy and molecular mobility in determining chemical reactivity were evaluated for insulin lyophilized with α, β -poly (*N*-hydroxyethyl)-L-aspartamide (PHEA), and compared with that for insulin lyophilized with trehalose, which had been found to have the ability to decrease the molecular mobility of insulin at low humidity. The ratio of the observed rate

constant k_{obs} to the chemical activation energy-controlled rate constant k_{act} (k_{obs}/k_{act}) at glass transition temperature (T_g) was estimated to be approximately 0.6 and 0.8 at 6% RH and 12% RH, respectively, indicating that the degradation rate is significantly affected by molecular mobility at lower humidity conditions. However, these k_{obs}/k_{act} values at T_g were larger than those for the insulin-trehalose system, and changes in the temperature-dependent slope around T_g were less obvious than those for the insulin-trehalose system. Thus, the contribution of molecular mobility to the degradation rate in the insulin-PHEA system appeared to be less intense than that in the insulin-trehalose system. The subtle change in the temperature-dependent slope around T_g observed in the insulin-PHEA system brought about a significant bias in shelf-life estimation when the reaction rate was extrapolated from temperatures above T_g according to the Arrhenius equation.

Key words: solid state stability, glass transition, lyophilization, amorphous, shelf life, molecular mobility.

Yoshioka, S., Miyazaki, T., Aso, Y.: **β -relaxation of insulin molecule in lyophilized formulations containing trehalose or dextran as a determinant of chemical reactivity.**

Pharm. Res., **23**, 961-966 (2006)

The purpose was to elucidate whether the degradation rate of insulin in lyophilized formulations is determined by matrix mobility, as reflected in glass transition temperature (T_g), or by β -relaxation, as reflected in rotating-frame spin-lattice relaxation time ($T_{1\rho}$). The storage stability of insulin lyophilized with dextran was compared to previously reported data for insulin lyophilized with trehalose. The degradation rate of insulin lyophilized with dextran was not significantly affected by the T_g of the matrix, even at low humidity, in contrast to that of insulin lyophilized with trehalose. The insulin-dextran system exhibited a substantially greater degradation rate than the insulin-trehalose system at a given temperature below the T_g . The difference in degradation rate between the insulin-dextran and insulin-trehalose systems observed at 12%RH was eliminated at 43%RH. In addition, the $T_{1\rho}$ of the insulin carbonyl carbon at low humidity (12%RH) was prolonged by the addition of trehalose, but not by the addition of dextran. This difference was eliminated at 23%RH, at which point the solid remained in the glassy state. These findings suggest that the β -relaxation of insulin is inhibited by trehalose at low humidity, presumably due to insulin-trehalose interac-

tion, and thus becomes a rate-determinant. In contrast, dextran, whose ability to interact with insulin is thought to be less than trehalose, did not inhibit the β -relaxation of insulin, and thus the chemical activation barrier (activation energy) rather than β -relaxation becomes the major rate-determinant. β -relaxation rather than matrix mobility appears to be more important in determining the stability of insulin in the glassy state in lyophilized formulations containing trehalose and dextran.

Key words: solid-state stability, molecular mobility, glass transition, insulin, β -relaxation.

Miyazaki, T., Yoshioka, S., Aso, Y. and Kawanishi, T.: **Crystallization rate of amorphous nifedipine analogues unrelated to the glass transition temperature.**

Int. Pharm. J., **336** : 191-195 (2007)

To examine the relative contributions of molecular mobility and thermodynamic factor, the relationship between glass transition temperature (T_g) and the crystallization rate was examined using amorphous dihydropyridines (nifedipine (NFD), m-nifedipine (m-NFD), nitrendipine (NTR) and nilvadipine (NLV)) with differing T_g values. The time required for 10% crystallization, t_{90} , was calculated from the time course of decreases in the heat capacity change at T_g . The t_{90} of NLV and NTR decreased with decreases in T_g associated with water sorption. The t_{90} versus T_g/T plots almost overlapped for samples of differing water contents, indicating that the crystallization rate is determined by molecular mobility as indicated by T_g . In contrast, differences in the crystallization rate between these four drugs cannot be explained only by molecular mobility, since the t_{90} values at a given T_g/T were in the order: NLV > NTR > NFD \approx m-NFD. A lower rate was obtained for amorphous drugs with lower structural symmetry and more bulky functional groups, suggesting that these factors are also important. Furthermore, the crystallization rate of NTR in solid dispersions with poly(vinylpyrrolidone) (PVP) and hydroxypropyl methylcellulose (HPMC) decreased to a greater extent than expected from the increased T_g . This also suggests that factors other than molecular mobility affect the crystallization rate.

Keywords: crystallization; amorphous state; nifedipine; glass transition; molecular mobility; excipients

Miyazaki, T., Yoshioka, S., Aso, Y.: **Physical stability of amorphous acetanilide derivatives improved**

by polymer excipients.

Chem. Pharm. Bull., **54**, 1207-1210 (2006)

Crystallization rates of drug-polymer solid dispersions prepared with acetaminophen (ACA) and p-aminoacetanilide (AAA) as model drugs, and polyvinylpyrrolidone and polyacrylic acid (PAA) as model polymers were measured in order to further examine the significance of drug-polymer interactions. The crystallization of AAA and ACA was inhibited by mixing those polymers. The most effective inhibition was observed with solid dispersions of AAA and PAA. The combination of AAA and PAA showed a markedly longer enthalpy relaxation time relative to drug alone as well as a higher T_g than predicted by the Gordon-Taylor equation, indicating the existence of a strong interaction between the two components. These observations suggest that crystallization is effectively inhibited by combinations of drug and polymer that show a strong intermolecular interaction due to hydrogen bonding and proton transfer between acidic and basic functional groups.

Key words: crystallization rate; solid dispersion; drug-polymer interaction; enthalpy relaxation; glass transition temperature

Ryo Kobayashi*, Yasuto Fujimaki, Tatsuzo Ukita*, Yukio Hiyama: **Monitoring of solvent-mediated polymorphic transition using in situ analysis tools.**

Organic Process Research & Development, **10** (6), 1219-1226 (2006)

In general, polymorphs have been identified by using off-line techniques such as X-ray diffraction, Raman spectroscopy, and infrared spectroscopy (IR). However, these techniques are unsuitable for process monitoring because they are slow and require sample preparation. In this study, the possibility of applying in situ techniques to the monitoring of solvent-mediated transitions was investigated. These in situ techniques include Raman spectroscopy, near-infrared spectroscopy (NIR), and focused beam reflectance measurement (FBRM), in which it is possible to perform measurements quickly and in a nondestructive manner. Raman spectroscopy is effective as a process analytical technology (PAT) tool for determining polymorphic transition because this technique is insensitive to aqueous solvents. NIR can be used for measurements on crystal polymorphs with sampling from a slurry; however, it is not effective if it is also off-line due to the interruption of the absorption band of water. Providing the particle size changes with the polymorphic transition, FBRM can be very useful as a PAT

tool for monitoring not only particle distribution size but also polymorphic transition. Raman spectroscopy provides an insight into the properties of crystallization, especially the rapid quantitative analysis of polymorphic transition. This technique offers a time-saving approach for the development of the crystallization process. In situ techniques such as Raman spectroscopy can be used during scale-up to understand and monitor crystallization processes.

Keyword: Process analytical technology, Bulk drug manufacturing process, Raman spectroscopy, Near infrared spectroscopy, Crystal polymorph

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Sakurai F.*¹, Kawabata K.*¹, Koizumi N.*¹, Inoue N.*², Okabe M.*², Yamaguchi T., Hayakawa T.*³ and Mizuguchi H.*¹: **Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice.**

Gene Ther., **13**, 1118-1126 (2006)

We previously demonstrated that systemic administration of adenovirus serotype 35 (Ad35) vectors to mice does not mediate efficient transduction in organs, probably because expression of the mouse analog of the subgroup B Ad receptor, human CD46 (membrane cofactor protein), is limited to the testis. Here, we describe the *in vitro* and *in vivo* transduction characteristics of Ad35 vectors by using homozygous and hemizygous human CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46. An Ad35 vector more efficiently transduced the primary dendritic cells and macrophages prepared from CD46TG mice than those from wild-type mice. *In vivo* transduction experiments demonstrated that CD46TG mice are more susceptible to Ad35 vector-mediated *in vivo* transduction than are wild-type mice. In particular, homozygous CD46TG mice, which express higher levels of CD46 in the organs than hemizygous CD46TG mice, tend to exhibit higher transduction efficiencies after intraperitoneal administration than hemizygous CD46TG mice. Intraperitoneal administration of Ad35 vectors resulted in efficient transduction into the mesothelial cells of the peritoneal organs in homozygous CD46TG mice. These results indicate that an Ad35 vector recognizes human CD46 as a cellular receptor in CD46TG mice. However, the *in vivo* transduction efficiencies of Ad35 vectors in CD46TG mice are much lower than those of conventional Ad5 vectors in wild-type mice

Keywords: adenovirus serotype 35 vector, human CD46, human CD46 transgenic mice

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Minamisawa S.^{*1}, Uemura N.^{*3}, Sato Y.^{*3}, Yokoyama U.^{*1}, Yamaguchi T, Inoue K., Nakagome M.^{*1}, Bai Y.^{*1}, Hori H.^{*1}, Shimizu M.^{*2}, Mochizuki S.^{*2}, Ishikawa Y.^{*3}: **Post-transcriptional downregulation of sarcolipin mRNA by triiodothyronine in the atrial myocardium.**

FEBS Lett., **580**, 2247-2252 (2006)

Thyroid hormone-mediated positive cardiotropic effects are differently regulated between the atria and ventricles. This regulation is, at least in part, dependent on sarcoplasmic reticulum (SR) proteins. Sarcolipin, a homologue of phospholamban, has been recently identified as an atrium-specific SR protein. The expression of sarcolipin mRNA was significantly decreased in the atria of mice with hyperthyroidism and in 3,5,3'-triiodo-L-thyronine-treated neonatal rat atrial myocytes. Promoter activity and mRNA stability analyses revealed that thyroid hormone post-transcriptionally downregulated the expression of sarcolipin mRNA. The atrium-specific effect of thyroid hormone may occur in part through the regulation of atrial sarcolipin gene expression.

Keywords : thyroid hormone, calcium, gene expression

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Zhao, Y.^{*1}, Nakagawa, T.^{*1}, Itoh, S., Inamori, K.^{*1}, Isaji, T.^{*1,2}, Kariya, Y.^{*3}, Kondo, A.^{*1}, Miyoshi, E.^{*1}, Miyazaki, K.^{*3}, Kawasaki, N., Taniguchi, N.^{*1,4} and Gu, J.^{*1,2}: **N-acetylglucosaminyltransferase III antagonizes the effect of N-acetylglucosaminyltransferase V on $\alpha 3 \beta 1$ integrin-mediated cell migration.**

J. Biol. Chem., **281**, 32122-32130 (2006)

N-acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of $\beta 1,6$ -GlcNAc branching of N-glycans, which contributes to metastasis. N-acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting Gl-

cNAc structure in N-glycans, resulting in the suppression of metastasis. It has long been hypothesized that the suppression of GnT-V product formation by the action of GnT-III would also exist in vivo, which will consequently lead to the inhibition of biological functions of GnT-V. To test this, we draw a comparison among MKN45 cells, which were transfected with GnT-III, GnT-V, or both, respectively. We found that $\alpha 3 \beta 1$ integrin-mediated cell migration on laminin 5 was greatly enhanced in the case of GnT-V transfectant. This enhanced cell migration was significantly blocked after the introduction of GnT-III. Consistently, an increase in bisected GlcNAc but a decrease in $\beta 1, 6$ -GlcNAc-branched N-glycans on integrin $\alpha 3$ subunit was observed in the double transfectants of GnT-III and GnT-V. Conversely, GnT-III knockdown resulted in increased migration on laminin 5, concomitant with an increase in $\beta 1,6$ -GlcNAc-branched N-glycans on the $\alpha 3$ subunit in CHP134 cells, a human neuroblastoma cell line. Therefore, in this study, the priority of GnT-III for the modification of the $\alpha 3$ subunit may be an explanation for why GnT-III inhibits GnT-V-induced cell migration. Taken together, our results demonstrate for the first time that GnT-III and GnT-V can competitively modify the same target glycoprotein and furthermore positively or negatively regulate its biological functions.

Keywords: N-acetylglucosaminyltransferase V, N-acetylglucosaminyltransferase III, $\alpha 3 \beta 1$ integrin

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Zhao, Y.^{*1}, Itoh, S., Wang, X.^{*1}, Isaji, T.^{*1,2}, Miyoshi, E.^{*1}, Kariya, Y.^{*3}, Miyazaki, K.^{*3}, Kawasaki, N., Taniguchi, N.^{*1,4} and Gu, J.^{*1,2}: **Deletion of core fucosylation on $\alpha 3 \beta 1$ integrin down-regulates its functions.**

J. Biol. Chem., **281**, 38343-38350 (2006)

The core fucosylation ($\alpha 1,6$ -fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently $\alpha 1,6$ -fucosylation has been further reported to be very crucial by the study of $\alpha 1,6$ -fucosyltransferase (*Fut8*)-knock-out mice, which shows the phenotype of emphysema-like changes in the lung and severe growth retardation. In this study, we extensively investigated the effect of core fucosylation on $\alpha 3 \beta 1$ integrin and found for the first time that *Fut8* makes an important contribution to the functions of this integrin. The role of core fucosylation in $\alpha 3 \beta 1$ integrin-mediated events has been studied by using

Fut8^{+/+} and *Fut8^{-/-}* embryonic fibroblasts, respectively. We found that the core fucosylation of $\alpha3\beta1$ integrin, the major receptor for laminin 5, was abundant in *Fut8^{+/+}* cells but was totally abolished in *Fut8^{-/-}* cells, which was associated with the deficient migration mediated by $\alpha3\beta1$ integrin in *Fut8^{-/-}* cells. Moreover integrin-mediated cell signaling was reduced in *Fut8^{-/-}* cells. The reintroduction of *Fut8* potentially restored laminin 5-induced migration and intracellular signaling. Collectively, these results suggested that core fucosylation is essential for the functions of $\alpha3\beta1$ integrin.

Keywords: $\alpha1,6$ -fucosyltransferase, $\alpha3\beta1$ integrin

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T. Suzuki, T. Nishimaki-Mogami, H. Kawai^{*1}, T. Kobayashi, Y. Shinozaki, Y. Sato, T. Hashimoto^{*2}, Y. Asakawa^{*2}, K. Inoue, Y. Ohno, T. Hayakawa, and T. Kawanishi: **Screening of novel nuclear receptor agonists by a convenient reporter gene assay system using GFP derivatives.**

Phytomedicine, **13**, 401-411 (2006)

Nuclear receptors represent a very good family of protein targets for the prevention and treatment of diverse diseases. In this study, we screened natural compounds and their derivatives, and discovered ligands for the retinoic acid receptors (RARs) and the farnesoid X receptor (FXR). In the reporter assay system of nuclear receptors presented here, two fluorescent proteins, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP), were used for detection of a ligand-based induction and as an internal control, respectively. By optimizing the conditions (e.g., of hormone response elements and promoter genes for reporter plasmids), we established a battery of assay system for ligands of RARs, retinoid X receptor (RXR) and FXR. The screening using the reporter assay system can be carried out without the addition of co-factors or substrates. As the result of screening of more than 140 compounds, several compounds were detected which activate RARs and/or FXR. Caffeic acid phenylethyl ester (CAPE), known as component of propolis from honeybee hives, and other derivatives of caffeic acid up-regulated the expression of reporter gene for RARs. Griefolin and ginkgolic acids, which are non-steroidal skeleton compounds purified from mushroom or ginkgolic leaves, up-regulated the expression of the reporter gene for FXR.

Keywords: FXR, RXR, reporter assay

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Harashima, M.^{*1}, Niimi, S., Koyanagi, H.^{*1}, Hyuga, M., Noma, S.^{*2}, Seki, T.^{*1}, Ariga, T.^{*1}, Kawanishi, T. and Hayakawa, T.^{*3}: **Change in annexin A3 expression by regulatory factors of hepatocyte growth in primaru cultured rat hepatocytes.**

Biol. Pharm. Bull., **29**, 1339-1343 (2006)

We have recently reported that annexin (Anx) A3 expression is necessary for hepatocyte growth in cultured rat hepatocytes seeded at half the subconfluent density on collagen. In the present study, we investigated the effects of various regulatory factors of hepatocyte growth on AnxA3 expression. AnxA3 expression was significantly reduced in hepatocytes cultured under various growth inhibitory conditions such as presence of dexamethasone, culture at subconfluent cell density, and on EHS-Matrigel and lactose-carrying styrene polymer. On the other hand, hepatocyte growth factor and epidermal growth factor, stimulators of hepatocyte growth, significantly increased AnxA3 expression in hepatocytes cultured on EHS-Matrigel. These results show close correlation between known stimulatory or inhibitory actions of various factors to hepatocyte growth and increase or decrease in AnxA3 expression, and suggest the involvement of AnxA3 in their regulation of hepatocyte growth.

Keywords: annexin A3, hepatocyte growth, primary cultured rat hepatocytes

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Watanabe, K.^{*}, Hyuga, S.^{*}, Hyuga, M., Kawanishi, T. and Hanawa, T.^{*}: **Agonistic or antagonistic action of Kampo medicines used for menopausal symptoms on estrogen receptor subtypes, ER α and ER β .**

J. Trad. Med., **23**, 203-207 (2006)

Kampo medicines are used for the therapy of menopausal symptoms as an alternative to HRT in Japan. Previously, we reported that there is the estrogen-like activity in some Kampo medicines. However, it is not clear whether the medicines act directly on estrogen receptors (ERs) α and β . We analyzed both agonistic and antagonistic actions of nine kinds of Kampo medicines used for the treatment of menopausal symptoms at the receptors using the Recep-

tor / Coactivator Ligand Assay. Hachimijiogan (TJ-7), kamisyoyosan (TJ-24), keishibukuryogan (TJ-25), and tokakujyokito (TJ-61) acted agonistically on ER β , but had almost no actions on ER α . Kakkonto (TJ-1) acted agonistically on both ER α and ER β . Tokisyakuyakusan (TJ-23), and unkeito (TJ-106) acted antagonistically on ER β , and orengedokuto (TJ-15) and nyoshinsan (TJ-67) acted antagonistically on both ER α and ER β . In the present study, we found for the first time that Kampo medicines act directly on the estrogen receptor α or β as the agonist or antagonist.

Keywords: estrogen receptor, menopause, HRT

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川崎ナナ, 原園 景, 川西 徹: 糖タンパク質性医薬品の試験法に関する研究—LC/MS/MSを用いたペプチドマッピング—.

医薬品研究, **37**, 438-447 (2006)

LC/MS/MSを用いたペプチドマッピングの糖タンパク質性医薬品の品質試験法としての応用可能性を検証した。その結果、ペプチド部分の配列はデータベースを利用することによって容易に確認できること、また、糖ペプチドのMS/MSスペクトルを選び出して解析することによって、ペプチド部分の配列と部位特異的な糖鎖構造の概略を確認できることが明らかになった。LC/MS/MSを用いたペプチドマッピングは、一次構造と糖鎖を含む翻訳後修飾を同時に確認できるものであり、品質試験法として応用可能であることが示唆された。

Keywords: LC/MS/MS, 糖タンパク質, 品質試験法

川崎ナナ, 伊藤さつき, 橋井則貴, 日向昌司, 川西 徹: 局方組換えタンパク質性医薬品の糖鎖試験法に関する研究—LC/MSⁿを用いた糖鎖プロファイリング—. 医薬品研究, **37**, 448-456 (2006)

LC/MSⁿを用いた糖鎖プロファイリングの糖鎖試験法としての応用可能性を検証した。その結果、LC/MSⁿを用いた糖鎖プロファイリングは、LC 上の溶出位置の違いだけでなく、質量の違い、更には糖鎖配列の違いから糖鎖を識別できることから、糖タンパク質性医薬品の糖鎖試験法として応用可能であることが示唆された。また、本分析法は、糖鎖の識別だけでなく、構造解析にも有用であり、新規糖タンパク質性医薬品の特性解析・品質評価、糖鎖改変タンパク質の糖鎖解析、製造方法変更時における同等性/同質性評価、並びにバイオ後続品の評価等にも有用であると思われる。

Keywords: LC/MS/MS, 糖鎖プロファイリング, 糖タンパク質, 品質試験法

Mizuguchi, H.^{*1}, Funakoshi, N.^{*2}, Hosono, T., Sakurai, F.^{*1}, Kawabata, K.^{*1}, Yamaguchi, T. and Hayakawa, T.^{*3}: **Rapid construction of small interfering RNA-expressing adenovirus vectors on the basis of direct cloning of short hairpin RNA-coding DNAs.**

Hum. Gene Ther., **18**, 74-80 (2007)

In the conventional method for constructing an adenoviral (Ad) vector expressing small interfering RNA (siRNA), short hairpin RNA (shRNA) -coding oligonucleotides are introduced downstream of a polymerase III (or polymerase II) -based promoter cloned into a shuttle plasmid. An siRNA expression cassette, which is cloned into the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid by in vitro ligation or homologous recombination in *Escherichia coli*, and the linearized plasmid is transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step plasmid manipulation is required. In this study, we developed a method by which shRNA-coding oligonucleotides can be introduced directly into the Ad vector plasmid. To do this, we constructed a new vector plasmid into which the human U6 promoter sequence was cloned in advance. Unique restriction enzyme sites were introduced at the transcription start site of the U6 promoter sequence in the vector plasmid. Luciferase and p53 genes were efficiently knocked down by Ad vectors generated by the new method and expressing siRNA against the target gene. This method should be useful for RNA interference-based experiments, and should make it easy to construct an siRNA-expressing Ad vector library for functional screening.

Keywords: adenovirus vector, siRNA, shRNA

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Koizumi N.^{*1}, Yamaguchi T., Kawabata K.^{*1}, Sakurai F.^{*1}, Sasaki T.^{*1}, Watanabe Y.^{*2}, Hayakawa T.^{*3} and Mizuguchi H.^{*1}: **Fiber-modified adenovirus vectors decrease liver toxicity through reduced interleukin 6 production.**

J. Immunol., **178**, 1767-1773 (2007)

Adenovirus (Ad) vectors are one of the most commonly used viral vectors in gene therapy clinical trials. However, they elicit a robust innate immune response and inflammatory responses. Improvement of the therapeutic index of Ad

vector gene therapy requires elucidation of the mechanism of Ad vector-induced inflammation and cytokine/chemokine production as well as development of the safer vector. In the present study, we found that the fiber-modified Ad vector containing poly-lysine peptides in the fiber knob showed much lower serum IL-6 and aspartate aminotransferase levels (as a maker of liver toxicity) than the conventional Ad vector after i.v. administration, although the modified Ad vector showed higher transgene production in the liver than the conventional Ad vector. RT-PCR analysis showed that spleen, not liver, is the major site of cytokine, chemokine, and IFN expression. Splenic CD11c⁺ cells were found to secrete cytokines. The tissue distribution of Ad vector DNA showed that spleen distribution was much reduced in this modified Ad vector, reflecting reduced IL-6 levels in serum. Liver toxicity by the conventional Ad vector was much reduced in this modified Ad vector, reflecting reduced IL-6 levels in serum. Liver toxicity by the conventional Ad vector was reduced by anti-IL-6R Ab, suggesting that IL-6 signaling is involved in liver toxicity and that decreased liver toxicity of the modified Ad vector was due in part to the reduced IL-6 production. This study contributes to an understanding of the biological mechanism in innate immune host responses and liver toxicity toward systemically administered Ad vectors and will help in designing safer gene therapy methods that can reduce robust innate immunity and inflammatory responses.

Keywords : adenovirus vector, interleukin 6, cytokine/chemokine

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Wada, Y.^{*1}, Azadi, P.^{*2}, Costello, C. E.^{*3}, Dell, A.^{*4}, Dwek, R. A.^{*5}, Geyer, H.^{*6}, Geyer, R.^{*6}, Kakehi, K.^{*7}, Karlsson, N. G.^{*8,9}, Kato, K.^{*10}, Kawasaki, N., Khoo, K. H.^{*11}, Kim, S.^{*12}, Kondo, A.^{*13}, Lattova, E.^{*13}, Mechref, Y.^{*15}, Miyoshi, E.^{*13}, Nakamura, K.^{*16}, Narimatsu, H.^{*17}, Novotny, M. V.^{*18}, Packer, N. H.^{*8}, Perreault, H.^{*14}, Peter-Katalinic, J.^{*18}, Pohlentz, G.^{*18}, Reinhold, V. N.^{*19}, Rudd, P. M.^{*5,20}, Suzuki, A.^{*21} and Taniguchi, N.^{*13,22}: **Comparison of the methods for profiling glycoprotein glycans-HUPO Human Disease Glycomics/Proteome Initiative multi-institutional study.**

Glycobiology, **17**, 411-422 (2007)

Mass spectrometry (MS) of glycoproteins is an emerging field in proteomics, poised to meet the technical demand for elucidation of the structural complexity and functions of the oligosaccharide components of molecules. Considering the divergence of the mass spectrometric methods employed for oligosaccharide analysis in recent publications, it is necessary to establish technical standards and demonstrate capabilities. In the present study of the Human Proteome Organisation (HUPO) Human Disease Glycomics/Proteome Initiative (HGPI), the same samples of transferrin and immunoglobulin-G were analyzed for N-linked oligosaccharides and their relative abundances in 20 laboratories, and the chromatographic and mass spectrometric analysis results were evaluated. In general, matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS of permethylated oligosaccharide mixtures carried out in six laboratories yielded good quantitation, and the results can be correlated to those of chromatography of reductive amination derivatives. For underivatized oligosaccharide alditols, graphitized carbon-liquid chromatography (LC) /electrospray ionization (ESI) MS detecting deprotonated molecules in the negative ion mode provided acceptable quantitation. The variance of the results among these three methods was small. Detailed analyses of tryptic glycopeptides employing either nano LC/ESI MS/MS or MALDI MS demonstrated excellent capability to determine site-specific or subclass-specific glycan profiles in these samples. Taking into account the variety of MS technologies and options for distinct protocols used in this study, the results of this multi-institutional study indicate that MS-based analysis appears as the efficient method for identification and quantitation of oligosaccharides in glycomic studies and endorse the power of MS for glycopeptide characterization with high sensitivity in proteomic programs.

Keywords: Mass spectrometry, transferrin, immunoglobulin-G

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Baba, M.^{*1}, Ma, B. Y.^{*1}, Nonaka, M.^{*1,2}, Matsuishi, Y., Hirano, M.^{*1,2}, Nakamura, N.^{*1,2}, Kawasaki, N., Kawasaki, N.^{*1} and Kawasaki, T.^{*1}: **Glycosylation-dependent interaction of Jacalin with CD45 induces T lymphocyte activation and Th1/Th2 cytokine secretion.**

J. Leukoc. Biol. **81**, 1002-1011 (2007)

Jacalin, an α -O-glycoside of the disaccharide Thomsen-Friedenreich antigen (galactose β -3 N-acetylgalactosamine, T-antigen) -specific lectin from jackfruit seeds, has been shown to induce mitogenic responses and to block infection by HIV-1 in CD4⁺ T lymphocytes. The molecular mechanism underlying Jacalin-induced T cell activation has not been elucidated completely yet. In the present study, protein tyrosine phosphatase (PTPase) CD45 was isolated from a Jurkat T cell membrane fraction as a major receptor for Jacalin through affinity chromatography and mass spectrometry. CD45, which is highly glycosylated and expressed exclusively on the surface of lymphocytes, is a key regulator of lymphocyte signaling, playing a pivotal role in activation and development. We found that the lectin induced significant IL-2 production by a CD45-positive Jurkat T cell line (JE6.1) and primary T cells. However, this effect did not occur in a CD45-negative Jurkat T cell line (J45.01) and was blocked completely by a specific CD45 PTPase inhibitor in Jurkat T (JE6.1) and primary T cells. Furthermore, we also observed that Jacalin caused a marked increase in IL-2 secretion in response to TCR ligation and CD28 costimulation and contributed to Th1/Th2 cytokine production by activating CD45. Jacalin increased CD45 tyrosine phosphatase activity, which resulted in activation of the ERK1/2 and p38 MAPK cascades. Based on these findings, we propose a new, immunoregulatory model for Jacalin, wherein glycosylation-dependent interactions of Jacalin with CD45 on T cells elevate TCR-mediated signaling, which thereby

up-regulate T cell activation thresholds and Th1/Th2 cytokine secretion.

Keywords: CD45, Jacalin, Th1/Th2 cytokine secretion

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Kanayasu-Toyoda, T., Suzuki, T., Oshizawa, T., Suzuki, T., Uchida, E., Hayakawa, T., and Yamaguchi, T. : **Granulocyte colony-stimulating factor promotes the translocation of protein kinase C ϵ in neutrophilic differentiation cells.**

J. Cell. Physiol. **211**, 189-96 (2007)

Previously, we suggested that the phosphatidylinositol 3-kinase (PI3K) -p70 S6 kinase (p70 S6K) pathway plays an important role in granulocyte colony-stimulating factor G-CSF-dependent enhancement of the neutrophilic differentiation and proliferation of HL-60 cells. While atypical protein kinase C (PKC) has been reported to be a regulator of p70 S6K, abundant expression of PKC ϵ was observed in myeloid and lymphoid cells. Therefore, we analyzed the participation of PKC ϵ in G-CSF-dependent proliferation. The maximum stimulation of PKC ϵ was observed from 15 min to 30 min after the addition of G-CSF. From 5 to 15 min into this lag time, PKC ϵ was found to translocate from the nucleus to the membrane. At 30 min it re-translocated to the cytosol. This dynamic translocation of PKC ϵ was also observed in G-CSF-stimulated myeloperoxidase-positive cells differentiated from cord blood cells. Small interfering RNA for PKC ϵ inhibited G-CSF-induced proliferation and the promotion of neutrophilic differentiation of HL-60 cells. These data indicate that the G-CSF-induced dynamic translocation and activation processes of PKC ϵ are important to neutrophilic proliferation.

Keywords: protein kinase C ϵ , granulopoiesis, G-CSF

Ng M.K.^{*}, Wu J.^{*}, Chang E.^{*}, Wang B.Y.^{*}, Katzenberg-Clark R.^{*}, Ishii-Watabe A., and Cooke J.P.^{*} : **A central role for nicotinic cholinergic regulation of growth factor-induced endothelial cell migration.**

Arterioscler. Thromb. Vasc. Biol., **27**, 106-112 (2007)

An endothelial nicotinic acetylcholine receptor (nAChR) participates in atherogenesis and tumorigenesis by promoting neovascularization. To date, the mechanisms of nAChR-mediated angiogenesis and their relationship to angiogenic factors, eg, VEGF and bFGF, are unknown. **METHODS AND RESULTS:** Nicotine induced dose-dependent human microvascular endothelial cell (HMVEC) migration, a key angiogenesis event, to an extent which was equivalent in

magnitude to bFGF (10 ng/mL) but less than for VEGF (10 ng/mL). Unexpectedly, nAChR antagonism not only abolished nicotine-induced HMVEC migration but also abolished migration induced by bFGF and attenuated migration induced by VEGF. Transcriptional profiling identified gene expression programs which were concordantly regulated by all 3 angiogens (nicotine, VEGF, and bFGF), a notable feature of which includes corepression of thioredoxin-interacting protein (TXNIP), endogenous inhibitor of the redox regulator thioredoxin. Furthermore, TXNIP repression by all 3 angiogens induced thioredoxin activity. Silencing thioredoxin by small interference RNA abrogated all angiogen-induced migration while silencing TXNIP strongly induced HMVEC migration. Interestingly, nAChR antagonism abrogates growth factor (VEGF and bFGF)-mediated induction of thioredoxin activity. CONCLUSIONS: Nicotine promotes angiogenesis via stimulation of nAChR-dependent endothelial cell migration. Furthermore, growth factor-induced HMVEC migration, a key angiogenesis event, requires nAChR activation — an effect mediated in part by nAChR-dependent regulation of thioredoxin activity.

Keywords: endothelial cells, nicotine, angiogenesis

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H. Kawai^{*1}, T. Suzuki, T. Kobayashi, A. Ishii-Watabe, H. Sakurai^{*2}, H. Ohata, K.^{*2} Honda^{*2}, K. Momose^{*2}, T. Hayakawa, and T. Kawanishi: **Caspase cascade proceeds rapidly after cytochrome c release from mitochondria in Tumor Necrosis Factor- α -induced cell death.**

J. Pharmacol. Sci., **103**, 159-167 (2007).

The caspase activation cascade and mitochondrial changes are major biochemical reactions in the apoptotic cell death machinery. We attempted to clarify the temporal relationship between caspase activation, cytochrome c release, mitochondrial depolarization, and morphological changes that take place during tumor necrosis factor (TNF)- α -induced cell death in HeLa cells. These reactions were analyzed at the single-cell level with 0.5 – 1 min resolution by using green fluorescent protein (GFP)-variant-derived probes and chemical probes. Cytochrome c release, caspase activation, and cellular shrinkage were always observed in this order within 10 min in all dying cells. This sequence of events was thus considered a critical pathway of cell death. Mitochondrial depolarization was also observed in all dying cells observed, but frequently occurred after caspase activation and cellular shrinkage. Mitochondrial depolariza-

tion is therefore likely to be a reaction that does not induce caspase activation and subsequent cellular shrinkage. Mitochondrial changes are important for apoptotic cell death; moreover, cytochrome c release, and not depolarization, is a key reaction related to cell death. In addition, we also found that the apoptotic pathway proceeds only when cells are exposed to TNF- α . These findings suggest that the entire cell death process proceeds rapidly during TNF- α exposure.

Keywords: tumor necrosis factor (TNF)- α , caspase, real-time imaging

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Hyuga, S.^{*1}, Hyuga, M., Nakanishi, H.^{*2}, Ito, H.^{*1}, Watanabe, K.^{*1}, Oikawa, T.^{*1} and Hanawa, T.^{*1}: **Maoto, Kampo medicine, suppresses the metastatic potential of highly metastatic osteosarcoma cells.**

J. Trad. Med., **24**, 51-58 (2007)

Maoto, Kampo medicine, has been shown to inhibit the motility of highly metastatic osteosarcoma, FBJ-LL cells. In the present study, maoto was found to suppress the liver-metastasis of FBJ-LL cells and had no effect on the primary tumor growth. The expression pattern of cytokines in the serum from the tumor-bearing mice given maoto approximated that of the serum from normal mice and was different from that in the serum obtained from the tumor-bearing mice given water. Maoto suppressed the activation of matrix metalloproteinases involved in metastatic processes. On the other hand, jumentaihoto, which has been reported to suppress cancer metastasis via activation of the immune system, had no effect on either liver-metastasis of FBJ-LL cells or primary tumor growth. These results suggest that maoto is a candidate for a novel inhibitor of metastasis, and that the inhibition mechanism of metastasis is different from that of jumentaihoto.

Keywords: Maoto, metastasis, MMP

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Sakai, S., Otake, R.^{*}, Toida, T.^{*}, Goda, Y. :

Identificaiton of the origin of chondroitin sulfate in “health foods” .

Chem. Pharm. Bull., **55**, 299-303 (2007)

Twelve “health foods” products containing chondroitin sulfate (CS) were purchased from the Japanese market and the origin of the CS was investigated by conducting disac-

charide compositional analysis after enzymatic depolymerization and by ¹H-NMR spectroscopy. Nine of the 12 products had labels indicating that the origin of the CS was shark cartilage. However, two of them were found to contain mammalian CS. Next, we compared the ratio of the sulfate group to the galactosamine residue after the acid hydrolysis of CS. The results suggest that all of the CS from sharks had a ratio of more than 1.0, while the CS from mammals had a ratio of less than 1.0. Since this comparative analysis does not require expensive purified enzyme, it would be an economical way to identify the origin of CS in “health foods”. Being able to determine the origin of the ingredients in natural products is very important for ensuring their quality, safety, and efficacy. Therefore, we think that regulatory requirements for accurately indicating the origin of “health foods” and effective enforcement of these requirements are needed.

Keywords: chondroitin sulfate, chemical compositional analysis, health food origin

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岡田 稔^{*3}, 寺林 進^{*3}, 嶋田康男^{*4}, 川崎武志^{*5}, 藪崎 晃^{*6}, 山本 豊^{*7}, 川合 保^{*8}, 川原信夫, 合田 幸広

生薬の残留有機塩素系農薬試験法の検討:

医薬品研究 37, 567-581 (2006)

第15改正日本薬局方 (JP15) に記載するオンジ、ケイヒ、サンシュユ、タイソウ、オウギ、カンゾウ、サイシン、ボタンピ、ソヨウ、チンピ、ニンジン、ピワヨウを対象とした残留有機塩素系農薬試験法について検討し、既存で規格のあるコウジン、ニンジン、センナも対象とするJP15記載試験法案を提案した。また、測定上の注意点等も記載した。

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Nakamura, Y.^{*1}, Yomura, K.^{*1}, Kammoto, T.^{*1}, Ishimatsu, M.^{*1}, Kikuchi, Y.^{*1}, Niitsu, K.^{*1}, Terabayashi, S.^{*1}, Takeda, S.^{*1}, Sasaki, H.^{*1}, Arimoto, K.^{*2}, Okada, M.^{*1}, Sekita, S.^{*3}, Satake, M.^{*4}, Goda, Y.: **Physicochemical quality evaluation of natural compounds isolated from**

crude drugs. Standard compounds for the official specification and testing method of “Processed Aconite Root” and “Powdered Processed Aconite Root” in Japanese Pharmacopoeia.

J. Nat. Med., **60**, 285-294 (2006)

Aconite root has high toxicity caused by diester alkaloids, thus it was necessary to define the limiting value of diester alkaloids used in medicine formulation. To give the quality of “Processed Aconite Root” and “Powdered Processed Aconite Root” in the Japanese Pharmacopoeia (14th edn, supplement), we established the official specification and evaluation methods of standard substances. High qualitative grade diester alkaloids, aconitine, hypaconitine, jesaconitine and mesaconitine, which were useful to evaluate the purity of processed aconite root and powdered processed aconite root, were prepared and evaluated for their stability. We studied the physicochemical specification and evaluation methods of these alkaloids. In addition, an “Aconitum diester alkaloids standard solution for purity”, which was used for the purity test, was prepared, and we also studied its physicochemical specification and evaluation methods. In addition, to evaluate the quality of processed aconite root and powdered processed aconite root, a TLC identification test was established. A monoester alkaloid of benzoylmesaconine hydrochloride was used as the reference standard in the latter test, and we also investigated its physicochemical specification and evaluation methods.

Keywords: processed aconite root, powdered processed aconite root, the Japanese Pharmacopoeia

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Matsumoto, T., Kikura-Hanajiri, R., Kamakura, H., Kawahara, N., Goda, Y.: **Identification of N-methyl-4-(3,4-methylenedioxyphenyl) butan-2-amine, distributed as MBDB.**

Journal of Health Science, **52**, 805-810 (2006)

N-methyl-1-(3,4-methylenedioxyphenyl) butan-2-amine (MDP-2-MB, MBDB) is a new homologue of *N*-methyl-1-(3,4-methylenedioxyphenyl) propan-2-amine (MDMA), which is strictly controlled as a narcotic. As part of our continuous survey on illegal designer drugs in the Japanese market, we found that *N*-methyl-4-(3,4-methylenedioxyphenyl) butan-2-amine (MDP-3-MB, HMDMA) was being

sold as MBDB. As this is the first time that HMDMA has been revealed to be in market distribution, and its physico-chemical data is thus far unreported, we describe the structure elucidation of HMDMA and comparative analysis with related compounds.

Keywords: HMDMA, MBDB, structure elucidation

Kawahara, N., Sakai, E.^{*1}, Itokazu, N., Satake, M.^{*2}, Goda, Y.: **Comparative study on testing methods and specification values for crude drugs used in monographs among four western pacific regional countries (Japan, China, Korea and Vietnam) (2) Comparative study on TLC and assay conditions.** *The Japanese Journal of Pharmacognosy*, **60** (2), 73-85 (2006)

Five expert working groups (EWG1-5) were established in the Sub-committee I Meeting of the Western Pacific Regional Forum for the Harmonization of Herbal Medicine (FHH) nomenclature and standardization. The task of EWG2 (Testing Methods used in Monographs) is to list the testing methods in each monograph. In a previous paper, we reported on the preparation of a comparative table of testing methods and specification values for crude drugs used in monographs among four western Pacific regional countries (Japan, China, Korea and Vietnam). In this paper, we report on the further preparation of comparative tables on TLC conditions for identification and chemical assay conditions for component quantification used in monographs and to obtain some knowledge from these comparative tables.

Keywords: FHH, Crude drug, Comparative table, TLC conditions, Assay conditions

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Hosoe, T.^{*1}, Fukushima, K.^{*2}, Takizawa, K.^{*2}, Itabashi, T.^{*1}, Kawahara, N., Vidotto, V.^{*3}, Kawai, K.^{*1}: **A New Antifungal Macrolide, Eushearilide, Isolated from *Eupenicillium shearii*.**

J. Antibiotics, **59** (9), 597-600 (2006)

In screening for antifungal substances, a new macrolide, eushearilide (1), was isolated from *Eupenicillium shearii* IFM54447. The structure of 1 was established to be 24-membered macrolide having a non-conjugated diene and a choline phosphate ester moiety on the basis of detailed investigation of NMR, UV, IR and MS spectral data. Compound 1 showed antifungal activity against various fungi

and yeasts, including human pathogens *Aspergillus fumigatus*, *Trichophyton* spp. and *Candida* spp.

Key words: macrolide, eushearilide, *Eupenicillium shearii*, antifungal activity

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Kim, I-H., Uchiyama, N., Kawahara, N., Goda, Y.: **Iridoid and cucurbitacin glycosides from *Neopicrorhiza scrophulariiflora*.**

Phytochemistry, **67** (24), 2691-2696 (2006)

Three iridoid glycosides, picrorosides A (1), B (2) and C (3), and a cucurbitacin glycoside, scrophoside A (4), were isolated from the rhizomes of *Neopicrorhiza scrophulariiflora* (Scrophulariaceae), along with two known iridoid glycosides, picrosides I (5) and II (6), and three known cucurbitacin glycosides (7-9). Their structures were elucidated on the basis of both chemical and spectroscopic data.

Key words: *Neopicrorhiza scrophulariiflora*, Scrophulariaceae, Iridoid glycoside, Cucurbitacin glycoside, Picrorosides A, B and C, Scrophoside A

Hirasawa, Y.^{*1}, Kobayashi, J.^{*2}, Obara, Y.^{*3}, Nakahata, N.^{*3}, Kawahara, N., Goda, Y., Morita, H.^{*1}: **A New Alkaloid from *Lycopodium hamiltonii* and Revised Stereostructure of Nankakurine A.**

Heterocycles, **68**, 2357-2364 (2006)

A new *Lycopodium* alkaloid, nankakurine B (2), has been isolated from the club moss *Lycopodium hamiltonii* together with nankakurine A (1). Stereochemistry of 2 was elucidated by combination of NOESY correlations and chemical transformation. Stereostructure of 1 was revised to be the same as that of 2. Nankakurine A (1) induced secretion of neurotrophic factors from human astrocytoma cells.

Key words: nankakurine A, nankakurine B, *Lycopodium* alkaloid, *Lycopodium hamiltonii*

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Kawahara, N., Kim, I-H., Goda, Y.: **Content of Sulfur Dioxides in Herbal Materials Obtained from the Japanese Market.**

Jpn. J. Food Chem., **13** (3), 105-108 (2006)

Sulfur dioxides and sulfites are registered in “The Japan’s Specifications and Standards for Food Additives” mainly used as bleach and anti-oxidants, and Food Sanitation Law prohibits the use to sesame, legumes and vegetables. In China, sulfur fumigation is performed for the purpose of bleaching, drying, insecticide and antibacterial to some crude drugs. Recently, it has been reported that large quantities of sulfur dioxides are detected from sulfur fumigated crude drugs. In the course of our study of the survey of impurity in herbal materials, we analyzed the content of sulfur dioxides for 31 kinds of crude drugs (5 companies, 151 herbal materials) purchased from the Japanese market. By using modified Rankine method, more than 1,000 ppm of sulfur dioxides was detected from three crude drugs (Dioscorea Rhizome, Gastrodia Tuber, Fritillaria Bulb) and also more than 500 ppm of sulfur dioxides was detected from seven crude drugs (Puerariae Radix, Lillii Bulbus, Zingiberis Rhizoma, Asparagi Tuber, Platycodi Radix, Mori Cortex, Forsythiae Fructus). Since herbal materials such as Dioscorea Rhizome, Puerariae Radix, Lillii Bulbus, Zingiberis Rhizoma are used as food and food additives, sulfur dioxide content of these materials should be noted at the viewpoint of food safety.

Key words: sulfur dioxides, sulfur fumigation, herbal materials

Zou, D-P., Kim, I-H., Kawahara, N., Goda, Y.: **Chemical Constituents of “Garden Balsam Extract” as a Natural Food Additive.**

Jpn. J. Food Chem., **13** (3), 114-117 (2006)

“Garden balsam extract”, a natural food additive, is commercially available as an ethanol extract of the whole plant of Garden balsam (*Impatiens balsamina* L.) and is officially approved in the “Lists of Existing Food Additives in Japan”. In our ongoing study to evaluate its quality and safety as a food additive, 14 components in “Garden balsam extract” were purified by the preparative HPLC. A new compound, (3R*,4R*)-3,4-dihydroxy-3,4-dihydronaphthalen-1 (2H)-one, was purified and characterized on the basis of the spectral evidence.

Key words: Garden balsam extract, natural food additive, *Impatiens balsamina*, naphthoquinone derivative

Anjiki, N., Yoshino, C., Kawahara, N., Goda, Y.: **Evaluation of the Taste of Kampo Formula by Taste-Sensing System (3), Taste of Ryokeijutsukanto.**

The Japanese Journal of Pharmacognosy, **61** (1), 21-27

(2007)

In the course of our studies to evaluate objectively the taste of Kampo formulae by taste-sensing system, we investigated the characteristic taste and taste factor of Ryokeijutsukanto of which the component crude drugs are Poria Sclerotium, Glycyrrhiza, Cinnamon Bark and Atractylodes rhizome or Atractylodes Lancea Rhizome. The results suggested that the following facts. 1) Glycyrrhiza mainly contributes the saltiness of Ryokeijutsukanto and Poria Sclerotium decreases it. 2) Atractylodes Rhizome contributes to the anionic bitterness in which Atractylodes Rhizome used-Ryokeijutsukanto shows, while Cinnamon Bark contributed to it in which Atractylodes Lancea Rhizome used-Ryokeijutsukanto shows. 3) Taste-sensing system does not recognize a difference of the taste of Ryokeijutsukanto and that of the mixed preparations consisting of the corresponding four single decoctions of the component crude drugs. 4) Any component crude drugs solely do not express the Ryokeijutsukanto-like taste in the decoction. Namely, the combination of the taste of the component drugs expresses that of Ryokeijutsukanto decoction.

Key words: taste evaluation, Kampo formula, Ryokeijutsukanto, taste-sensing system

Kawahara, N., Itokazu, N.^{*1}, Satake, M.^{*2}, Goda, Y.: **Comparative Study on Testing Methods and Specification Values for Crude Drugs in Pharmacopoeias among Four Western Pacific Regional Countries (Japan, China, Korea and Vietnam) (III) Comparative Study on General Testing Methods for Crude Drugs.**

The Japanese Journal of Pharmacognosy, **61** (1), 44-57 (2007)

The Western Pacific Regional Forum for the Harmonization of Herbal Medicines (FHH) has three Sub-Committees (Sub-C). Of them, Sub-C I deals with the nomenclature and standardization and consists of five Expert Working Groups (EWGs 1-5). The task of EWG 5 is to list the information on general testing methods for crude drugs described in the general test section of each Pharmacopoeia among four countries (Japan, China, Korea and Vietnam). In this paper, we show the results of the task work. In the sections of sampling, foreign matter, loss on drying, total ash, acid-insoluble ash, extract content, essential oil content, arsenic limit test and heavy metals limit test, there are many similarities among the four Pharmacopoeias. However, the method of microscopic examination in the

Pharmacopoeias of China and Vietnam were completely different from those of Japan and Korea. Namely, the former describe detailed techniques and observation points.

Keywords: FHH, Crude drug, Comparative table, General testing methods

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Kim, I. H., Umezawa, M., Kawahara, N., Goda, Y.: **The constituents of the roots of *Ampelopsis japonica***. *J. Nat. Med.*, **61**, 224-225 (2007)

Six compounds, schizandriside, resveratrol, (+) -catechin, (-) -epicatechin, (+) -galocatechin, and (-) -epicatechin gallate, were isolated from the roots of *Ampelopsis japonica* (Vitaceae). Their structures were determined on the basis of both chemical and spectroscopic data.

Key words: *Ampelopsis japonica*, Vitaceae, Schizandriside, Resveratrol

Abe, I.^{*1}, Morita, H.^{*2}, Oguro, S.^{*1}, Noma, H.^{*1}, Wanibuchi, K.^{*1}, Kawahara, N., Goda, Y., Noguchi, H.^{*1}, Kohno, T.^{*2}: **Enzymatic Formation of An Unnatural Nonaketide Naphthopyrone by A Mutant of Plant Type III Polyketide Synthase**.

J. Am. Chem. Soc., **129** (18), 5976-5980 (2007)

Pentaketide chromone synthase (PCS) from *Aloe arborescens* is a novel plant-specific type III polyketide synthase (PKS) that produces 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA. On the basis of the crystal structures of wild-type and M207G mutant PCS, the F80A/Y82A/M207G triple mutant was constructed and shown to produce an unnatural novel nonaketide naphthopyrone by sequential condensations of nine molecules of malonyl-CoA. This is the first demonstration of the formation of a nonaketide by the structurally simple type III PKS. A homology model predicted that the active-site cavity volume of the triple mutant is increased to 4 times that of the wild-type PCS.

Key words: Pentaketide chromone synthase, *Aloe arborescens*, type III polyketide synthase, naphthopyrone

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Anjiki, N.^{*}, Togashi, M.^{*}, Yoshimitsu, M.^{*}, Kawahara, N., Mikage, M.^{*}: **Evaluation of the Crude Drugs by means of Colorimeter. Part 6. Correlation between the Color and Total Polyphenol Content of**

Geranium Herb.

Journal of Traditional Medicines, **24** (2), 67-71 (2007)

The color of Geranium Herb, which is a quite popular folk medicine for intestinal disorders such as diarrhea, was investigated for the quality evaluation by means of colorimeter. The antidiarrheal effect of Geranium Herb is considered to be due to tannin, which is a kind of polyphenols. On various Geranium Herb, we examined the correlation between the total polyphenol content and color of both the powder and water extract treated with the iron (III) chloride TS. As the result, we found that Geranium Herb with a higher total polyphenol content tended to show lower color index L^* value in the water extract treated with the iron (III) chloride TS. In addition, the total polyphenol quantity was high, while L^* value was low in the foliar part. On the other hand, total polyphenol quantity was low, while L^* value was high in the stem part. Therefore, when the effect of total polyphenol is expected, Geranium Herb with a low ratio of stem part is thought to be of good quality, and it is considered that color index L^* value of the Geranium Herb water extract treated with the iron (III) chloride TS is available in the quality evaluation for Geranium Herb.

Key words: Geranium Herb, quality evaluation, colorimeter, CIE 1976 $L^*a^*b^*$ color system

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Maruyama, T., Kawahara, N., Yokoyama, K.^{*1}, Makino, Y.^{*2}, Fukiharu, T.^{*3}, Goda, Y.: **Phylogenetic relationship of psychoactive fungi based on rRNA gene for a large subunit and their identification using the TaqMan assay (II)**.

Forensic Sci. Int., **163**, 51-58 (2006)

‘Magic mushroom (MM)’ is the name most commonly given to psychoactive fungi containing the hallucinogenic components: psilocin (1) and psilocybin (2). We investigated the rRNA gene (internal transcribed spacer (ITS) and large subunit (LSU)) of two *Panaeolus* species and four *Psilocybe* species fungi (of these, two are non-psilocybin species). On the basis of sequence alignment, we improved the identification system developed in our previous study. In this paper, we describe the new system capable of distinguishing MMs from non-psilocybin *Psilocybe* species, its application data and the phylogeny of MM species.

Keywords: Magic mushroom; rRNA gene; TaqMan PCR; Genus *Psilocybe*; Genus *Panaeolus*

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Uchiyama, N., Kiuchi, F. *², Ito, M. *¹, Honda, G. *¹, Takeda, Y. *³, Khodzimatov, O. K. *⁴, Ashurmetov, O. A.

*⁴: **Trypanocidal Constituents of *Dracocephalum komarovi*.**

Tetrahedron, **62**, 4355-4359 (2006)

Trypanocidal constituents of *Dracocephalum komarovi* were investigated. Under guidance of the in vitro trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease, two new diterpenes, dracocequinones A (1) and B (2), and two known triterpene acids, ursonic acid and ursolic acid, were isolated as trypanocidal constituents, in addition to previously reported diterpenes, cyclocoulterone (4), komaroviquinone (5), dracocephalone A (6) and komarovispirone (7). Furthermore a new diterpene, komarovinone A (3), was isolated, together with four known terpenes. Among these compounds, komaroviquinone (5) showed the most potent activity with minimum lethal concentration of 0.4 μ M. Structure elucidation of the new diterpenes 1-3 was described.

Keywords: *Dracocephalum komarovi*, Diterpene, *Trypanosoma cruzi*

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Shinozaki, Y., Sato, Y., Koizumi, S., Ohno, Y., Nagao, T. and Inoue, K.: **Retinoic acids acting through retinoic acid receptors protect hippocampal neurons from oxygen-glucose deprivation-mediated cell death by inhibition of c-jun-N-terminal kinase and p38 mitogen-activated protein kinase.**

Neuroscience, **147**, 153-163 (2007)

Retinoic acids (RAs), including all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), play fundamental roles in a variety of physiological events in vertebrates, through their specific nuclear receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR). Despite the physiological importance of RA, their functional significance under pathological conditions is not well under-

stood. We examined the effect of ATRA on oxygen/glucose-deprivation/reperfusion (OGD/Rep)-induced neuronal damage in cultured rat hippocampal slices, and found that ATRA significantly reduced neuronal death. The cytoprotective effect of ATRA was observed not only in cornu ammonis (CA) 1 but also in CA2 and dentate gyrus (DG), and was attenuated by selective antagonists for RAR or RXR. By contrast, in the CA3 region, no protective effects of ATRA were observed. The OGD/Rep also increased phosphorylated forms of c-jun-N-terminal kinase (P-JNK) and p38 (P-p38) in hippocampus, and specific inhibitors for these kinases protected neurons. ATRA prevented the increases in P-JNK and P-p38 after OGD/Rep, as well as the decrease in NeuN and its shrinkage, all of which were inhibited by antagonists for RAR or RXR. These findings suggest that the ATRA signaling via retinoid receptors results in the inhibition of JNK and p38 activation, leading to the protection of neurons against OGD/Rep-induced damage in the hippocampus.

Keywords: retinoid, apoptosis, MAP kinase

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Onohara, N. *¹, Nishida, M. *¹, Inoue, R. *², Kobayashi, H. *¹, Sumimoto, H. *³, Sato, Y., Mori, Y. *⁴, Nagao, T. and Kurose, H. *¹: **TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy.**

EMBO J., **25**, 5305-5316 (2006)

Angiotensin (Ang) II participates in the pathogenesis of heart failure through induction of cardiac hypertrophy. Ang II-induced hypertrophic growth of cardiomyocytes is mediated by nuclear factor of activated T cells (NFAT), a Ca²⁺-responsive transcriptional factor. It is believed that phospholipase C (PLC)-mediated production of inositol-1,4,5-trisphosphate (IP₃) is responsible for Ca²⁺ increase that is necessary for NFAT activation. However, we demonstrate that PLC-mediated production of diacylglycerol (DAG) but not IP₃ is essential for Ang II-induced NFAT activation in rat cardiac myocytes. NFAT activation and hypertrophic responses by Ang II stimulation required the enhanced frequency of Ca²⁺ oscillation triggered by membrane depolarization through activation of DAG-sensitive TRPC channels, which leads to activation of L-type Ca²⁺ channel. Patch clamp recordings from single myocyte revealed that Ang II activated DAG-sensitive TRPC-like currents. Among DAG-activating TRPC channels (TRPC3, TRPC6, and TRPC7), the activities of TRPC3 and TRPC6 channels correlated with Ang II-induced NFAT activation and hyper-

trophic responses. These data suggest that DAG-induced Ca^{2+} signaling pathway through TRPC3 and TRPC6 is essential for Ang II-induced NFAT activation and cardiac hypertrophy.

Keywords: angiotensin II, TRP channels, cardiomyocytes

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Nagamatsu, Y.^{*1}, Nishida, M.^{*1}, Onohara, N.^{*1}, Fukutomi, M.^{*1}, Maruyama, Y.^{*2}, Kobayashi, H.^{*1}, Sato, Y. and Kurose, H.^{*1}: **Heterotrimeric G protein $G_{\alpha_{13}}$ -induced induction of cytokine mRNAs through two distinct pathways in cardiac fibroblasts.**

J. Pharmacol. Sci., **101**, 144-150 (2006)

Overexpression of constitutively active (CA) $-G_{\alpha_{13}}$ significantly increased the expression of interleukin (IL) -1β and IL-6 mRNAs and proteins in rat cardiac fibroblasts. IL- 1β mRNA induction by CA- $G_{\alpha_{13}}$ was suppressed by diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, but not by BAPTA-AM, an intracellular Ca^{2+} chelator. In contrast, IL-6 mRNA induction by CA- $G_{\alpha_{13}}$ was suppressed by BAPTA-AM but not by DPI. However, both IL- 1β and IL-6 mRNA induction was suppressed by nuclear factor κB (NF- κB) inhibitors. The CA- $G_{\alpha_{13}}$ -induced NF- κB activation was suppressed by DPI and BAPTA-AM, but not C3 toxin and the Rho-kinase inhibitor Y27632. IL-6 mRNA induction by CA- $G_{\alpha_{13}}$ was suppressed by SK&F96365 (1- $[\beta$ -[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride), an inhibitor of receptor-activated nonselective cation channels, and the expression of CA- $G_{\alpha_{13}}$ increased basal Ca^{2+} influx. These results suggest that $G_{\alpha_{13}}$ regulates IL- 1β mRNA induction through the reactive oxygen species-NF- κB pathway, while it regulates IL-6 mRNA induction through the Ca^{2+} -NF- κB pathway.

Keywords: G protein, interleukin, reactive oxygen species

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Yoshida, T.^{*1*2*3}, Inoue, R.^{*4}, Morii, T.^{*5}, Takahashi, N.^{*1}, Yamamoto, S.^{*1}, Hara, Y.^{*1}, Tominaga, M.^{*2*3}, Shimizu, S.^{*6}, Sato, Y. and Mori, Y.^{*1}: **Nitric oxide activates TRP channels by cysteine S-nitrosylation.**

Nat. Chem. Biol., **2**, 596-607 (2006)

Transient receptor potential (TRP) proteins form plasma-membrane cation channels that act as sensors for diverse

cellular stimuli. Here, we report a novel activation mechanism mediated by cysteine S-nitrosylation in TRP channels. Recombinant TRPC1, TRPC4, TRPC5, TRPV1, TRPV3 and TRPV4 of the TRPC and TRPV families, which are commonly classified as receptor-activated channels and thermosensor channels, induce entry of Ca^{2+} into cells in response to nitric oxide (NO). Labeling and functional assays using cysteine mutants, together with membrane sidedness in activating reactive disulfides, show that cytoplasmically accessible Cys553 and nearby Cys558 are nitrosylation sites mediating NO sensitivity in TRPC5. The responsive TRP proteins have conserved cysteines on the same N-terminal side of the pore region. Notably, nitrosylation of native TRPC5 upon G protein-coupled ATP receptor stimulation elicits entry of Ca^{2+} into endothelial cells. These findings reveal the structural motif for the NO-sensitive activation gate in TRP channels and indicate that NO sensors are a new functional category of cellular receptors extending over different TRP families.

Keywords: nitric oxide, TRP channels calcium

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Zhan, L.^{*1}, Honma, M., Wang, L.^{*1}, Hayashi, M., Wu, D.^{*2}, Zhang, L.^{*2}, Rajaguru, P.^{*3}, Suzuki, T.: **Microcystin-LR is not mMutagenic in vivo in the λ /lacZ tTransgenic mMouse (MutaTMMouse).**

Genes and Environment, **28**: 68-73 (2006)

The water pollution of toxic cyanobacteria (blue-green algae) is causing a serious public health problem in many parts of the world. Microcystin-LR (MCLR) is a potent cyclic heptapeptidic hepatotoxin produced by the cyanobacterium *Microcystis aeruginosa*. MCLR presents acute and chronic hazards to human health and has been linked to primary liver cancer in humans chronically exposed to this peptide toxin through drinking water. To assess the in vivo mutagenicity of MCLR, the λ /lacZ transgenic mice (MutaTMMouse) were treated with MCLR (1 mg/kg per week x 4) and examined for mutant frequencies (MFs) in the lacZ and cII genes of liver and lungs. Micronucleus induction in peripheral blood cells was also assessed. Co-mutagenic effect of MCLR was studied in combination with N-nitrosodiethylamine (DEN). MCLR did not increase

either MFs of the target genes in liver and lungs or micronucleus frequency in the peripheral blood cells of the $\Delta/lacZ$ transgenic mouse. While DEN treatment increased MFs significantly, the co-administration of MCLR did not potentiate its mutagenicity. We conclude that pure MCLR has no in vivo mutagenicity as it failed to induce gene mutation and micronucleus in transgenic mouse. Its tumor promoting effect is independent of its interaction to DNA.

Keywords: Microcystin-LR, N-nitrosodiethylamine, MutaTMMouse

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中島晴信*, 宮野直子*, 松永一朗*, 中島ナオミ*, 鹿庭正昭: 大阪府下における抗菌製品の市販実態調査—1991年から2004年—

大阪府立公衆衛生研究所所報, **44**, 85-116 (2006)

1991年度から2004年度まで, 大阪府下における家庭用抗菌製品の市販実態, 製品表示の調査を継続して実施してきた。その結果をもとに, 抗菌製品の分類表を独自に作成するとともに, データベース化し, 抗菌製品の種類・数量の推移等について評価・解析を進めてきた。

Keywords: antimicrobial agent, commercially available product research, database system

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伊佐間和郎, 鹿庭正昭, 土屋利江: 金属製アクセサリ—類等に含有するカドミウムの分析調査。

中毒研究, **19**, 409-411 (2006)

日本で市販されている金属製アクセサリ—類等に含有する有害元素の分析調査を行った。122検体(98製品)における有害4元素(カドミウム, ヒ素及び水銀)の濃度を蛍光X線分析(XRF)によって測定した。EUのRoHS指令の規制値である0.01%を超えるカドミウムを含有する検体は48点(39.3%)あり, 1%を超えるカドミウムを含有する検体も9点(7.4%)あった。0.01%を超えるカドミウムを含有する48検体における有害元素の酸溶出量を誘導結合プラズマ質量分析(ICP-MS)によって測定した。10 μ gを超えるカドミウムを溶出した検体は28点(58.3%)あり, 100 μ gを超えるカドミウムを溶出した検体も8点(16.7%)あった。10 μ gを超えるヒ素または水銀を溶出する検体は無かった。

Keywords: metal accessory, cadmium

Tamai, M., Isama, K., Nakaoka, R. and Tsuchiya, T.: **Synthesis of a novel beta-tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties.**

J. Artif. Organs, **10**, 22-28 (2007)

To promote the osteogenic properties of osteoblasts, we synthesized a hydroxyapatite (HAp) with beta-tricalcium phosphate (beta-TCP) biphasic calcium phosphate containing Nb ions (NbTCP/HAp). NbTCP/HAp was prepared by annealing precipitates obtained by coprecipitation of an aqueous solution of Ca (NO₃)₂ and a mixture of (NH₄)₂HPO₄ and aqueous Nb solution. The precipitates can be regarded as a calcium-deficient HAp, the PO₄ sites of which are partly occupied by Nb ions. NbTCP/HAp was successfully synthesized by thermal decomposition of the precipitates. NbTCP/HAp enhanced the calcification of normal human osteoblasts (NH₂Ost), and the amount of calcified tissue increased in proportion to the Nb ion concentration in the NbTCP/HAp. The alkaline phosphatase (ALP) activity of NH₂Ost was also enhanced by NbTCP/HAp. Because Nb ions significantly enhance the ALP activity of NH₂Ost, calcification by NbTCP/HAp is considered to be due to enhancement of ALP activity induced by Nb ions dissolved from NbTCP/HAp. These results indicate that NbTCP/HAp can be an effective bone repair material.

Keywords: tissue engineering, calcium phosphate, Nb ion

Wakata, A.*¹, Matsuoka, A., Yamakage, K.*²他15名: **SFTG international collaborative study on in vitro micronucleus test IV. Using CHL cells.**

Mutat. Res., **607**, 88-124 (2006)

Fourteen laboratories participated under the coordination by the SFTG (the French branch of the European Environmental Mutagen Society). Nine coded substances with different modes of action and at different levels were assessed in the micronucleus (MN) test, using a common protocol. Mitomycin C was a positive control. In order to help to define a standard protocol on CHL cells, short and long treatment periods followed by various recovery times, with or without cytochalasin B (Cyt B), were compared. After an evaluation of the acceptability of the assays, the tested chemicals were classified as negative, positive, or equivocal. Mannitol and clofibrate were negative. Bleomycin was positive, with an increase in the number of MN cells in both mono- and bi-nucleate cells when using Cyt B. This was also shown for the anagens colchicine, diethylstilbe-

strol and griseofulvin, as expected. Urethane was equivocal only after long treatment with Cyt B, and negative in all other treatment schedules. In any case, no genotoxic compound would have been missed with schedules including a short and a long treatment time, whether the treatment was followed by a recovery period or not and whether Cyt B was used or not. These results show that CHL cells were suitable for accurately detecting clastogens and aneugens of various types in the in vitro MN test.

Keywords: cytochalasin B, clastogens, aneugens

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Matsumoto, T.^{*1,*2}, Yung, Y.C.^{*1}, Fischbach, C.^{*1}, Kong, H.J.^{*1}, Nakaoka, R. and Mooney, D.J.^{*1}: **Mechanical Strain Regulates Endothelial Cell Patterning in Vitro.**

Tissue Engineering, **13**, 207-217 (2007)

Blood vessels of the vertebrate circulatory system typically exhibit tissue-specific patterning. However, the cues that guide the development of these patterns remain unclear. We investigated the effect of cyclic uniaxial strain on vascular endothelial cell dynamics and sprout formation in vitro in two-dimensional (2D) and three-dimensional (3D) culture systems under the influence of growth factors. Cells preferentially aligned and moved in the direction perpendicular to the major strain axis in monolayer culture, and mechanical strain also regulated the spatial location of cell proliferation in 2D cell culture. Cells in 3D cell culture could be induced to form sprouts by exposure to appropriate growth factor combinations (vascular endothelial growth factor and hepatocyte growth factor), and the strain direction regulated the directionality of this process. Moreover, cyclic uniaxial strain inhibited branching of the structures formed by endothelial cells and increased their thickness. Taken together, these data support the importance of external mechanical stimulation in the regulation of endothelial cell migration, proliferation, and differentiation into primitive vessels.

Keywords: mechanical strain, cell patterning, growth factor combinations

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Nakaoka, R., Hsiong, S.X.^{*1} and Mooney, D.J.^{*2}: **Regulation of chondrocyte differentiation level via co-**

culture with osteoblasts.

Tissue Engineering, **12**, 2425-2433 (2006)

The close apposition of osteoblasts and chondrocytes in bone, and their interaction during bone development and regeneration suggest they may each regulate the other's growth and/or differentiation. In these studies, osteoblasts and chondrocytes were co-cultured in vitro, with both direct and indirect contact. Proliferation of the co-cultured chondrocytes was enhanced by soluble factors produced from the osteoblasts and the differentiation level of the chondrocytes was influenced by the differentiation level of the osteoblasts. In addition, the chondrocytes regulated differentiation of the co-cultured osteoblasts by both soluble factors and direct contact. These data support the possibility of direct, reciprocal instructive interactions between chondrocytes and osteoblasts in a variety of normal processes, and further suggest that it may be necessary to account for this signaling in the regeneration of complex tissues comprised of both cartilage and mineralized tissue.

Keywords: tissue engineering, growth plate, hypertrophic differentiation,

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Umeda-Sawada, R., Fujiwara, Y.^{*1}, Ushiyama, I.^{*1}, Sagawa, S.^{*1}, Morimitsu, Y.^{*1}, Kawashima, H.^{*2}, Ono, Y.^{*2}, Kiso, Y.^{*2}, Matsumoto, A.^{*3} and Seyama, Y.^{*1}: **Distribution and metabolism of dihomo-gamma-linolenic acid (DGLA, 20:3n-6) by oral supplementation in rats.**

Biosci. Biotechnol. Biochem., **70** (9), 2121-2130 (2006)

We compared the dietary effects of dihomo- γ -linolenic acid (DGLA) contained in the DGLA oil produced by a fungus with γ -linolenic acid (GLA) on the fatty acid composition. Wistar rats were fed with three kinds of oil for two weeks as follows: (i) control group: corn oil; (ii) GLA group: borage oil; (iii) DGLA group: DGLA oil/safflower oil = 55:45. The DGLA concentrations in the liver, serum, and brain of the DGLA group were higher than those of the GLA oil group. We also examined the dose effect of DGLA. The DGLA levels in the liver, serum, and brain significantly increased with increasing dosage of DGLA in the diet. DGLA administration significantly increased the ratio of PGE₁/PGE₂ in the rat plasma. The mechanism for GLA administration to improve atopic ec-

zema is thought to involve an increase in the concentration of DGLA metabolized from GLA, so these results suggest that the dietary effect of DGLA would be more dominant than GLA.

Keywords: dihomono- γ -linolenic acid (DGLA), arachidonic acid, delta 5 and delta 6 desaturase

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Sawada, R., Ito, T. and Tsuchiya, T.: **Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells.**

J. Artif. Organs, **9**, 179-184 (2006)

We investigated the expression levels of several genes related to cell proliferation in human mesenchymal stem cells (hMSCs) during in vitro culture for use in clinical applications. In this study, we focused on the relationship between hMSC proliferation and their transforming growth factor β (TGF β) signaling during in vitro culture. The proliferation rate of hMSC gradually decreased and the marked changes in hMSC morphology were not observed in 3 months of in vitro culture. The mRNA expressions of TGF β 1, TGF β 2, and TGF β receptor type I (TGF β RI) in hMSCs increased with the length of cell culture. There had been no change in the TGF β 3, TGF β RII, and TGF β RIII mRNA expressions by the 12th passage from the primary culture (for about 3 months). The mRNA expressions of Smad3 increased, but those of c-myc and nucleostemin decreased with the length of hMSC in vitro culture. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1-2 months in vitro culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGF β family signaling.

Keywords: stem cells, cell proliferation, TGF β signaling

Sun, X.^{*1}, Kurosu, S.^{*} and Shintani, H.: **The expanded application of most probable number to quantitative evaluation of extremely low microbial count.**

PDA J., **60**, 124-134 (2006)

This paper is about the evaluation of the extremely low microbial counts from the field by expanding the most probable number (MPN) methodology when the data fol-

low Poisson distribution in order to achieve more accurate estimation with limited number of data. The MPN values are generally larger than the arithmetic average, indicating a higher sensitivity of the data assay. This approach is justified because Poisson distribution is the mathematical background of the MPN procedure with the Halvorson and Ziegler equation as its foundation. It is considered that the MPN methodology has a potential application for quality control of the extremely low level microbial counting environment in the clean rooms above class B level. However, further studies on the precision and sampling plan based on careful mathematical analysis will help to refine the approach.

Keywords: most probable number, clean room, Poisson distribution

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Banu, N. and Tsuchiya, T.: **Markedly different effects of hyaluronic acid and chondroitin sulfate-A on the differentiation of human articular chondrocytes in micromass and 3-D honeycomb rotation culture.**

J. Biomed. Mater. Res., **80**, 257-267 (2007)

A source of morphologically and functionally available human cartilagenous tissue for implantation is required in the field of tissue engineering. To achieve this goal, we evaluated the effects of hyaluronic acid (HA-810 and 1680 kDa), and chondroitin sulfate (CS-A 16 and C-34 kDa) on human articular chondrocytes (HC) in micromass and rotation culture conditions. Cell proliferation was increased by CS-A 16 kDa under micromass and rotation cultures, while cell differentiation was increased under rotation but not micromass conditions. Proliferation and differentiation due to CS-C 34 kDa were very similar to the control under both culture conditions. With HA, cell proliferation was increased depending on the molecular weight under micromass and rotation conditions. In contrast, chondrocyte differentiation was enhanced under rotation conditions, but decreased under micromass conditions depending on the molecular weight of HA. In both culture conditions, aggrecan gene was continuously expressed. However, the collagen type II gene was more weakly expressed in rotation than the micromass culture conditions. Thus, the chemical structures of polysaccharides, and the culture condition, rotation or micromass, caused differences in chondrogenesis. Keywords: human articular cartilage, hyaluronic acid, chon-

droitin sulfate

Jung, D.-Y., Kang, Y.-B.^{*}, Tsuchiya T. and Tsutsumi, S.^{*} :
A novel non-destructive method for measuring elastic moduli of cultivated cartilage tissues.

Key Engineering, **342-343**, 853-856. (2007)

Accurate measurement of the mechanical properties of artificial or cultivated cartilage is a major factor for determining successive regeneration of defective soft tissues. In this study, we developed a novel method that enabled the bulk modulus (k-modulus) to be measured nondestructively using the relationship between volume and pressure of living soft tissues. In order to validate this method we estimated the bulk modulus of soft silicone rubbers using our new method and a conventional method. The results showed a 5 ~ 10% difference between the results obtained with the two methods. Our method was used subsequently to measure the mechanical properties of cultivated cartilage samples (collagen gel type), that had been incubated for four weeks in the presence or absence of human articular chondrocytes (HACs). Our experiments showed that cultivated cartilage tissues grown in the presence of HACs had a higher bulk modulus (120 ± 20 kPa) than samples grown without HACs (90 ± 15 kPa). The results indicated that our novel method offered an effective method for measurement of volume changes in minute living soft tissues, with the measurements having a high degree of accuracy and precision. Furthermore, this method has significant advantages over conventional approaches as it can be used to rapidly and accurately evaluate the strength of soft tissues during cultivation without causing damage to the specimen.

Keywords: mechanical property, human articular chondrocyte

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Nagira, T., Nagahata-Ishiguro, M. and Tsuchiya, T.: **Effects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression.**

Biomaterials, **28**, 844-850 (2007)

Sulfated hyaluronan (SHya), which is composed of a sulfated group and hyaluronan (Hya), has high activity on and biocompatibility with cells. When normal human epidermal keratinocytes (NHEKs) were incubated in dishes coated with SHya, cell proliferation was suppressed in a dose-dependent manner. The expression levels of keratin 1 and loricrin mRNAs, as detected by real-time RT-PCR, were increased significantly. The expressions of Wnt mRNAs,

which play important roles in cell proliferation and differentiation, were modulated. Wnt4 and Wnt6 mRNA expressions were increased compared to controls, while expression of Wnt5a was similar to the control and that of Wnt7a mRNA was decreased. In addition, the expression of Notch mRNAs, which play a critical role in keratinocyte differentiation, were affected. Notch3 mRNA was increased significantly, while Notch1 mRNA was decreased compared to controls, and expression of Notch2 was similar to that of control. These results suggested that a SHya-coated scaffold might be useful for regulating cell activity in tissue engineering.

Keywords: sulfated hyaluronan, normal human epidermal keratinocyte, differentiation

Ahmed, S. and Tsuchiya, T.: **A mouse strain difference in tumorigenesis induced by biodegradable polymers.**

J. Biomed. Mater. Res., **79A**, 409-417 (2006)

The use of poly-L-lactic acid (PLLA) surgical implants for repair of bone fractures has gained popularity in the past decade. The aim of this study was to evaluate the in vivo effect of PLLA plates on subcutaneous tissue in two mouse strains, BALB/cJ and SJL/J, which have higher and lower tumorigenicity, respectively. Gap-junctional intercellular communication and protein expression of connexin 43 were significantly suppressed, whereas secretion of transforming growth factor-1 and expression of extracellular matrix, insulin-like growth factor binding protein 3, and cysteine-rich intestinal protein 2 were significantly increased in PLLA-implanted BALB/cJ mice when compared with BALB/cJ controls. Finally, tumors were formed after implantation of cultured cells from the more-tumorigenic BALB/cJ, but not SJL/J, mice into nude mice.

Key Words: poly-L-lactic acid, gap-junctional intercellular communication, transforming growth factor- β 1

Uchiyama, S., Matsushima, E., Tokunaga, H., Otsubo Y.^{*1}, Ando.M.^{*2}: **Determination of Phthalaldehydes in Air Using 2,4-dinitrophenylhydrazine-Impregnated Silica Cartridge and High-Performance Liquid Chromatography.**

Journal of Chromatography A, **1116**, 165-171 (2006)

A new method is described for the determination of orthophthalaldehyde in air used for the disinfection of various instruments in hospital. Orthophthalaldehyde in air was collected with a silica gel cartridge impregnated with acidified

2,4-dinitrophenylhydrazine (DNPH-cartridge) and derivatives were analyzed by HPLC. The derivatization was examined by comparing the process with three phthalaldehyde isomer. In the case of iso- and tere-phthalaldehyde, derivatives synthesized with excess of aldehyde consisted mainly of mono-derivatives, and derivatives synthesized with excess of DNPH consisted mainly of bis-derivative. In the case of orthophthalaldehyde, derivative consisted of only bis-derivative. Orthophthalaldehyde was completely retained by the DNPH-cartridge during air sampling, however, the derivatization reaction was incomplete and unreacted orthophthalaldehyde was flushed from the cartridge during the subsequent solvent extraction process. Untreated orthophthalaldehyde and DNPH reacted again in the extraction solvent solution. Immediately after the solvent extraction, both mono- and bis-DNPH hydrazone derivatives were present in the solution. However, over time, the mono-derivative decreased and bis-derivative increased until only the bis-derivative was left allowing accurate determination of the orthophthalaldehyde concentration. The transformation of mono-derivative to bis-derivative was faster in polar solvents. Transformation was found to occur most quickly in acetonitrile solvent and was completed in 4 h in this case. It was possible to measure orthophthalaldehyde in air as bis-derivative using a DNPH impregnated silica cartridge and HPLC analysis.

Keywords: orthophthalaldehyde, 2,4-dinitrophenylhydrazine, HPLC, workplace air

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Marcos,R. ^{*1} Martinez,V. ^{*1} Hernandez,A. ^{*1} Creus,A. ^{*1} Sekara,C. ^{*2} Tokunaga,H. Quinteros,D^{*3}: **Metabolic profile in workers occupationally exposed to arsenic: Role of GST polymorphisms.**

J.Occup.Environ. Med., **48**, 334-341 (2006)

Chronic exposure to inorganic arsenic involves a biotransformation process that leads to the main excretion of organic methylated metabolites, such as monomethylarsonic acid (MMA) and demethylarsinic acid (DMA), as well as the parental inorganic species. Interindividual variation in arsenic metabolism has been extensively reported, and polymorphisms in genes involved in such process could be related to changes in the arsenic excretion profile and the response to chronic exposures. Our analysis of the metabolic profiles in three groups of workers exposed to different arsenic exposure levels showed high amounts of

inorganic arsenic and MMA in the most-exposed workers versus the least-exposed workers, in whom high amounts of DMA were observed. With respect to the role of different genetic polymorphisms in the glutathione S-transferase (GST) genes in the modulation of the urinary profiles, for the overall population only a tendency was just observed between GSTM1 null and MMA excretion as well as between GSTP1 val/val and DMA excretion.

Keywords: arsenic, arsenic metabolites, GST polymorphisms

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Hanioka, N.^{*1}, Obika, N.^{*1}, Nishimura, M.^{*2}, Jinno, H., Tanaka-Kagawa, T., Saito, K.^{*1}, Kiryu, K.^{*2}, Naito, S.^{*2}, Narimatsu, S.^{*1}: **Inducibility of UDP-glucuronosyltransferase 1As by β -naphthoflavone in HepG2 cells.**

Food Chem. Toxicol., **44**, 1251-1260 (2006)

UDP-glucuronosyltransferases (UGTs) are conjugation enzymes, which are regulated in a tissue-specific manner by endogenous and environmental factors. In this study, we focused on UGT1A isoforms (UGT1A1, UGT1A6 and UGT1A9), mainly expressed in the human liver, and examined the inducibility of UGT1As by beta-naphthoflavone (BNF) in human hepatoma HepG2 cells. The cells were pretreated for 72 h with BNF at concentrations of 25, 50 and 100 microM. 7-Ethyl-10-hydroxycamptothecin (SN-38) glucuronidation, used as a probe for UGT1A1, showed sigmoidal kinetics with a Hill coefficient (n) of 1.2-1.3 in control and BNF-pretreated HepG2 cells. The Vmax values were significantly increased 3.6- to 4.3-fold by BNF, whereas there was no significant change in the S50 values by BNF at any concentration examined. On the other hand, 4-methylumbelliferone (4-MU) glucuronidation as a probe for UGT1A6 and UGT1A9 in the control and BNF-pretreated HepG2 cells exhibited a biphasic kinetic pattern. Although Km1 values for the low-Km phase were similar between the control and BNF-pretreated HepG2 cells, Km2 values for the high-Km phase of BNF-pretreated HepG2 cells were reduced to 54-69% of control HepG2 cells. The values of Vmax1 and Vmax2 for the low- and high-Km phases, respectively, were significantly increased 1.9- to 2.6-fold by BNF at 25 and/or 50 microM but not 100 microM. With respect to Vmax (Vmax1 and Vmax2) and Vmax/Km (Vmax1/Km1 and Vmax2/Km2), the values were significantly increased 2.0- to 3.2-fold by BNF at all concentrations examined. Further-

more, real-time reverse transcription polymerase chain reaction using TaqMan probes demonstrated that BNF concentration-dependently induced mRNA levels of UGT1A1 but not UGT1A6 or UGT1A9 in HepG2 cells (1.3- to 6.0-fold). These results suggest that the inducibility of UGT1A isoforms in HepG2 cells by BNF is different from other aryl hydrocarbon receptor agonists previously reported, and should provide useful information for the prediction of drug-drug interactions and toxicological assessment of environmental chemicals.

Keywords: UDP-glucuronosyltransferase (UGT), β -naphthoflavone (BNF), Inducibility

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Hanioka, N.^{*1}, Takeda, Y.^{*1}, Jinno, H., Tanaka-Kagawa, T., Naito, S.^{*2}, Koeda, A.^{*3}, Shimizu, T.^{*3}, Nomura, M.^{*3}, Narimatsu, S.^{*1}: **Functional characterization of human and cynomolgus monkey UDP-glucuronosyltransferase 1A6 enzymes.**

Chem. Biol. Interact., **164**, 136-145 (2006)

UDP-glucuronosyltransferase 1A6 (UGT1A6) is a major isoform in the human liver that glucuronidates numerous drugs, environmental chemicals and endogenous substrates. In this study, human and cynomolgus monkey UGT1A6 cDNAs (humUGT1A6 and monUGT1A6, respectively) were cloned, and the corresponding proteins were heterologously expressed in yeast cells to identify the functions of primate UGT1A6s. The enzymatic properties of UGT1A6 proteins were characterized by the kinetic analysis of serotonin (5-hydroxytryptamine, 5-HT) and 4-methylumbelliferone (4-MU) glucuronidation. humUGT1A6 and monUGT1A6 showed 96% identity in their nucleotide and amino acid sequences. Immunoblotting analysis using an antibody raised against human UGT1A6 showed that protein staining intensities were different between human and cynomolgus monkey UGT1A6 enzymes in microsomal fractions from livers and yeast cells, although both enzymes were detectable. The apparent K (m) value (15 mM) for 5-HT glucuronidation of cynomolgus monkey liver microsomes was significantly higher than that (8.6mM) of human liver microsomes, whereas V (max) values were lower in cynomolgus monkeys (2.8 nmol/min/mg protein) than in humans (8.6 nmol/min/mg protein). No significant species difference was observed in K (m) (approximately

90 microM) or V (max) (approximately 25 nmol/min/mg protein) values for liver microsomal 4-MU glucuronidation. In yeast cell microsomes, K (m) values (approximately 6mM) for 5-HT glucuronidation by recombinant UGT1A6s were similar, while a V (max) value (0.1nmol/min/mg protein) of monUGT1A6 was significantly lower than that (0.7 nmol/min/mg protein) of humUGT1A6. In 4-MU glucuronidation, both K (m) (210 microM) and V (max) (3.5 nmol/min/mg protein) values of monUGT1A6 were significantly higher than those of humUGT1A6 (K (m), 110 microM; V (max), 1.5nmol/min/mg protein). These findings suggest that the enzymatic properties of UGT1A6 were extensively different between humans and cynomolgus monkeys, although humUGT1A6 and monUGT1A6 showed high homology at the amino acid level. The information gained in this study should help with in vivo extrapolation and to assess the toxicity of xenobiotics.

Keywords: UDP-glucuronosyltransferase (UGT), Serotonin (5-HT), Primates

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五十嵐良明, 豊田和弘^{*1}, 小林郁夫^{*2}, 土居 寿^{*2}, 米山隆之^{*2}, 浜中人士^{*2}, 土屋利江: 生体適合性を改良したチタン-ジルコニウム合金: ラット埋植試験におけるチタン-ジルコニウム合金の純チタンおよびジルコニウムと比較した組織反応性と感作性.

日本金属学会誌, **71**, 395-401 (2007)

チタン-ジルコニウム合金の生体適合性を純チタン, ジルコニウム及び陽性対照としてのクロムと比較した. 8ヶ月間, ラットの皮下に埋植した. 血液学的検査ではいくつかの項目で変化があるものの, 埋植した材料によると考えられるものはなかった. 幼若化因子による脾臓リンパ球増殖反応は, 各試料埋植群においてほぼ同程度であった. チタン-ジルコニウム合金片周囲の線維カプセル膜の厚さはクロムに比べて有意に薄く, 線維カプセル内への炎症性細胞の浸潤の程度も純チタンやジルコニウム群よりも少なく, 組織反応強度の得点は試験した材料の中で最も低かった. 更に, チタンやジルコニウム溶液を塗布しても皮膚反応は示さず, 感作も起こらなかった. 以上, チタン-ジルコニウム合金は純チタンなどよりも整形外科用インプラント材料として生体適合性に優れていることがわかった.

Keywords: titanium alloy, biocompatibility, hypersensitivity

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Tahara, M., Kubota, R., Nakazawa, H.^{*}, Tokunaga, H., and Nishimura, T. : **Analysis of active oxon forms of organophosphorus pesticides in water samples using gas chromatography with mass spectrometric detection.**

J. Health Sci., **52**, 313-319 (2006)

We established a method for the simultaneous quantitative analysis of nine organophosphorus pesticides (OPs) and their active oxon forms in water samples using gas chromatography with mass spectrometric detection with solid-phase extraction (SPE). In this method, the lower limit of detection for the nine oxons ranged from 0.5 to 20 ng/ml. Each calibration curve had good linearity, with correlation coefficients (R²) greater than 0.991. In comparing three SPE cartridges, the recovery rate of these compounds extracted from water was highly reproducible using a cartridge of packed silica bonded with C18. The limit of quantification ranged from 2.5 to 200 ng/ml at 500-fold concentrations. When the OPs were examined after chlorination treatment to simulate the water treatment process, they decomposed rapidly and were converted to their oxon forms as primary reaction products of chlorination. Under these established analytical conditions, the behavior of oxons formed in the environment and after water treatment can be determined accurately.

Keywords : oxon, Water Quality Standard, chlorination

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斎藤 勲^{*}, 上野英二^{*}, 大島晴美^{*}, 松本 浩^{*}, 佐々木久美子, 米谷民雄 : **HPLCによる食品中メトプレンの分析法.**

食品衛生学雑誌, **47**, 173-177 (2006)

GC-FIDを用いるメトプレン試験法を見直すための検討を行った。試料からアセトニトリル抽出し、塩析により水層分離後、アセトニトリル層を少量のヘキサンで洗浄、次いでフロリジルカラムで精製してHPLV-UVで測定した。小麦など7種類の試料からの平均回収率は74.6 ~ 82.8%と良好であった。さらに本法を6機関で評価したところ、5種類の試料からの平均回収率は79.4 ~ 84.6%, 併行再現性及び室間再現性の相対標準偏差はそれぞれ2.3 ~ 8.8%, 8.8 ~ 23.6%であった。1機関でらかせいからの回収率が低かったために室間再現性が高くなったのを除いて良好な結果が得られた。検出限界は0.001 ~ 0.02 μg/gであった。

Keywords : methoprene, HPLC, food

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Tsutsumi, T., Amakura, Y., Okuyama, A.^{*1}, Tanioka, Y.^{*2}, Sakata, K.^{*2}, Sasaki, K., Maitani, T.: **Application of an ELISA for PCB 118 to the screening of dioxin-like PCBs in retail fish.**

Chemosphere, **65**, 467-473 (2006)

A commercially available ELISA kit was evaluated for the determination of TEQs of dioxin-like PCBs in retail fish. The ELISA was highly specific for PCB 118, which is generally the most abundant dioxin-like PCB isomer found in fish. The quantitative limit of the ELISA for PCB 118 was 10 ng ml⁻¹ in the standard curve. Good recoveries of PCB 118 (78.7–112.3%) were obtained for spiked purified fish extracts according to the ELISA. No significant interference of the matrix was observed in the ELISA when this purification procedure was used. Recovery tests in which PCB 118 was added to fish samples also resulted in acceptable recoveries (60.2–82.3%) in the ELISA following purification. The ELISA results for fish samples correlated well with the TEQs of dioxin-like PCBs obtained by HRGC/HRMS (r = 0.92, n = 26). These data indicate that the ELISA kit is suitable for screening retail fish for the TEQs of dioxin-like PCBs.

Keywords: dioxin-like PCBs, ELISA, fish

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*2 Daiichi Fine Chemical Co., Ltd.

Tsutsumi, T., Amakura, Y., Sasaki, K., Maitani, T.: **Dioxin concentrations in the edible parts of Japanese common squid and saury.**

J. Food Hyg. Soc. Japan, **48**, 8-12 (2007)

We examined the concentrations of PCDDs, PCDFs and dioxin-like PCBs in muscle and gut tissues from Japanese common squid and saury. The TEQ concentrations in the squid gut samples (1.0 to 14 pg-TEQ/g fresh weight, n=3) were 50-fold larger than those in the muscle tissues (0.020 to 0.22 pg-TEQ/g fresh weight, n=3) taken from the same samples. By contrast, the TEQ concentrations in the saury gut samples (0.35 to 0.63 pg-TEQ/g fresh weight, n=3) were only 1.1 to 1.7-fold greater than those in the muscle tissues (0.33 to 0.37 pg-TEQ/g fresh weight, n=3) from the same samples. The TEQ contents in the squid gut tissues ranged from 60 to 990 pg-TEQ/squid, accounting for about 95% of the total dioxin content of the edible parts of the samples. By contrast, the TEQ contents in the saury gut

tissues ranged from 4.4 to 12 pg-TEQ/saury, accounting for below 25% of the total dioxin content of the edible parts of the samples. These tissues showed comparable PCDD/PCDF-congener and dioxin-like PCB-isomer profiles in both species. The results indicate that squid gut tissues occasionally contain high levels of dioxins, and consumption of this food stuff could potentially significantly increase dietary intake of dioxins.

Keywords: dioxins, Japanese common squid, saury

武川哲也*, 宮原 誠, 米谷民雄: **微生物による香辛料の放射線照射検知スクリーニング法の検知.**

防菌防黴, 35, 251-257 (2007)

照射香辛料の検知法の一つに, 微生物学的方法がある. ここでは収穫から2ヶ月以内の10種類の香辛料を対象とし, 3, 7, 10 kGyの電子線を照射してその芽胞菌数および生菌数のD値の変化を調べた. 生菌数等の試験は食品衛生法記載の方法に準拠した.

大部分の香辛料は線量に従って生菌数のD値が変化することが分かった. この結果, パプリカ, オールスパイス, コリアンダー, オレガノ, パセリは, 生菌数D値が3kGy以上の場合に照射されている可能性が高いことが分かった. また, カシア, シナモンは逆に, 生菌数D値が2 kGy以下の場合に照射されている可能性が高いことが分かった. 一方, 黒胡椒とローレルは芽胞菌数および生菌数のD値共に照射線量による変化は殆どなく, D値による照射の有無の判定は困難であった. 従って, 黒胡椒, ローレルを除く多くの香辛料で, D値を調べることで, 照射・非照射の判定が可能である.

Keywords: food irradiation; detection method; microbial

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後藤典子^{*1}, 山崎正夫^{*2}, 関口正之^{*2}, 等々力節子^{*3}, 宮原 誠: **非照射香辛料に混合した照射香辛料の熱ルミネッセンス法による検知.**

Radioisotopes, 56, 103-113 (2007)

照射香辛料と非照射香辛料を用いて混ぜ合わせ, これを混合試料とし, どの程度の混合比まで判定が可能か調べた. 5.4 kGy照射黒コショウを2, 5, 10, 20% (w/w) 含有する混合試料をそれぞれ5試料ずつ, 合計4組(20個) 作製した. 照射した黒コショウを2%混合した5個の混合試料はいずれも照射による発光極大が認められなかった. 混合比をふやして, 照射試料を5又は10%含有の混合試料では, それぞれの混合試料5個の結果は一致した結果を示さず, 「照射」, 「一部照射」, 「どちらでもない」と判定された. さらに混合比を増やして, 照射試料を20%含有した混合試料は5試料とも「照射」と判

定された. 5.0 kGy照射パプリカを5%含有の混合試料は5試料すべて「照射」と判定された. 照射試料を0.2%以上含有する混合試料は5つの試料すべてについて158~250°Cの範囲に発光極大が認められた.

以上の結果では, スパイスの種類によって「照射」と判定できる混合割合が異なることを示した. TL発光量の積算温度範囲を変えて, 照射・非照射判定に影響するか調べた. 本実験の70~400°Cと被爆線量測定TLD100に適用される約150~250°Cを比較すると, TL比は後者の方が小さくなった. しかし, いずれの場合も「照射」と判定され, 積分範囲の影響はなかった. TLの発光量はUV照射の影響を受けると増加する可能性が示唆されていたので, 照射していない黒コショウとパプリカに紫外線を照射し, TL測定しても放射線照射と判定されることはなかった.

Keywords: food irradiation; detection method; thermoluminescence

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石井里枝*, 堀江正一*, 村山三徳, 米谷民雄: **LC/MS/MSによるハチミツおよびローヤルゼリー中のテトラサイクリン系抗生物質の分析.**

食品衛生学雑誌, 47, 277-283 (2006)

高速液体クロマトグラフィー-質量分析計(LC/MS/MS)を用いたハチミツおよびローヤルゼリー中のオキシテトラサイクリン(OTC), クロルテトラサイクリン(CTC), テトラサイクリン(TC)の3種のテトラサイクリン系抗生物質(TCs)の簡便で精度の高い分析法を検討した. LC/MS/MS条件はポジティブモード, LC条件はカラムにL-column ODSを, 移動相に0.01%ギ酸-アセトニトリルを用いた. 前処理法はハチミツについては精製水で希釈後, ローヤルゼリーについては2%メタリン酸-メタノール混液(6:4)で除タンパク後, それぞれ Oasis HLB, Sep Pak C18で精製した. 本法による定量下限値はハチミツでTCおよびOTCが5 ng/g, CTCが10 ng/g, ローヤルゼリーでTCおよびOTCが25 ng/g, CTCが50 ng/gであった. また, 添加回収率はいずれも75~120%であった.

Keywords: tetracyclines, honey, royal jelly

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Yamaguchi, A.^{*1}, Shimizu, K.^{*1}, Mishima, T.^{*1}, Aoki, N.^{*2}, Hattori, H.^{*1}, Sato, H.^{*1}, Ueda, N.^{*1}, Watanabe, T., Hino, A.^{*3}, Akiyama, H., and Maitani, T.: **Detection Method of Genetically Modified Papaya using duplex**

PCR.

J. Food Hyg. Soc. Japan, **47**, 146-150 (2006)

A simple and rapid method for the identification of genetically modified (GM) papaya, derived from Line 55-1, was developed after several modifications of the Japanese official PCR method. Genomic DNA was directly extracted from the fresh fruit without the lyophilization step using a commercial silica-based kit. To develop a duplex PCR method which simultaneously detects the GM papaya specific gene and the intrinsic papain gene, the papain 2-5' / 3' (184 amplicon size; 184 bp) primer pair for the detection of the papain gene was newly designed within the region of the products (211 bp) amplified using the papain 1-5' / -3' primer pair adopted in the Japanese official PCR method. To detect the GM papaya specific gene, the primer pair, Nos C-5' / CaM N-3' described in the Japanese official method, was used. The DNA sequences of the GM papaya gene and the intrinsic papain gene were co-amplified using the PCR method in a single tube. The developed duplex PCR method can allow the simultaneous detection of the products using agarose gel electrophoresis or microchip electrophoresis. The proposed method was simple and rapid for the GM papaya identification.

Keywords: genetically modified papaya, microchip electrophoresis, duplex PCR

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*2 Japan Food Research Laboratories, Tama

*3 National Food Research Institute

Akiyama, H., Watanabe, T., Kikuchi, H., Sakata, K., Tokichita, S., Hayashi, H., Hino, A.,^{*1} Teshima, R., Swada, J., and Maitani, T.: **A Detection Method of CryIAC Protein for Identifying Genetically Modified Rice using the Lateral Flow Test Assay.**

J. Food Hyg. Soc. Japan, **47**, 111-114 (2006)

We examined the lateral flow strip assay for identifying unauthorized genetically modified (GM) rice. The GM rice expresses the *Bacillus thuringiensis* (Bt) toxin, CryIAC protein, which confers tolerance to insects. The recombinant CryIAC protein was prepared from the inclusion body of the *E. coli* strain inserted in the CryIAC gene using gel filtration chromatography. The lateral flow strip assay for the identification of GM cotton which also expresses the CryIAC protein, was applied to the unpolished rice and polished rice spiked with the recombinant CryIAC protein. The spiked recombinant CryIAC protein can be clearly detected at the level of 0.012 μ g/g in both the unpolished and

polished rice. After loading of the extract on the strip, 60 minutes as the stand time is necessary to clearly detect the CryIAC protein. The detection limit was approximately estimated to be 12 ng CryIAC protein per gram of rice. These results suggest that the lateral flow strip assay for GM cotton could detect the CryIAC protein expressed in GM rice.

Keywords: genetically modified rice; recombinant DNA; lateral flow strip assay; detection method; CryIAC

*1 National Food Research Institute

Toyota, A.,^{*1,2} Akiyama, H., Suhimura, M.^{*1}, Watanabe, T., Sakata, K., Siramasa, Y., Kitta, K.^{*2}, Hino, A.^{*2}, Esaka, M.^{*2}, and Maitani, T.: **Quantification of 35S Promoter and MON810 Maize Construct- Specific Gene in Maize Using a Combination of a Capillary-Type Real- Time PCR System and a Plasmid Reference Standard.**

Biosci. Biotech. Biochem., **70**, 2965-2973 (2006)

For rough qualitative analysis of genetically modified maize contents, rapid methods for measurement of the copy numbers of the cauliflower mosaic virus 35S promoter region (P35S) and MON 810 construct-specific gene (MON 810) using a combination of a capillary-type real-time PCR system with a plasmid DNA were established. To reduce the characteristic differences between the plasmid DNA and genomic DNA, we showed that pretreatment of the extracted genomic DNA by a combination of sonication and restriction endonuclease digestion before measurement is effective. The accuracy and reproducibility of this method for MON 810 content (%) at a level of 5.0% MON 810 mixed samples were within a range from 4.26 to 5.11% in the P35S copy number quantification. These methods should prove to be a useful tool to roughly quantify GM maize content.

Keywords: genetically modified maize; capillary-type real-time PCR system

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*3 Graduate School of Biosphere Sciences, Hiroshima University

Amakura, Y.^{*1}, Kondo, K., Akiyama, H., Ito, H.^{*2}, Hatanoto, T.^{*2}, Yoshida, T.^{*1}, and Maitani, T.: **Conjugated Ketonic Fatty Acids from *Pleurocybella porrigens*.** *Chemical & Pharmaceutical Bulletin.*, **54**, 1213-1215 (2006)

Three novel conjugated long-chain fatty acids (1–3) were yielded from the aqueous methanol extracts of *Pleurocybella porrigens* together with nine known constituents including (8E,10E) - 7, 12- dioxo- 8, 10- octadecadienoic acid (ostopanic acid) (4) . The structures of new fatty acids were characterized as (14RS) - (10E,12E) - 14- hydroxy- 9- oxo- 10, 12- octadecadienoic acid (1) , (12RS) - (8E,10E) - 12- hydroxy- 7- oxo- 8, 10- octadecadienoic acid (2) , and (10E,12E) - 9, 14- dioxo- 10, 12- octadecadienoic acid (3) using spectroscopic methods. **Keywords:** *Pleurocybella porrigens*; Sugihiratake; Tricholomataceae; conjugated ketonic fatty acid

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Amakura, Y.^{*1} Kondo, K., Akiyama, H., Ito, H., Hatano, T., Yoshida, T., and Maitani, T.: **Characteristic Long-Chain Fatty Acid of *Pleurocybella porrigens*.**

J. Food Hyg. Soc. Japan, **47**, 178-181 (2006)

As part of investigation on chemical constituents of *Pleurocybella porrigens* (Japanese name: Sugihiratake) , we analyzed long-chain fatty acids composition of this mushroom using HPLC with photo-diode array detector. The fatty acid of the major UV detected peak was isolated and identified as α -eleostearic acid with conjugated triene moiety based on the spectroscopic methods. Triolein was also obtained in the course of fractionation process. α -Eleostearic acid is suggested to be a characteristic fatty acid of *P. porrigens* because it was not detectable in eight other edible mushrooms examined. Free long-chain fatty acids in *P. porrigens* and other edible mushrooms were analyzed by HPLC after treatment with acidic 2-nitrophenylhydrazine hydrochloride. Oleic acid was characterized as the main fatty acid in *P. porrigens*, and saturated long-chain fatty acids such as linoleic acid, palmitic acid and stearic acid, together with α -eleostearic acid, were also detected.

Keywords: *Pleurocybella porrigens*, Sugihiratake, long-chain fatty acid, α -eleostearic acid, triolein

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Sasaki, H.^{*1}, Akiyama, H., Yoshida, Y.^{*1}, Kondo, K., Amakura, Y.^{*2}, Kasahara, Y., and Maitani, T.: **Sugihirat-**

ake Mushroom (Angel' s Wing Mushroom) -induced Cryptogenic Encephalopathy may Involve Vitamin D Analogues.

Biol. Pharm. Bull., **29**, 2514-2518 (2006)

In autumn 2004, many Japanese patients with renal failure developed cryptogenic encephalopathy by consuming sugihiratake mushroom, a Japanese delicacy. To elucidate the relationship between the cryptogenic cases and this mushroom, we conducted a multivariate analysis of metabolites in 'Probably Toxic' sugihiratake collected from the area of encephalopathy outbreaks, and 'Probably Safe' sugihiratake collected from unaffected areas using UPLC/ToF MS. The results indicate that the presence of milligram quantities of vitamin D-like compounds per 10 g of dried sugihiratake from the areas of encephalopathy outbreaks. Two hypotheses to induce the encephalopathy are proposed: the found metabolites are (1) vitamin D agonists, which induce acute and severe hypercalcemia and/or hyperammonemia and/or vitamin D toxicity, or (2) vitamin D antagonists, which induce acute and severe hypocalcemia.

Keywords: sugihiratake; vitamin D; encephalopathy; ultra performance liquid chromatography (UPLC) ; time-of-flight (ToF) -MS; multivariate analysis

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Yamakawa, H.^{*1}, Akiyama, H., Endo, Y. ^{*1}, Miyatake, K. ^{*1}, Sakata, K., Sakai, S., Moriyama, T.^{*2}, Urisu, A.^{*3}, and Maitani, T.: **A Specific Detection of Soybean Residues in Processed Foods Using Polymerase Chain Reaction.**

Biosci. Biotech. Biochem., **71**, 269-272 (2007)

A sensitive qualitative detection method for soybeans in foods using the polymerase chain reaction (PCR) was developed. For the specific detection of soybeans with a high specificity, the primer pair Gym 81/ Gym 82 was designed on the gene encoding glycine max repetitive sequence. The trace amount of soybean in the commercial food products could be qualitatively detected using this method.

Keywords: food allergy; soybean; Glycine max L.; detection method; PCR

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*¹ Fujita Health University The Second Teaching Hospital

Taguchi, H., Watanabe, S., Hirao, T., Akiyama, H., Sakai, S., Watanabe, T., Matsuda, R., Urisu, A., and Maitani, T.:

Specific Detection of Potentially Allergenic Kiwifruit in Foods Using Polymerase Chain Reaction.

J. Agric. Food Chem., **55**, 1649-1655 (2007)

Kiwifruit (*Actinidia deliciosa* and *Actinidia chinensis*) is allergenic to sensitive patients, and, under Japanese regulations, it is one of the food items that are recommended to be declared on food labeling as much as possible. To develop PCR-based methods for the detection of trace amounts of kiwifruit in foods, two primer pairs targeting the ITS-1 region of the *Actinidia* spp. were designed using PCR simulation software. On the basis of the known distribution of a major kiwifruit allergen (actinidin) within the *Actinidia* spp., as well as of reports on clinical and immunological cross-reactivities, one of the primer pairs was designed to detect all *Actinidia* spp. and the other to detect commercially grown *Actinidia* spp. (i.e., kiwifruit, *Actinidia arguta*, and their interspecific hybrids) except for *Actinidia polygama*. The specificity of the developed methods using the designed primer pairs was verified by performing PCR experiments on 8 *Actinidia* spp. and 26 other plants including fruits. The methods were considered to be specific enough to yield target-size products only from the target *Actinidia* spp. and to detect no target-size products from nontarget species. The methods were sensitive enough to detect 5-50 fg of *Actinidia* spp. DNA spiked in 50 ng of salmon testis DNA used as a carrier (1-10 ppm of kiwifruit DNA) and 1700 ppm (w/w) of fresh kiwifruit puree spiked in a commercial plain yogurt (corresponding to ca. 10 ppm of kiwifruit protein). These methods would be expected to be useful in the detection of hidden kiwifruit and its related species in processed foods.

Keywords: Food allergy; kiwifruit; *Actinidia* spp.; internal transcribed spacer; ITS; PCR

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Kondo, K., Watanabe, A., Iwanaga, Y., Abe, I.^{*1}, Tanaka, H.^{*1}, Nagaoka, M.H., Akiyama, H., Maitani, T.: **Analysis of agaritine in mushrooms and in agaritine-administered mice using liquid chromatography-tandem mass spectrometry.**

J. Chromatogr B., **834**, 55-61 (2006)

A sensitive and specific method for quantifying a genotoxic hydrazine, agaritine, has been developed using liquid chromatography-electrospray ionization tandem mass spectrometry (MS). The recoveries of agaritine from the spiked mushroom samples and spiked mouse plasma were

60.3-114 and 74.4%, respectively. The intra-day precision values for the spiked mushrooms were 5.5 and 4.2%, and the inter-day precision values were 15.0 and 23.0%, respectively. The limit of quantification was 0.01 microg/g (in mushrooms) and 0.01 microg/ml (in plasma).

Keywords: agaritine, mushroom, LCMS

^{*1} University of Shizuoka, School of Pharmaceutical Sciences.

Kondo, K., Watanabe, A., Iwanaga, Y., Abe, I.^{*1}, Tanaka, H.^{*1}, Nagaoka, M.H., Akiyama, H., Maitani, T.: **Determination of genotoxic phenylhydrazine agaritine in mushrooms using liquid chromatography-electrospray ionization tandem mass spectrometry.**

Food Addit Contam., **23**, 1179-1186 (2006)

A new method with good sensitivity and specificity for detecting and quantifying genotoxic hydrazines, agaritine and 4- (hydroxymethyl) phenylhydrazine (HMPH), was developed using liquid chromatography-electrospray tandem mass spectrometry (MS). *Agaricus* spp. contained 1247 and 2017 $\mu\text{g g}^{-1}$ agaritine. Other species of mushroom had no agaritine. We also directly analysed HMPH, an active free hydrazine form of genotoxic agaritine and obtained direct evidence of its absence from mushrooms. A precursor ion scan confirmed that agaritine derivatives, which could exert similar toxicity, were absent.

Keywords: agaritine, mushroom, LCMS, HMPH

^{*1} University of Shizuoka, School of Pharmaceutical Sciences.

Matsunami, K.^{*1}, Takamori, I.^{*1}, Shinzato, T.^{*2}, Aramoto, M.^{*3}, Kondo, K., Otsuka, H.^{*1}, Takeda, Y.^{*4}: **Radical-Scavenging Activities of New Megastigmane Glucosides from *Macaranga tanarius* (L.) MULL.-ARG.**

Chem.Pharm.Bull., **54**, 1403-1407 (2006)

Four new megastigmane glucosides, named macarangiosides A?D (2?5), together with mallophenol B, laurosides E, methyl brevifolin carboxylate, and hyperin and isoquercitrin as a mixture were isolated from the leaves of *Macaranga tanarius* (L.) MULL.-ARG. (Euphorbiaceae). Their structures were elucidated by spectroscopic and chemical analyses. Macarangioside A?C (2?4) and mallophenol B were galloylated on glucose moiety and possessed the potent 2,2-diphenyl-picrylhydrazyl (DPPH) radical-scavenging activity.

Keywords: radical scavenging, megastigmane glucosides,

DPPH

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^{*4} Faculty of Integrated Arts and Sciences, The University of Tokushima

Watanabe, T., Tokishita, S., Spiegelhalter, F.^{*1}, Furui, S.^{*2}, Kitta, K.^{*2}, Hino, A.^{*2}, Matsuda, R., Futo, S.^{*3}, Akiyama, H., and Maitani, T.: **Development and Evaluation of Event-specific Qualitative PCR Methods for Genetically Modified Bt10 Maize.**

J. Agric. Food Chem., **55** (4), 1274-1279 (2006)

In 2005 it was reported that the genetically modified (GM) maize strain or 'event' called Bt10 had been distributed inadvertently in the United States over the previous four years. In order to ensure that grain for food and feed production did not contain trace amounts of Bt10 maize and complied with the applicable regulation, highly sensitive and specific detection of Bt10 maize was required. Accordingly, we developed a novel qualitative PCR system for specific detection of Bt10 maize. Moreover, we amply evaluated the performance characteristics of two PCR systems, our own and the one provided by the developer of Bt10, Syngenta Co. Ltd. It was confirmed that both the qualitative PCR systems can specifically detect Bt10 maize, and the results of a single-laboratory examination suggested that the limit of detection was approximately less than 0.05% for both methods. To evaluate the reproducibility of the methods, we organized an inter-laboratory study with the participation of 6 laboratories and analysis of 240 blind test samples. In this paper, we report, for the first time, the statistical analysis of the qualitative PCR data obtained from the inter-laboratory study. The results of this analysis also revealed that there was no significant difference in the sensitivity between the two aforementioned methods and that the limit of detection of both the methods was less than 0.05%. Thus, we conclude that both of the methods are equally suitable for correct identification and sensitive detection of the unapproved GM maize Bt10 event in test samples.

Keywords: unapproved, genetically modified, maize, Bt10, Qualitative PCR, detection method.

^{*1} Eurofins GeneScan Inc.

^{*2} National Food Research Institute

^{*3} FASMAC Co., Ltd.

渡邊敬浩, 時下祥子, 笠間菊子^{*1}, 鈴木達也^{*1}, 大島赴夫^{*1}, 菊地博之, 日野明寛^{*2}, 穂山 浩, 米谷民雄: **遺伝子組換えトウモロコシ (GA21ならびにMON810系統) の定量PCR法を対象とした外部精度管理試験.**

日本食品化学学会誌, **13** (1), 18-28 (2006)

安全性審査を終了したGMトウモロコシ系統 (GA21ならびにMON810系統) の定量PCR法を対象とした外部精度管理を目的とし, 試料の妥当性 (均一性ならびに安定性) について検証した後, 共同試験を実施した. 共同試験用の試料には, GA21ならびにMON810試料を重量換算でそれぞれ1.0%となるよう混合した試料 (GA21L), GA21試料を5.0%, MON810試料を1.0%となるよう混合した試料 (GA21H) を調製した. 共同試験に参加した33機関から回収した分析結果を基に統計処理を行い, 問題が認められた場合には, その要因について明らかにするために, 調査項目も参照しながら詳細な解析を行った. その結果, 他機関に比べ, 分析精度が明らかに低下していた機関においては, DNAの収量ならびに質および, 内在性遺伝子の測定値に問題があることが明らかになった. この結果から, DNA抽出操作あるいはPCR試薬の調製に改善すべき問題点があるものと推察された.

Keywords: genetically modified maize, PCR, testing method, laboratory-performance study

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渡邊敬浩, 時下祥子, 菊地博之, 坂田こずえ, 日野明寛^{*1}, 穂山 浩, 米谷民雄: **定量PCR法による遺伝子組換えトウモロコシの定量分析に適用される4種のDNA抽出法の比較検討.**

日本食品化学学会誌, **13** (2), 63-71 (2006)

厚生労働省ならびに農林水産省の両省により, 安全性審査を終了した遺伝子組換え (GM) トウモロコシを対象とした定量分析法として, 定量PCR法が定められている. また厚生労働省は, 定量PCRに供するDNAを抽出・精製する方法について, 3種の方法を示している. 一方, 定量PCR法の妥当性確認試験においては, 上記3種の方法とは異なるDNA抽出法が採用されており, 農林水産省はこの方法を, 定量PCR法に適用可能なDNAを抽出するための方法として示している. しかし, これら異なる4種のDNA抽出法が分析結果 (定量値) に与える影響についてはこれまでに明らかにされていない. そこで, 100% GMトウモロコシ試料 (MON810ならびにGA21系統) および, それらを含む2種の混合試料から各DNA抽

出法を用いて抽出されたDNAの質ならびに収量, DNA分解の程度, さらに定量PCR法により得られる定量値について詳細な比較解析を行った。

Keywords: DNA extraction method, genetically modified maize, quantitative polymerase chain reaction, testing method.

*1 (独) 食品総合研究所

Hayashi, Y. and Matsuda, R.: **An expression of uncertainty in calibration using stepwise or separate dilution of a stock solution.**

Anal. Sci., **22**, 889-894 (2006)

ストック溶液から検量線用試料を希釈して作るときの, 検量線の信頼区間を求める理論式を, 実際の問題に適用した。

Keywords: uncertainty, calibration

Ijuin, K.^{*1}, Kusu, F.^{*2}, Matsuda, R. and Hayashi, Y.: **Classification of drugs according to stochastic properties of drug supply at a pharmacy.**

Jpn. J. Pharm. Health Care Sci., **32**, 489-496 (2006)

薬局の薬剤販売量の時系列の自己相関関数及びパワースペクトル密度を計算し, それらに基づいて薬剤を分類した。その結果, 時系列の確率的性質と, 薬効分類との間に関連があることが明らかとなった。

Keywords: drug supply, auto correlation, power spectrum density

*1 Tanashi Yakuhin Co.Ltd.

*2 Tokyo University of Pharmacy & Life Science

Takahashi, M.^{*1}, Ijuin, K.^{*2}, Iwaki, K.^{*3}, Matsuda, R., Hayashi, Y. and Yajima, T.^{*1}: **Stochastic study on the sales pattern of influenza anti-viral agents at pharmacies.**

J. Health Sci., **52**, 431-435 (2006)

抗インフルエンザ薬の薬局での販売量時系列間の相互相関関数から, 地域間の流行の推移を推定した。また, モデル時系列により, 相互相関関数から得られる2事象の時間関係は, 重心の距離を示していることを明らかにした。

Keywords: Tamiflu, crosscorrelation

*1 Toho University

*2 Tanashi Yakuhin Co.Ltd.

*3 Ohu University

Takahashi, M.^{*1}, Kobari, T.^{*2}, Ijuin, K.^{*3}, Iwaki, K.^{*4}, Ishii, F.^{*5}, Matsuda, R., Hayashi, Y. and Yajima, T.^{*1}:

Smoothing of Correlation Functions in the Estimation of the Order of Influenza Infection between Adults and Children.

J. Health Sci., **52**, 435-442 (2006)

大人用と子供用の抗インフルエンザ薬の薬局での販売量時系列を平滑化し, 解析への影響を議論した。

Keywords: Tamiflu, crosscorrelation, smoothing

*1 Toho University

*2 Kosumo Chouzai Yakkyoku Co.Ltd.

*3 Tanashi Yakuhin Co.Ltd.

*4 Ohu University

*5 Meiji Pharmaceutical University

Kobari, T.^{*1}, Takahashi, M.^{*2}, Ijuin, K.^{*3}, Takeuchi, H.^{*4}, Iwaki, K.^{*5}, Ishii, F.^{*6}, Matsuda, R., Hayashi, Y. and Yajima, T.^{*2}: **Quantitative epidemiological understanding of influenza propagation process in Tokyo and environs.**

J. Health Sci., **52**, 637-641 (2006)

東京近辺の薬局におけるタミフル製剤の売り上げ時系列の相互相関関数から, インフルエンザの感染経路と伝播速度を求めた。

Keywords: Tamiflu, influenza propagation, crosscorrelation

*1 Kosumo Chouzai Yakkyoku Co.Ltd.

*2 Toho University

*3 Tanashi Yakuhin Co.Ltd.

*4 Triad Japan Co.Ltd.

*5 Ohu University

*6 Meiji Pharmaceutical University

Hasegawa, H.^{*}, Shinohara, Y.^{*}, Hashimoto, T.^{*}, Matsuda, R. and Hayashi, Y.: **Prediction of measurement uncertainty in isotope dilution Gas Chromatography/Mass Spectrometry.**

J. Chromatogr. A, **1136**, 226-230 (2006)

GC/MSにおける同位体希釈法の精度を理論的に予測し, 実験により検証した。

Keywords: GC/MS, isotope dilution method, precision

* Tokyo University of Pharmacy & Life Science

Choi, D. H.^{*1}, Katakura, Y.^{*1}, Matsuda, R., Hayashi, Y., Hirobe, M.^{*2}, Goda, Y.^{*2}, Ninomiya, K.^{*1} and Shioya, S.^{*1}: **Validation of a method for predicting the precision, limit of detection and range of quantitation in competitive ELISA.**

Analytical Sciences, **23**, 215-218 (2007)

各操作の精度から, 競合ELISA法全体の分析精度

を予測する方法を提案し、市販ELISAキットを用いて検証した。

Keywords: ELISA, prediction of precision

*1 Osaka University

*2 Japan Envirochemicals Ltd.

Matsuda, R., Yoshioka, Y., Akiyama, H., Aburatani, K.^{*1}, Watanabe, Y.^{*1}, Matsumoto, T.^{*2}, Morishita, N.^{*2}, Sato, H.^{*3}, Mishima, T.^{*3}, Gamo, R.^{*4}, Kihira, Y.^{*5} and Maitani, T.: **Interlaboratory evaluation of two kinds of ELISA kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods.**

JAOAC Int., **89**, 1600-1608 (2006)

食品中の特定原材料5品目(卵, 乳, 小麦, そば, 落花生)を検知する2種類のELISAキットの性能を, 5種類の食品を試料とし, 10機関による機関間バリデーションにより評価した. この結果, 両キット共に良好な室間精度を示した.

Keywords: food allergen, ELISA, interlaboratory validation

*1 Morinaga Institute of Biological Science

*2 R&D Center, Nippon Meat Packers

*3 Japan Food Research Laboratories

*4 Nippon Gene Co., Ltd.

*5 Oriental Yeast Co., Ltd.

Zhang, F.^{*1}, Sun, P.^{*2}, Muñoz, E.^{*1}, Chi, L.^{*1}, Sakai, S., Toida, T.^{*3}, Zhang, H.^{*4}, Mousa, S.^{*4} and Linhardt, R. J.^{*1}: **Microscale isolation and analysis of heparin from plasma using an anion exchange spin column.**

Anal. Biochem., **353**, 284-286 (2006)

Heparin and low-molecular weight heparin can be recovered from human plasma using a simple procedure involving protease digestion and strong anion-exchange chromatography on a spin column, followed by salt release and methanol precipitation. The recovered heparin is free of most contaminants, containing only 0.87 μ g/ml of the endogenous chondroitin sulfate plasma peptidoglycan, bikunin. Approximately 90% of heparin, at a concentration of 4 μ g/ml, was recovered from 2 ml of plasma and in sufficient purity to be quantified by carbazole assay and to have its molecular weight properties and disaccharide composition determined.

Keywords: heparin, low-molecular weight heparin, anion-exchange spin column

*1 Rensselaer Polytechnic Institute

*2 Zhejiang University of Technology

*3 Graduate School of Pharmaceutical Sciences, Chiba Uni-

versity

*4 Albany College of Pharmacy

Sakai, S., Akiyama, H., Sato, Y., Yoshioka, Y., Linhardt, R. J.^{*1}, Goda, Y., Maitani, T. and Toida, T.^{*2}: **Chondroitin Sulfate Intake Inhibits the IgE-mediated Allergic Response by Down-regulating Th2 Responses in Mice.**

J. Biol. Chem., **281**, 19872-19880 (2006)

Chondroitin sulfate (CS) was administered orally to BALB/c mice immunized intraperitoneally with ovalbumin (OVA) and/or dinitrophenylated OVA. The titers of antigen-specific IgE and IgG1 in mouse sera were determined. The antigen-specific IgE production by mice fed ad libitum with CS was significantly inhibited. We also examined the effect of feeding CS on immediate-type hypersensitivity. One hour after antigen stimulation, the ears of mice fed with CS swelled less than those of the control mice. Furthermore, the rise in serum histamine in the mice fed with CS under active systemic anaphylaxis was significantly lower than that in the controls. We next examined the pattern of cytokine production by splenocytes from mice followed by re-stimulation with OVA in vitro. The splenocytes from the mice fed with CS produced less interleukin (IL) -5, IL-10, and IL-13 than those from the control group. In contrast, the production of interferon-gamma and IL-2 by the splenocytes of mice fed with CS was not significantly different from those in the control mice. In addition, the production of transforming growth factor-beta from the splenocytes of mice fed with CS was significantly higher than that of the control mice. Furthermore, we showed that the percentages of CD4+ cells, CD8+ cells, and CD4+CD25+ cells in the splenocytes of mice fed with CS are significantly higher than those of the control. These findings suggest that oral intake of CS inhibits the specific IgE production and antigen-induced anaphylactic response by up-regulating regulatory T-cell differentiation, followed by down-regulating the Th2 response.

Keywords: chondroitin sulfate, Th1/Th2 balance, splenocytes

*1 Rensselaer Polytechnic Institute

*2 Graduate School of Pharmaceutical Sciences, Chiba University

Nagaoka, M.H., Yamazaki, T., Nishimura, T. and Maitani T.: **Application of the popliteal lymph node assay (PLNA) for evaluation of the antigenicity of**

water-soluble food colors.

Japanese Journal of Food Chemistry, **13**, 6-10 (2006)

The mouse popliteal lymph node assay (PLNA) has been proposed as an immunotoxicological test to predict the allergenicity of chemicals without additional adjuvant. Although a PLN response in the primary PLNA is also observed in association with non-specific activation induced by some irritants, the PLN response in the secondary PLNA in previously sensitized animals is used to detect memory immune responses without using adjuvant. In this study PLNA was applied to evaluate the antigenicity of water-soluble food colors. The PLN cellularity indices of individual dyes were calculated from the cell counts of exposed and control PLNs of mice. The xanthene dyes Food Red No.3, Food Red No.104, and Food Red No.105, but not Food Red No.106 gave a high PLN cellularity index. The azo dyes and triphenylmethane dyes were not associated with any increase in PLN cellularity index. Indigo Carmine (Food Blue No.2) also had a high PLN cellularity index. The reactivity of the PLNA correlated well with the chemical structure category of the synthetic dyes. The high PLN cellularity indices were considered to be associated with protein binding.

Keywords: popliteal lymph node assay, PLNA, LLNA, synthetic dye, food additive

Analysis of inorganic arsenic in foods by hydride generation-cold trap-atomic absorption spectrophotometry.

Metal Ions in Biology and Medicine, **9**, 75-77 (2006)

The JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has set a PTWI (provisional tolerable weekly intake) value of arsenic at a quantity of more toxic inorganic arsenic, since the toxicity of arsenic in foods differs greatly between inorganic arsenic and organic arsenic. To determine the inorganic arsenic contents in food samples such as seaweed, rice and water samples, a speciation analysis method by hydride generation-cold trap-atomic absorption spectrometry was applied. To extract inorganic arsenic efficiently, arsenic in foods was extracted with mixed acids (nitric acid and perchloric acid). When some water samples containing germanium as organic germanium compound at the high concentrations were applied to this system, the peak of germane was obviously detected at an earlier retention time than that of arsine in spite of the use of lamp for arsenic detection. Thus, arsine could be detected separately from germane by hydride generation-cold

trap-atomic absorption spectrometry, even if both arsenic and germanium were present.

Keywords: inorganic arsenic, food, speciation, hydride generation-cold trap-atomic absorption spectrometry

Decrease of arsenic in edible brown algae *Hijikia fusiforme* by the cooking process.

Applied Organometallic Chemistry, **20**, 585-590 (2006)

A type of edible sea brown algae, *Hijikia fusiforme*, contains a high concentration of inorganic arsenic. In July 2004, the British Food Standard Agency (FSA) advised people not to eat a type of seaweed called Hijiki because it contained high levels of arsenic. We examined the removal of inorganic arsenic compounds in *Hijikia fusiforme* by performing a soaking procedure with pure water, and the excretion of arsenic contained in Hijiki was investigated in mice. The total arsenic was measured by hydride generation-atomic absorption spectrometry (HG-AAS), and the speciation analysis of arsenic was monitored by high-performance liquid chromatograph coupled with inductively coupled plasma mass spectrometry (HPLC/ICP-MS). It was observed that 28.2-58.8% (w/w) of the total arsenic in edible alga *Hijikia fusiforme* was eluted with water, and 49.3-60.5% (w/w) of arsenic in the residue of Hijiki was dissolved by cooking. Thus, 88.7-91.5% (w/w) of arsenic in Hijiki is removable by the cooking process. When Hijiki was given to mice, dimethylarsinic acid (DMAA) was mainly metabolized in urine. It became evident that soaking with water and cooking are effective for removing arsenic in edible brown algae.

Keywords: *Hijikia fusiforme*, arsenic, HPLC/ICP-MS, cooking process, seaweed

*1 Tokyo University of Pharmacy and life Sciences

*2 National Fisheries University

The effects of retentivity in footpads on evaluation of the antigenicity of water-soluble substances by PLNA.

Japanese Journal of Food Chemistry, **13**, 109-113 (2006)

The mouse popliteal lymph node assay (PLNA) has been applied as an immunotoxicological test to predict the allergenicity of chemicals. We applied this test for evaluating the antigenicity of water-soluble dyes. In our

previous study, dyes which did not increase the PLN cellularity index showed an immediate disappearance from the footpads of mice. Food Red No.106 (R106) dissolved in water, did not increase the PLN cellularity index. Food Red No.105 (R105) dissolved in water, showed a strong PLN cellularity index. In this study, we investigated the effect of prolonged retention of dyes in the footpad on the antigenicity of dyes detected by PLNA. Each dye sample was applied to PLNA in an undissolved form using adjuvant, alum (aluminum hydroxide) adjuvant or incomplete Freund's adjuvant (IFA), so as to be retained in the footpads for a prolonged period. The undissolved forms of R106 were retained in the footpads and did not increase the PLN cellularity index, regardless of which adjuvant was used. Thus, the relationship between the prolonged retention of dyes in the footpad and the antigenicity of dyes was not observed. Our study suggests that the antigenicity by PLNA depends on chemical structures of dyes, not on retention period in the footpads

Keywords: popliteal lymph node assay, PLNA, IFA, alum, adjuvant

Yamamoto, M.^{*1}, Zhu, C.^{*2}, Yi, L.^{*2}, Rong, Z.^{*2}, Miura, Y.^{*1}, Izumi, M.^{*2}, Nakajima, S.^{*1}, Tanamoto, K., Shimizu, S.^{*3}, Baba, N.^{*1}: **Synthesis of Lipid Derivatives of Pyrrole Polyamide and Their Biological Activity.**

Biosci. Biotechnol. Biochem., **71**, 1078-82 (2007)

Novel fatty acyl and phospholipid derivatives of pyrrole polyamide were synthesized. Their cytotoxicity against a cancer cell line of MT-4 cells and those infected by human immunodeficiency virus (HIV) was examined. Although no anti-HIV activity was found, their cytotoxicity against the cancer cells was significantly enhanced by introducing a lipophilic group into the pyrrole polyamide.

Keywords: AIDS, anti-HIV activity, pyrrole polyamide

^{*1} Okayama University

^{*2} Beijing Institute of Technology, China

^{*3} Kyoto University

Kitamura, Y.^{*1}, Iwasaki, T.^{*1}, Mifune, M.^{*1}, Saito, Y.^{*1}, Sato, K.^{*1}, Yomota, C., Tanamoto, K.: **Standard infra-red absorption spectrum of betaine and optimal conditions for its measurement.**

J. Food. Hyg. Soc. Japan, **47**, 232-236 (2006)

The infrared absorption (IR) spectrum is often used as a standard reference in identification tests of food additives in Japan. In the case of betaine, many different IR

spectra have been reported and, therefore, it is necessary to establish an IR spectrum that is reproducible and reliable enough to be used as a standard for identification. In the present study, suitable conditions to obtain a standard IR spectrum were examined from various viewpoints, including pretreatment, selection of method, and measuring technique. The KBr disk method, which has generally been used to identify betaine, was found to be humidity-dependent, and there was also an interaction between betaine and KBr. A reproducible IR spectrum suitable as a standard could be obtained by drying betaine at 105 degrees C for 3 hours over phosphorus pentoxide, and then measuring the IR spectrum by the liquid paraffin (Nujol) paste method.

Keywords: standard IR spectrum, betaine, food additives

^{*1} Okayama University

Sasaki, C. Vårum, K. M.^{*1}, Itoh, Y.^{*2}, Tamoi, M.^{*3}, Fukamizo, T.^{*3}: **Rice chitinases: sugar recognition specificities of the individual subsites.**

Glycobiology, **16**, 1242-1250 (2006)

Sugar recognition specificities of class III (OsChib1a) and class I (OsChia1cΔChBD) chitinases from rice, *Oryza sativa* L. were investigated by analyzing ¹H- and ¹³C-nuclear magnetic resonance spectra of the enzymatic products from partially N-acetylated chitosans. The reducing end residue of the enzymatic products obtained by the class III enzyme was found to be exclusively acetylated, whereas both acetylated and deacetylated units were found at the nearest neighbor to the reducing end residue. Both acetylated and deacetylated units were also found at the nonreducing end residue and its nearest neighbor of the class III enzyme products. Thus, only subsite (-1) among the contiguous subsites (-2) to (+2) of the class III enzyme was found to be specific to an acetylated residue. For the class I enzyme, the reducing end residue was preferentially acetylated, although the specificity was not absolute. The nearest neighbor to the acetylated reducing end residue was specifically acetylated. Moreover, the nonreducing end residue produced by the class I enzyme was exclusively acetylated, although there was a low but significant preference for deacetylated units at the nearest neighbor to the nonreducing end. These results suggest that the three contiguous subsites (-2), (-1), and (+1) of the class I enzyme are specific to three consecutive GlcNAc residues of the substrate. In rice plants, the target of the class I enzyme might be a consecutive GlcNAc sequence probably in the cell wall of fungal pathogen, whereas the class III enzyme might act toward

an endogenous complex carbohydrate containing GlcNAc residue.

Keywords: chitinase, *Oryza sativa* L., partially N-acetylated chitosan

*¹ Norwegian University of Science and Technology

*² Akita Research Institute of Food and Brewing

*³ Kinki university

Sugimoto, N., Yomota, C., Furusho, N., Sato, K., Yamazaki, T., Tanamoto, K.: **Application of liquid chromatography-nuclear magnetic resonance spectroscopy for the identification of ethyldimethylpyrazine, a food flavouring agent.**

Food Add. Contam., **23**, 1253-1259 (2006)

The application of liquid chromatography-nuclear magnetic resonance spectroscopy (LC-NMR) for the direct identification of ethyldimethylpyrazine, a food flavouring agent, has been studied. The commercial product is a mixture of two regio-isomers, 2-ethyl-3,5-dimethylpyrazine (**1**) and 2-ethyl-3,6-dimethylpyrazine (**2**); however, the exact composition of the mixture is unknown. Structural characterization by LC-MS and GC-MS was not possible because both regio-isomers yield the same molecular related ion and ion fragmentation. To rapidly identify the two regio-isomers, the product was analyzed by LC-NMR with on-flow and fraction loop modes. From the results, the structure elucidations of the two regio-isomers could be carried out without the need to isolate the isomers by the usual procedures.

Keywords: LC-NMR, dimethylethylpyrazine, flavouring agent

金 哲龍, 多田敦子, 杉本直樹, 佐藤恭子, 増田愛乃*, 山形一雄*, 山崎 壮, 棚元憲一: **既存添加物ウルシロウの成分分析.**

食品衛生学雑誌, **47**, 167-172 (2006)

既存添加物ウルシロウの成分組成についてはまだ明らかにされていない。そこで、安全性試験試料のウルシロウ製品を用い成分分析を行った。ウルシロウの構成脂肪酸は、パルミチン酸、オレイン酸、ステアリン酸であった。主成分はトリグリセリドであり、Glycerol tripalmitate (30.7%), glycerol dipalmitate monooleate (21.2%), glycerol dioleate monopalmitate (2.1%), glycerol monooleate monopalmitate monostearate (2.6%), glycerol dipalmitate monostearate (5.6%), glycerol distearate monopalmitate (1.4%) を含んでいた。また、glycerol dipalmitate monooleateの脂肪酸結合位置が異なる異性体をLC/MS/MS分析により分別定量した。

Keywords: Urushi wax, triglyceride, LC/MS

* 日本大学

菅野慎二, 河村葉子, 六鹿元雄, 棚元憲一: **ラップフィルムおよびキャップシーリング中のエポキシ化大豆油およびエポキシ化亜麻仁油の分析.**

食品衛生学雑誌, **47**, 89-94 (2006)

可塑剤または安定剤として使用されるエポキシ化大豆油 (ESBO) およびエポキシ化亜麻仁油 (ELO) について、ラップフィルムおよびキャップシーリング中の分析法を検討した。アセトン-ヘキサソール (3:7) で抽出してアルカリ分解およびメチル化後、ジオキソラン誘導体としてGC/MSにより測定した。本法の回収率は92.6 ~ 104.4%と良好であり、定量限界はラップフィルムでESBO 0.01 mg/gおよびELO 0.02 mg/g, キャップシーリングで0.04 mg/gおよび0.08 mg/gであった。我が国の市場に流通する製品を調査したところ、PVC製ラップフィルムではESBOまたはELOが34.7 ~ 82.8 mg/g, PVDC製ラップフィルムではELOが8.60および11.4 mg/g, PVC製キャップシーリングではESBOが5.47 ~ 399 mg/gとすべての製品から検出された。含有量の少ないものは安定剤, 多いものは可塑剤として添加されたものと推定された。

Keywords: wrapping film, cap sealing, epoxidized soybean oil

尾崎麻子*, 川崎智恵, 河村葉子, 棚元憲一: **食品用紙製品からのビスフェノールA及びベンゾフェノン類の溶出.**

食品衛生学雑誌, **47**, 99-104 (2006)

食品用板紙製品15検体およびバージンパルプ紙製品6検体について、ビスフェノールA, ベンゾフェノン, 4-(ジメチルアミノ)ベンゾフェノン, ミヒラーズケトンおよび4,4'-ビス(ジエチルアミノ)ベンゾフェノンの食品擬似溶媒への溶出を検討した。バージンパルプ紙製品では一部でビスフェノールAとベンゾフェノンを含有していたが、いずれの化合物も溶出は認められなかった。一方、食品用板紙製品はいずれも再生紙を用いており、全ての製品でビスフェノールAやベンゾフェノン類の含有が認められた。ビスフェノールAは20%エタノール, ベンゾフェノン類は95%エタノールで溶出量が高くなる傾向がみられたが、それらの溶出量はほとんど20 ng/mL以下, 平均値では5 ng/mL以下であり、紙製品の使用状況, 接触食品の摂取量, 各化合物のTDIやNOAEL等を考慮すれば安全性に懸念がないと判断された。

Keywords: paper, bisphenol A, benzophenones

* 大阪市環境科学研究所

Ohno, H.^{*}, Kawamura Y.: **Analysis of vinylidene chloride and 1-chlorobutane in foods packaged with polyvinylidene chloride casing films by headspace GC/MS.**

Food Add. Contam., **23**, 839-844 (2006)

In the previous paper, we investigated the headspace GC/MS analysis of residual vinyl chloride and vinylidene chloride (VDC) in polyvinyl chloride and polyvinylidene chloride (PVDC) products. During that experiment, an unknown peak was detected from some PVDC casing films. In this paper, the peak was identified as 1-chlorobutane (1-CB). Next, a headspace GC/MS method was developed for the simultaneous determination of VDC and 1-CB in foods packaged with PVDC casing films. The recoveries of VDC and 1-CB in foodstuffs were 94.5-103.9% and 85.8-120.3%, respectively. Among 13 samples tested, VDC was detected at 0.001-0.020 μ g/g in 11 foodstuffs, and 1-CB was detected at 0.004-0.040 μ g/g in all. VDC was detected at 0.04 μ g/g in one casing film, and 1-CB was detected in all casing films. The results suggested that these compounds migrated from the casing films into the foodstuffs.

Keywords: vinylidene chloride, 1-chlorobutane, PVDC

^{*} Nagoya City Public Health Research Institute

菅野慎二, 河村葉子, 六鹿元雄, 棚元憲一: **瓶詰キャップシーリング中のエポキシ化大豆油の調査.**

食品衛生学雑誌, **47**, 196-199 (2006)

我が国の市販瓶詰の金属キャップに塗布されたシーリング103検体について, 形状, 材質, エポキシ化大豆油 (ESBO) 含有量, 共存可塑剤などを調査した. シーリングの材質は97%がポリ塩化ビニルであり, ごく一部がポリエチレンおよびアクリル樹脂であった. ESBOは全検体から0.006 ~ 42.4%の含有量で検出され, ベビーフード, ジャム用では高く, 飲料用では低かった. また, ラグキャップやプレスオンツイストキャップでは高く, ピルファーブルーキャップでは低く, スクリューキャップではばらつきが大きかった. スクリューキャップおよびラグキャップの一部からはフタル酸ジ (2-エチルヘキシル) (DEHP), フタル酸ジイソデシル (DIDP) などの可塑剤が検出され, これらのシーリングのESBO含有量はESBOのみの1/10以下と低かった. 今回の調査により, 我が国で流通するほぼすべてのキャップシーリングがESBOを含有し, 80%が主可塑剤として使用されていることが判明した.

Keywords: cap sealing, bottled food, epoxidized soybean oil

河村葉子, 菅野慎二, 六鹿元雄, 棚元憲一: **瓶詰食品中のエポキシ化大豆油の分析.**

食品衛生学雑誌, **47**, 196-199 (2006)

食品中のエポキシ化大豆油 (ESBO) の分析法について検討したところ, 食品由来の脂質によりESBOのエステル分解が阻害されやすく, また内標準の11,14-ジエポキシエイコサン酸エチルはより阻害を受けやすいためばらつきがみられた. そこで, 試料量を減じ標準添加法で定量したところ, 添加回収率は87.1および98.9%と良好であり, 定量限界は5.0 μ g/gであった. 我が国の市販瓶詰食品について調査を行ったところ, 瓶詰ベビーフードは14検体のいずれもESBOが検出されなかった. 我が国の瓶詰ベビーフードの脂質含有量や流動性が低いため, ESBOの移行が起こりにくかったものと推定された. 一方, 脂質含有量が高いレバーペースト, パスタソース, ラー油漬けメンマおよびラー油の4検体からは25.7 ~ 494.0 μ g/gのESBOが検出された. しかし, これらの食品によるESBO摂取量は, EUのTDIである1 mg/kg体重/日を上回ることはなく, 安全性に問題はないと判断された.

Keywords: bottled food, epoxidized soybean oil, cap sealing

Boonmar, S.^{*1}, Morita, Y.^{*2}, Fujita, M.^{*2}, Sangsuk, L.^{*3}, Suthivarakom, K.^{*3}, Padungtod, P.^{*4}, Maruyama, S.^{*5}, Kabeya, H.^{*5}, Kato, M.^{*2}, Kozawa, K.^{*2}, Yamamoto, S., and Kimura H.^{*6}: **Serotypes, antimicrobial susceptibility, and gyr A gene mutation of Campylobacter jejuni isolates from humans and chickens in Thailand.**

Microbiol. Immunol., **51**, 531-537 (2007)

In Thailand, 51% (36/70) Campylobacter jejuni isolates from humans and 68% (47/69) isolates from poultry were classified into 10 Penner serotype (serotype B, C, R, E, G, A, K, D, I, and L) and 9 serotypes (serotype A, C, I, K, B, E, S, D, and L), respectively. The rate of antimicrobial drug resistance to nalidixic acid, ciprofloxacin, ampicillin, tetracycline, and erythromycin shown by human isolates were 96%, 96%, 29%, 57%, and 14%, while that shown by poultry isolates were 77%, 77%, 22%, 26%, and 17%, respectively. All quinolone-resistant strains contained a mutation in the gyrA gene (T86 to I86), suggesting that the strains were already widespread in Thailand.

keywords: Antimicrobial drug resistance, Campylobacter, gyr A mutation

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Igimi, S., Yamasaki, M.^{*1}, Yamamoto, S. and Amano, F.^{*2}: **An anti-Salmonella antibody prevents the *Salmonella enterica* serovar Enteritidis from infecting a human intestinal epithelial cell line, Caco-2, by interacting with flagella.**

Bioscience Microflora, **25**, 117-119 (2006)

A polyclonal antibody of *Salmonella enterica* serovar Enteritidis (SE), designated RY542, inhibited SE from attaching to and invading the human intestinal epithelial cell line, Caco-2, in a dose-dependent manner. A major immunoreactive band of 55 kDa obtained in western blotting using RY542 was identified as FliC. An affinity-purified anti-flagellin antibody from RY542 similarly inhibited the infection. These results suggest that RY542 is a neutralizing antibody that blocks the infection of SE in Caco-2 cells by interacting with flagella, and that flagellin may potentially be useful as a component of a Salmonella vaccine.

Keyword : Salmonella Enteritidis, neutralizing antibody, Caco-2, flagella

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Toshiyuki Tsutsui^{*1} and Fumiko Kasuga: **Assessment of impact of cattle testing strategies on human exposure to BSE agents in Japan.**

Int. J. Food Microbiol., **107**, 256-264 (2006)

In Japan, cattle screening tests for BSE are conducted at slaughterhouses for surveillance purposes and as a meat safety measure, but the public health impacts of such testing and the subsequent removal of positive animals from the food chain have not been quantitatively assessed. We evaluated the influence of removing specified risk materials and the alternation of age limits for testing cattle at the slaughterhouse on human exposure to the BSE agent in Japan by constructing a probabilistic risk model. A stochastic model using Monte Carlo simulation was constructed in order to estimate the BSE infectivity destined for the food chain from a single BSE-infected animal at slaughter. The impact of different testing strategies and risk material removal were then compared. Murine intra-cerebral ID50 (m.i.c. ID50) units were used as units for BSE infectivity. Sensitivity analysis was conducted for key input variables by changing values within plausible ranges. The expected

fraction of BSE-infected cattle presented for slaughter that would be detected by screening tests was 20%, even if all slaughtered cattle were tested. The removal of risk materials reduced the median value estimate of infectivity destined for human consumption by 95%. Cattle screening tests reduced the infectivity further, but reduction efficacy did not differ among the various testing strategies. Sensitivity analysis indicated that the characteristics of BSE infectivity accumulation during the incubation period, extension of the incubation period, and lowering the detection limit of screening tests had no significant impact on relative infectivity reduction, which remained stable irrespective of testing strategy or changes in these parameters.

This study suggests that the impact of changing the age limit for testing cattle on beef safety is small, provided that the removal of risk materials is conducted properly.

Keywords: BSE, Stochastic model, Japan; Testing strategies

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Asakura, H., Ishiwa, A., Arakawa, E.^{*1}, Makino, S.^{*2}, Okada, Y., Yamamoto, S., Igimi, S.: **Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state.**

Environ Microbiol., **9**, 869-879 (2007)

Vibrio cholerae is an aetiological agent of cholera that inhabits marine and estuarine environments. It can survive harsh environments by entering the viable but non-culturable (VBNC) state, but the related changes in gene expression have not been described. Here, we experimentally induced the VBNC state in *V. cholerae* O1, by incubation in artificial seawater at 4 degrees C. Bacterial cells that were incubated for 70 days retained their membrane integrity and were pathogenic, colonizing the gut of iron-dextran-treated mice, even though they formed no colonies on tryptic soy agar (TSA) or TSA amended with pyruvate. We therefore used this stage of cells as the VBNC bacteria. We compared the global transcription pattern of the VBNC cells with that of stationary-phase cells grown in rich medium. A total of 100 genes were induced by more than fivefold in the VBNC state, and the modulated genes were mostly those responsible for cellular processes. Furthermore, real-time RT-PCR analysis verified the changes in the expression levels, showing that the VC0230 [iron (III) ABC transporter], VC1212 (polB), VC2132 (fliG) and VC2187 (flaC) mRNAs were increased in the non-culturable state. Thus, these genes may be suitable markers for the detection of VBNC *V. cholerae*. To our knowledge, this

is the first report of a comprehensive transcriptome analysis of *V. cholerae* in the VBNC state. The significance of this gene expression profile compared with those of in vivo isolates and non-stressed bacteria (culturable in vitro) is its potential to provide information about the public health risk from dormant bacteria.

Keywords: *Vibrio cholerae*, viable but non-culturable, transcriptome

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Okada, Y., Okada, N.*¹, Makino, S-I.*², Asakura, H., Yamamoto, S., Igimi, S.: **The sigma factor, RpoN (σ54) is involved in osmotolerance in *Listeria monocytogenes*.**

FEMS Microbiol. Lett. **263**, 54-60 (2006)

食品媒介感染症原因菌リステリアのシグマ因子のひとつであるRpoNのコード遺伝子を欠失させた変異株を作成し、その食塩耐性能における役割を明らかにした。変異株では、高食塩濃度下での増殖に重要なカルニチンの利用能が親株に比べ著しく低下していた。一方カルニチントランスポーターのコード遺伝子*opuC*の発現及び菌体内へのカルニチン取り込み活性には親株と差が見られず、カルニチン利用能の低下は細胞膜構成成分の変化によるものと思われた。

Keywords: *Listeria monocytogenes*, osmotolerance, sigma factor

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飯田真理子*^{1,3}, 朝倉 宏, 牧野壮一*², 岡村 登*³, 伊藤健一郎*¹: **我が国における腸管病原性大腸菌O157:H45の付着関連遺伝子*eae*, *EAF*, *bfpA*の保有状況.** *感染症学雑誌*, **80**, 531-533 (2006)

わが国に広く分布すると考えられる腸管病原性大腸菌O157:H45について、病原因子の保有状況を調査した。局在性付着に關与する*bfpA*はほぼ共通に認められ、同遺伝子型は1, 4a, ならびに4bであった。本因子の保有に基づく、凝集性試験は本血清型に属するEPECの病原性を図る上で有用かもしれない。

Keywords: enteropathogenic *Escherichia coli*, *bfpA*, O157:H45

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Asakura, H., Kawamoto, K.*¹, Igimi, S., Yamamoto, S.,

Makino, S.*²: **Enhancement of mice susceptibility to infection with *Listeria monocytogenes* by the treatment of morphine.**

Microbiol Immunol. **50**, 543-547 (2006)

The effect of morphine on the susceptibility of BALB/c mice to diarrheagenic *Escherichia coli*, *Shigella flexneri*, *Listeria monocytogenes*, *Salmonella* Enteritidis, *Yersinia enterocolitica*, was examined via the intraperitoneal inoculation. Morphine treatment increased the susceptibility to *S. Enteritidis* and *L. monocytogenes*, resulting in bacteremia and central nervous system (CNS) invasion (for *L. monocytogenes*), while the infection with other bacteria did not show the systemic dissemination in the morphine treated mice. Notably, *L. monocytogenes* infection caused 100% mortality with a mean survival time (MST) of 1.3 days in morphine-treated mice, but untreated mice did not die. The present data suggested that individuals using heroin or treated with morphine derivatives might be at high risk for listeriosis, especially those who are immunocompromised. Recent increasing consumption of morphine may propose the necessity for further epidemiological surveillance on infectious diseases.

Keywords: morphine, *Listeria monocytogenes*, mice susceptibility

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Hansman, G. S.*¹, Oka, T.*¹, Okamoto, R.*², Nishida, T.*², Toda, S.*², Noda, M., Sano, D.*³, Ueki, Y.*⁴, Imai, T.*³, Omura, T.*³, Nishio, O.*¹, Kimura, H.*¹, Takeda, N.*¹: **Human Sapovirus in Clams, Japan.**

Emerg Infect Dis. **13**, 620-622 (2007)

Human sapovirus was detected in 4 of 57 clam packages by reverse transcription-PCR and sequence analysis. This represents the first finding of sapovirus contamination in food. Closely matching sequences have been detected in stool specimens from patients with gastroenteritis in Japan, which indicates a possible food-to-human transmission link.

Keywords: clam, food-to-human transmission, sapovirus

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中 峰松*, 清水 晃*, 河野潤一*, 五十君静信: **市販ミンチ肉における黄色ブドウ球菌汚染調査と分離株の性状.** *日本食品微生物学会雑誌*, **23**, 217-222 (2006)

Between April and October 2005, 120 raw minced meat

samples comprising 40 pork, 40 beef, and 40 chicken samples purchased from 40 supermarkets in Hyogo (n=20) and Osaka (n=20) prefectures were examined for contamination with *Staphylococcus aureus*. The rate of isolation was 75.0% (30/40) for pork, 65.0% (26/40) for beef and 80.0% (32/40) for chicken meat. The most probable number (MPN) of the samples contaminated was widely distributed from 0.3 to >110/g. Seventy-nine (89.8%) of the positive samples had MPN values of less than 46/g. The majority of pork and beef isolates were classified as belonging to both human and K-β+CV:A biotypes. Also, most chicken isolates belonged to both poultry and human biotypes. Seven (23.3%) of 30 pork isolates, 4 (15.4%) of 26 beef isolates and 9 (28.1%) of 32 chicken isolates produced one or two enterotoxins. Overall, the 20 enterotoxigenic isolates produced B (n=10), C (n=5), A (n=4) and AD (n=1). Enterotoxin-type B was dominant in isolates from chicken meat. Eighty-two (93.2%) of the 88 isolates tested were differentiated into 8 coagulase types. All isolates except one (type I) were widely distributed from types II to VIII. Samples positive for *S. aureus* were detected in 38 of the 40 supermarkets investigated. Interestingly, identical coagulase types, biotypes or enterotoxin types were found in isolates from all 2 or 3 kinds of meat samples retailed at the same supermarkets, indicating clonal spread of the same strain within meat-processing plants by cross-contamination.

Keyword : *Staphylococcus aureus*, food contamination, biotype

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清水 晃*, 松村浩介^{*1}, 藤尾公輔^{*1}, 河野潤一^{*1}, 北井智^{*2}, 五十君静信: 綿棒を用いたふき取り増菌培養法による市販豚および牛スライス肉における黄色ブドウ球菌汚染調査と分離株の性状.

日本食品微生物学会雑誌, **23**, 242-246 (2006)

多数の検体を短時間で処理するための一つの方法として、検体からのサンプリングに滅菌綿棒を用いた拭き取り法と選択増菌培地を組み合わせさせた方法、すなわち綿棒増菌培養法を用いて、鶏肉における黄色ブドウ球菌の全国汚染実態調査を実施した。その結果、鶏肉444検体中292検体 (65.8%) から、また調査したスーパーマーケット145店舗中131店舗 (90.3%) の検体から黄色ブドウ球菌を検出しており、市販鶏肉が全国的に広く本菌に汚染されていることを明らかにした。今回は、同様な方法を用いて、市販の豚および牛スライス肉における黄色ブドウ球菌の汚染調査と分離株の各種性状を調べた。また直

接平板培養法と綿棒増菌培養法の検出率の比較を行ったので、その成績も併せて報告する。

Keyword : *Staphylococcus aureus*, food contamination, meat

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Kajikawa, A., Satoh, E.^{*1}, Leer, R. J.^{*2}, Yamamoto, S., and Igimi, S.: **Intragastric immunization with recombinant *Lactobacillus casei* expressing flagellar antigen confers antibody-independent protective immunity against *Salmonella enterica* serovar Enteritidis.**

Vaccine, **25**, 3599-3605 (2007)

A recombinant *Lactobacillus casei* expressing a flagellar antigen from *Salmonella enterica* serovar Enteritidis was constructed and evaluated as a mucosal vaccine. Intragastric immunization of the recombinant strain conferred protective immunity against *Salmonella* infection in mice. This immunization did not result in antigen-specific antibody in either feces or sera but induced the release of IFN-gamma on restimulation of primed lymphocytes ex vivo. The results suggested that the protective efficacy provided by flagellin-expressing *L. casei* is mainly attributable to cell-mediated immune responses. In addition, an adjuvant-type effect of the antigen delivery system with *L. casei* was also observed.

Keyword : *Lactobacillus casei*, vaccine, recombinant

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Igarashi A., Ohtsu S., Muroi M., Tanamoto K: **Effects of possible endocrine disrupting chemicals on bacterial component-induced activation of NF-κB.** *Biol. Pharm. Bull.* **29**, 2120-2122 (2006)

Effects of thirty-seven possible endocrine disrupting chemicals (EDCs) on lipopolysaccharide (LPS) - or bacterial lipopeptide (Pam₃CSK₄) -induced activation of NF-κB were investigated. Alachlor, benomyl, bisphenol A, carbaryl, kelthane, kepone, octachlorostyrene, pentachlorophenol, nonyl phenol, *p*-octylphenol and ziram inhibited both LPS- and Pam₃CSK₄-induced activation of NF-κB. Simazine inhibited only LPS-induced activation. On the other hand, diethylhexyl adipate and 4-nitrotoluene tended to enhance the activation induced by Pam₃CSK₄ and LPS, respectively. These results indicate that some agrochemicals have the potential to inhibit macrophage function and suggest that endocrine disruptors may influence the devel-

opment of bacterial infections.

Keywords: Endocrine disruptors, NF- κ B, lipopolysaccharide.

野田多美枝*、村上光一*、浜崎光宏*、石黒靖尚*、宮原美知子：赤痢菌型別検査方法としてのAmplified Fragment Length Polymorphism (AFLP) 法の有用性の検討。

感染症学雑誌、80, 513-521 (2006)

分子疫学的解析法の一つであるamplified fragment length polymorphism (AFLP) 法が、赤痢菌に対する型別方法として有用であるか否かを検討した。赤痢菌51株を、AFLP法とコリシン型別法およびパルスフィールドゲル電気泳動法 (PFGE法) の3法で型別し、「型別能力」、「再現性」、「識別能力」、「解釈の容易さ」および「実行の容易さ」の5項目について比較を行った。その結果AFLP法は、型別能力が100%、識別能力がSimpson's Indexにて1.000と優れているのは半面、結果の解釈の容易さ、実行の容易さ、さらに再現性が劣っていることが明らかになった。このうち再現性は、ATCC株3株について3回繰り返して全工程を実施し、結果を比較したところ、AFLP法は株によりDice係数を用いた相似値で81.9%から90.5%であり、PFGE法の92.3%~100%に比べ劣っていた。しかし、再現性が若干劣っていても系統樹を作成してクラスター解析を行うには問題はないと考えられた。

Keywords: *Shigella*, AFLP, bacterial strain typing

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Matsutani, S. : Mechanism of the transcription stimulated by the internal region of IS1 and the product of the *artA* gene.

WSEAS Transactions on Biology and Biomedicine, 4, 321-329 (2006)

The internal region of the bacterial insertion sequence IS1 acts as a *cis*-element to increase RNA synthesis from the IS1 promoter and exogenous promoters located upstream. The product of the *artA* gene in the *Escherichia coli* F factor, works with the IS1 internal region to stimulate transcription. Here, it was found that an IS1 internal sequence cloned in the opposite orientation to the upstream promoter can also stimulate gene expression. The *cis*-element sequence which was inserted upstream of the promoter seemed not to stimulate transcription. Furthermore, two-hybrid systems were constructed in *E. coli*, and it was suggested that the ArtA protein participates in transcription initiation, and associates with the *E. coli* RNA polymerase alpha subunit. It is possible that ArtA or the protein it in-

teracts with binds to the IS1 internal region, and helps to tether RNA polymerase near the promoter.

Keywords: *E. coli* RNA polymerase, transcription initiation, transposon

工藤由起子、尾上洋一*¹、中川 弘*²、高橋淳子*³、小西典子*³、高鳥浩介：液卵製造工程のモニタリングによる微生物的問題点の調査とその改善について。

日本食品衛生学会誌、47, 119-126 (2006)

From January to November, 2003, bacterial contamination were contamination was surveyed in a small egg breaking factory that produced non pasteurized liquid egg. Egg Test egg samples were taken on from various stages of an egg processing operation and from the attached production facility. *Salmonella* Enteritidis was isolated from liquid egg yolk and liquid egg white on October, but *Salmonella* was not found in other all samples (50 liquid egg samples, 21 containers and 94 attached production facilities and gloves). The data suggest that the contamination rate although (3.8%) (2/52) is lower than those in previous reports. of liquid eggs are contaminated with *Salmonella*, contamination of the eggs with *Salmonella* is a rare event in this factory. Bacterial standard plate counts, gram positive bacterial counts and gram negative bacterial counts were also examined. Levels of bacterial standard plate counts, gram positive bacterial counts and gram negative bacterial counts I were ranged from during 2 log CFU/g to 5 log CFU/g, from 2 log CFU/g to 3 log CFU/g, from 2 log CFU/g to 5 log CFU/g, respectively. Liquid egg Containers containers for liquid egg returned from customers was contaminated with had counts of bacteria in 8 log CFU bacterial counts/per a container. However, washing and using with sanitizer contained sodium hypochlorite reduced the bacterial counts.

Keywords: liquid egg, *Salmonella*, bacterial contamination

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Hara-Kudo, Y., Ohtsuka, K.*¹, Onoue, Y.*², Otomo, Y.*³, Furukawa, I.*², Yamaji, A., Segawa, Y. and Takatori, K. : *Salmonella* prevalence, total microbial and spore populations in imported spices to Japan.

J. Food Prot., 69, 2519-2523 (2006)

A total of 259 samples of 40 kinds of spices were tested for *Salmonella* contamination. As a result, *Salmonella* enterica serotypes Weltevreden and Senftenberg were isolated

from a black pepper and red pepper sample, respectively. The contamination level was <30 MPN/100g. Furthermore, the bacterial contamination was determined also. The mean aerobic bacterial count was more than 5.39 log cfu/g in turmeric, garam masala, curry powder and paprika. The mean bacterial spore count was more than 4.33 log cfu/g in turmeric and curry powder. The mean aerobic bacterial count in the two *Salmonella*-isolated samples was 6.93 log cfu/g. This study suggests that treatments to decrease pathogens and food storage at low temperature, including spices, are important to control *Salmonella* infection.

Keywords: *Salmonella*; spice; isolation; contamination; pepper

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Hara-Kudo, Y., Nemoto, J.^{*1}, Ohtsuka, K.^{*2}, Segawa, Y., Takatori, K., Kojima T.^{*1} and Ikedo, M.^{*1} : **Sensitive and rapid detection of Vero toxin-producing *Escherichia coli* using Loop-mediated isothermal amplification.**

J. Med. Microbiol., **56**, 398-406 (2007)

We developed a loop-mediated isothermal amplification (LAMP) assay to rapidly detect Vero toxin (VT) -producing *Escherichia coli* rapidly (within 60 min) . The 24 strains of VT -producing *E. coli* were successfully amplified, but not 6 strains of non-VT-producing *E. coli* were not, nor were and 46 bacterial species other than *E. coli*. The sensitivity of the LAMP assay was found to be >0.7 cfu/test using serogroups O157, O26 and O111 of VT-producing *E. coli*; this sensitivity is greater than that obtained by polymerase chain reaction (PCR) assay. Furthermore, the LAMP assay was examined for its ability to detect VT-producing *E. coli* in food because of the difficulty of detection in food. The recovery of VT-producing *E. coli* by LAMP assay from beef and radish sprouts inoculated with the pathogen was high, similar to that obtained using culture methods with direct plating and/or plating after immuno-magnetic separation. Although PCR assay was unable to recover VT-producing *E. coli* from half of the radish samples, LAMP assay was successful in most samples. In addition, VT-producing *E. coli* was successfully detected in cultures of the beef samples by LAMP assay, but not by the culture method. The LAMP products in naturally-contaminated beef samples were analyzed to confirm the specific amplification of the VT gene, and were found to show

a specific ladder band pattern on agar gel after electrophoresis. Additionally the sequences of the LAMP products were coincided well with the expected sequences of the VT gene. These results indicate that the proposed LAMP assay is a rapid, specific and sensitive method of detecting the VT-producing *E. coli*.

Keywords: vero toxin-producing *E. coli*, LAMP, rapid, sensitive, detection

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Takahashi, H., Konuma, H.^{*1} and Hara-Kudo, Y. : **Development of a quantitative real-time polymerase chain reaction method to enumerate total bacterial count in ready-to-eat fruits and vegetables.**

J. Food Prot., **69**, 2504-2508 (2006)

A newly developed real-time PCR assay rapidly quantifies the total bacterial numbers in contaminating ready-to-eat vegetables and fruits as compared to the standard plate count method. Primers targeting the rpoB gene, that encodes for the β -subunit of the bacterial RNA polymerase, common to most bacterial species was used instead of the 16S rRNA gene that has multiple copies and varies among bacterial species. A primer pair specific for rpoB was confirmed to amplify rpoB in wide-range of bacterial species by using 49 strains isolated from five kinds of fruits and vegetables. We purchased fruits and vegetables from retail shops and enumerated the bacteria associated with them using the real-time PCR and compared this to the number found by the culture method. We found a high correlation between the threshold PCR cycle number when compared to the plate count culture number. Further, the predominant bacterial flora detected by the culture method was compared with those detected by the real-time PCR assay using a denaturing gradient gel electrophoresis analysis. The results from the two methods were almost identical. The real-time PCR assay developed in this study can enumerate the dominant bacterial species in ready-to-eat fruits and vegetables. Keywords: real-time PCR, quantification, bacterial count, ready-to-eat, vegetable

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Yoneyama, N.^{*1}, Hara-Kudo, Y. and Kumagai, S.^{*1} : **Effects of heat-degraded sugars on survival and growth of *Vibrio parahaemolyticus* and other bacteria.**

J. Food Prot., **70**, 373-377 (2007)

We studied the effects of autoclaved (121 °C, 15 min) sugar solutions on the survival and growth of *Vibrio parahaemolyticus* and other bacteria. The growth and survival of *V. parahaemolyticus* in Luria-Bertani media and phosphate buffer, respectively, were inhibited by the addition of D-glucose autoclaved in pH 8.0 phosphate buffer. The bactericidal effect of autoclaved D-glucose was very small when autoclaved in pH 7.0 phosphate buffer, but larger effects were observed when autoclaved in the buffer at an alkaline pH. The autoclaving of D-glucose in CH₃COONa, NaHCO₃, and Na₂HPO₄ solutions at pH 7.6 to 8.5 also generated bactericidal effects, but it was not the case when D-glucose was autoclaved in Na₂SO₄, (NH₄)₂SO₄, or NH₄Cl solution at pH 8.0. The same effects as autoclaved D-glucose were observed in autoclaved lactose, D-fructose, and D-ribose. The bactericidal effects of autoclaved D-glucose were also noted in *Salmonella* Enteritidis, *Listeria monocytogenes*, and *E. coli* strains, but the effects were smaller than those seen in *V. parahaemolyticus* and *V. vulnificus*. The growth of *V. parahaemolyticus* in clam extracts was also inhibited by the addition of autoclaved D-glucose, indicating that heat-treated reduced sugars can exert bactericidal effects in foods.

Keywords: *Vibrio parahaemolyticus*, sugar, survival, growth

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Goto, M.^{*1}, Takahashi, H., Segawa, Y., Hayashidani, H.^{*2}, Takatori, K. and Hara-Kudo, Y. : **Real-time PCR method for quantification of *Staphylococcus aureus* in milk.**

J. Food Prot., **70**, 90-96 (2007)

A reproducible real-time PCR method that targets the putative transcriptional regulator gene of *Staphylococcus aureus* was developed to quantify this microorganism in milk samples. On the basis of partial sequences of this gene determined from *S. aureus* strains, we designed the specific primers and probe for use in a quantitative PCR assay. These specificities were confirmed with 25 strains of *S. aureus* and 35 strains of other bacteria. A real-time PCR assay with serial 10-fold dilutions of purified DNA and pure culture was conducted. It was possible to construct standard curves with a high correlation coefficient ($r_2 = 0.99$) in the range of 50 ng to 50 fg for purified DNA and 107 to 101 CFU/ml for a pure culture. The constructed standard curve for milk samples was similar to that for the pure culture, and the quantification of *S. aureus* in the range of 107 to 101 CFU/ml was possible. Moreover, to

determine how our real-time PCR method would perform under actual analytical conditions, we quantified the DNA from *S. aureus* after two types of heat treatments were used for the pasteurization of milk. The amount of DNA found was affected after heat treatment at 63°C for 30 min (low-temperature long-time method) but not at 72 °C for 15 s (high-temperature short-time method) . The results indicate that the real-time PCR method developed in this study is effective for monitoring *S. aureus* contamination in milk because of its high specificity and sensitivity.

Keywords: real-time PCR, quantification, *Staphylococcus aureus*, milk

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酒井綾子、尾関由姫恵^{*1}、佐々木洋介^{*2}、鈴木千尋、増井康子、相原真紀、菊池 裕、高鳥浩介 : **DNA塩基配列を利用した真菌の同定 : 国産玄米から分離された *Fusarium* の種レベルの同定.**

食品衛生学雑誌, **47**, 268-276 (2006)

国産玄米から分離した *Fusarium* を材料として DNA 塩基配列による真菌の同定を行い、その実践上の有用性と限界について検討した。rRNA 遺伝子内に存在する 3 領域、D2、ITS1 および ITS2 の塩基配列を解読し、米国の NCBI が管理する GenBank データベースに登録されている DNA 塩基配列と照合した。D2 領域については、Applied

Biosystems Fungal Library とも照合した。 *Fusarium* を種レベルで同定または推定するには、少なくとも 3 領域すべてをデータベースと照合する必要があった。今回同定に供した株の約半数は、塩基配列のみから種の同定が可能であった。残りの株は、1 株を除いて、DNA 塩基配列に基づいて候補となる種を絞ることができ、形態学的手法による同定が容易になった。

Keywords: fungal identification, DNA sequence, *Fusarium*

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Maragos, C.M.^{*1}, Busman, M.^{*1}, and Sugita-Konishi, Y. : **Production and characterization of a monoclonal antibody that cross-reacts with the mycotoxins nivalenol and 4-deoxynivalenol.**

Food Additives and Contaminants, **23** (8) , 816-825 (2006)

Nivalenol (NIV) is a mycotoxin produced by certain fungi that are pathogenic to important cereal crops, in particular maize, wheat, and barley. This toxin, 3 α ,4 β ,7 α ,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one, is found

worldwide and is closely related to 4-deoxynivalenol (DON or vomitoxin) a mycotoxin associated with outbreaks of Fusarium Head Blight in North America. Literature on the toxicity of NIV suggests it is similar, if not more toxic, than DON. Despite the development of rapid immunologically-based assays for detecting DON, such assays have not existed for detecting NIV without chemical modification of the analyte. This report describes the development of a monoclonal antibody (Mab) using a NIV-glycine protein conjugate. The Mab is specific for an acetylated form of DON (3-Ac-DON) and cross reacts with both DON and NIV at relevant concentrations without the need to chemically modify the toxin. In a competitive indirect (CI) ELISA format the concentrations of toxins able to inhibit color development by 50% (IC₅₀) were 1.7 ng ml⁻¹, 15.8 ng ml⁻¹, 27.5 ng ml⁻¹, 68.9 ng ml⁻¹, and 1740 ng ml⁻¹ for the mycotoxins 3-Ac-DON, DON, NIV, 15-Ac-DON, and fusarenon-X respectively. The antibody was also used to develop a competitive direct (CD) ELISA for DON and NIV, with IC₅₀'s of 16.5 ng ml⁻¹ (DON) and 33.4 ng ml⁻¹ (NIV). These assays are capable of detecting both DON and NIV simultaneously, a property that may be useful in regions where these toxins co-occur or in formats, such as immunoaffinity columns, where co-isolation of both toxins is desirable.

Keywords: nivalenol, deoxynivalenol, antibody

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Sugita-Konishi, Y., Nakajima, M.^{*1}, Stabata, S.^{*2}, Ishikuro, E.^{*3}, Tanaka, T.^{*4}, Narozuki, H.^{*5}, Itoh, Y., Aoyama, K.^{*5}, Fujita, K.^{*6}, Kai, S.^{*7}, Kumagai, S.^{*8}: **Occurrence of Aflatoxins, Ochratoxin A and Fumonisins in Retailed Foods in Japan.**

J. Food Protection, **69** (9), 1365-1370 (2006)

We conducted a survey of Aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), Ochratoxin A (OTA), Fumonisins B1 (FB1), B2 (FB2) and B3 (FB3) contaminations in various foods retailled in Japan in 2004-2005. The mycotoxins were analyzed by HPLC, LC/MS or HPTLC. Aflatoxin was detected in 10 out of 21 peanut butter samples, the highest level of AFB1 being 2.59 µg/kg. Aflatoxin contamination was not observed in other corn products, corn, peanuts, buckwheat flour, dried buckwheat noodle, rice and sesame oil. OTA was detected in oatmeal, wheat flour, rye, buckwheat flour, raw coffee, roasted coffee, raisin, beer and wine, but not in rice, corn

products. OTA levels in detected samples were below 0.8 µg/kg. Fumonisins were detected in popcorn, frozen corn, corn flake and corn grits. Highest levels in these samples were 354.0, 94.0 and 64.0 µg/kg, respectively, for FB1, FB2 and FB3.

Key words: total aflatoxin, ochratoxin A, total fumonisin

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Sugita-Konishi, Y., Tanaka, T.^{*1}, Nakajima, M.^{*2}, Fujita, K.^{*3}, Norizuki, H.^{*4}, Mochizuki, N.^{*5}, Takatori, K.: **The Comparison of two clean-up procedures, multi-functional column and immunoaffinity column, for HPLC determination of ochratoxin A in cereals, raisins and green coffee beans.**

Talanta, **69**, 650-655 (2006)

To evaluate a clean-up method of detecting ochratoxin A (OTA) by HPLC, the performances of two different clean-up columns, an immunoaffinity column and a multifunctional column were compared in an inter-laboratory study. As samples, un-contaminated wheat, corn grits, green coffee beans and naturally contaminated raisins were used. The recovery test was performed at two different concentrations of OTA (0.5 µg/kg and 5.0 µg/kg) except for naturally contaminated raisins. Using the immunoaffinity column, the recovery rates, and relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R) for wheat, corn grits and green coffee beans ranged 59.0 - 85.8 %, 4.2-7.8 % and 22.9-29.2 %, respectively. For naturally contaminated raisins, recovery, RSD_r and RSD_R were 84.1 %, 1.8 % and 5.1 %, respectively. Using the multifunctional column, the recovery rates, RSD_r and RSD_R for wheat, corn grits and green coffee beans ranged 80.8 - 185.0 %, 0.7-6.9 % and 15.2-33.9 %, respectively. For naturally contaminated raisins, the recovery, RSD_r and RSD_R were 128.7%, 1.1 % and 3.7 %, respectively. The results suggest that a multifunctional column could be used to detect OTA in wheat and corn grits at a concentration as low as 0.5 µg/kg, however it was difficult to detect OTA in green coffee beans and raisins at such a low level. Although an immunoaffinity column could be used for all

the test samples in this study from a low level to a high level, the recovery rates were lower than with a multifunctional column.

Key words: Ochratoxin A; immunoaffinity column; multifunctional column

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Sugita-Konishi, Y., Bong Joo Park, B.J., Kazuo Kobayashi-Hattori, K. ^{*1}, Tanaka, T. ^{*2}, Chonan, T. ^{*3}, Yoshikawa, K. ^{*1}, Kumagai, S. ^{*4} : **Effect of Cooking Process on the Deoxynivalenol Content and its Subsequent Cytotoxicity in Wheat Products.**

Biosci. Biotechnol. Biochem., **70** (7), 1764-1768 (2006)

The retention of deoxynivalenol in noodles and bread made from naturally-contaminated flour was examined by a chemical analysis (HPLC) and bioassays. The retention level of deoxynivalenol obtained from both assays was reduced by boiling process, although only the bioassays showed it to have been reduced by baking. This study is the first to estimate the exposure to deoxynivalenol from the consumption of the final products of wheat flour in Japan.

Key words: bread; cytotoxicity; deoxynivalenol; high-performance liquid chromatography; noodle

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Fukuhara, K., Oikawa, S. ^{*1}, Hakota, N. ^{*1}, Sakai, Y. ^{*2}, Hiraku, Y. ^{*1}, Shoda, T., Saito, S. ^{*2}, Miyata, N. ^{*3}, Kawanishi, S. ⁺, Okuda, H.: **9-Nitroanthracene Derivative as a Precursor of Anthraquinone for Photodynamic Therapy.**

Bioorg. Med. Chem., **15**, 3869-3873 (2007)

Anthraquinones are typical photosensitizers used in photodynamic therapy (PDT). However, systemic toxicity is a major problem for anthraquinones due to their ability not only to bind DNA but also to cause oxidative stress even without photoirradiation. To avoid such disadvantages in cancer therapy, we designed and synthesised a novel 9-nitroanthracene derivative (9NA) as a precursor of anthraqui-

none. Under photoirradiation, 9NA is converted into anthraquinone via generation of nitric oxide as confirmed by ESR. Under irradiation, strong DNA cleavage specifically at guanine under photoirradiation was observed, characteristic of DNA-cleaving reactions by photoirradiated anthraquinones. We propose development of 9NA as an alternative approach towards PDT that reduces the systemic toxicity of anthraquinone.

Key words: photodynamic therapy, anthraquinone, nitroanthracene

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Suhara, Y. ^{*1}, Kurihara, M., Kittaka, A. ^{*2}, Ichikawa, Y. ^{*1}: **Efficient synthesis of carbopeptoid oligomers and their conformational studies.**

Tetrahedron, **62**, 8207-8217 (2006)

The ready access to a new class of carbohydrate mimetics was demonstrated by the synthesis of tetrameric carbopeptoids, in which glycosidic bonds were replaced with amide linkages. We herein describe the detailed synthetic method of β (1 \rightarrow 2) - and β (1 \rightarrow 6) -linked carbopeptoids starting from each d-glucosamine and d-glucose derivative. The building blocks were polymerized using BOP reagent and DIEA to form a homooligomer. These produced carbopeptoids are resistant to glycosidases and have interesting biological activity. With conformational analysis by molecular modeling calculation, β (1 \rightarrow 2) -linked decamer showed a typical 16-helix form as a mimic of β -peptide. Therefore, our polysaccharide analogues have potential as peptide foldamers.

Key words: carbopeptoid, 16-helix

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Fujishima, T. ^{*1}, Tsutsumi, R. ^{*2}, Negishi, Y. ^{*3}, Fujii, S. ^{*1}, Takayama, H. ^{*2}, Kittaka, A. ^{*2}, Kurihara, M.: **Methyl-introduced A-ring analogues of 1 α ,25-dihydroxyvitamin D₃: synthesis and biological evaluation.**

Anticancer Res., **26**, 2633-2636 (2006)

The hormonally-active metabolite of vitamin D, 1 α ,25-dihydroxyvitamin D₃, has a wide variety of biological activities, which makes it a promising therapeutic agent for the treatment of cancer, psoriasis and osteoporosis. Insights into

the structure-activity relationships of the A-ring of 1 are needed to assist the development of more potent and selective analogues, as well as to define the molecular mode of action. All possible A-ring stereoisomers of 2-methyl-1,25-dihydroxyvitamin D₃ and 2,2-dimethyl-1,25-dihydroxyvitamin D₃, which differ in stereochemistry at the C1-, C2- and C3-positions, were designed and efficiently synthesized by employing the convergent method. Biological evaluation of the analogues, in terms of the vitamin D receptor-binding affinity and HL-60 cell differentiation-inducing activity, as well as the transcriptional potency in ROS 17/2.8 cells, revealed the importance of substituents at the C2-position in certain orientations.

Key words: Vitamins, hormones, receptors, chemical synthesis

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Sugiyama, T.^{*1}, Imamura, Y.^{*1}, Hakamata, W., Kurihara, M., Kittaka, A.^{*2}: **Sequence-specific recognition of double-stranded DNA by cooperative strand invasion**

Nucleic Acids Symposium Series, **50**, 157-158 (2006)

A remarkable feature of peptide nucleic acid (PNA) is its ability to recognize some sequences within duplex DNA by strand invasion. In order to improve binding properties of PNA triplex invasion, we tested the effect of cooperativity on the sequence specificity. A PNA targeting six bases within duplex DNA stringently recognized 12 base-pair homopurine site at a single base level.

Key words: cooperative strand invasion, peptide nucleic acid

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Demizu, Y.^{*1}, Tanaka, M.^{*1}, Nagano, M.^{*1}, Kurihara, M., Doi, M.^{*2}, Maruyama, T.^{*3}, Suemune, H.^{*1}: **Controlling 310-helix and α -helix of short peptides in the solid state.**

Chem. Pharm. Bull. **55**, 840-842 (2007)

L-Leu hexapeptide containing α -aminoisobutyric acid (Aib) forms a right-handed (P) 310-helix, whereas that containing cyclic α,α -disubstituted amino acid Ac-5cdOM assumes a right-handed (P) α -helix in the solid state.

Key words: α , α -disubstituted amino acid, peptide; helix,

conformation, secondary structure

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Hakamata, W., Muroi M.^{*1}, Nishio, T.^{*2}, Oku, T.^{*2}, Takatsuki, A.^{*3}, Osada, H.^{*1}, Fukuhara, K., Okuda, H., Kurihara, M.: **N-Linked oligosaccharide processing enzymes as molecular targets for drug discovery.**

J. Appl. Glycosci., **53**, 149-154 (2006)

N-Linked oligosaccharide processing enzymes are key enzymes in the biosynthesis of N-linked oligosaccharides. These enzymes are a molecular target for inhibition by anti-viral agents that interfere with the formation of essential glycoproteins required in viral assembly, secretion and infectivity. We think that the molecular recognition of three kinds of glucosidases (family 13 and family 31 α -glucosidases and endoplasmic reticulum glucosidases) are different. Therefore, glycon and aglycon specificity profiling of glucosidases was an important approach for the research of glucosidase inhibitors. We carried out the profiling of glucosidases using small molecules as a probe. Moreover, we designed and synthesized three types of glucosidase inhibitors. These compounds were evaluated with regard to their ability to inhibit glucosidases in vitro, and were also tested in a cell culture system. We found some compounds having glucosidase inhibitory activity and anti-viral activity.

Key words: α -glucosidases, ER glucosidases, inhibitor, anti-viral activity

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Hakamata, W., Yamamoto, E.^{*1}, Muroi, M.^{*2}, Mochizuki, M.^{*1}, Kurihara, M., Okuda, H., Fukuhara, K.: **Design and synthesis of α -glucosidase inhibitor having DNA cleaving activity.**

J. Appl. Glycosci., **53**, 255-260 (2006)

Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. The present study was designed to explore small molecule apoptosis inducer for antitumor agent. The synthesis of 4-sulfonylphenyl α -D-glucopyranoside derivatives 1-6 and 4-(sulfonylamino) phenyl α -D-glucopyranoside derivatives 7-12, endoplasmic reticulum (ER)-targeted small molecules that design for the purpose of apoptosis inducer from ER stress by ER glucosidase in-

hibition and DNA damage, are described. Compounds 6 and 12, with a terminal 2-naphthyl group, indicated inhibitions of α -glucosidases from *S. cerevisiae* (IC50 = 51.7 μ M and IC50 = 74.1 μ M) and *B. stearothermophilus* (IC50 = 60.1 μ M and IC50 = 89.1 μ M). Moreover, compound 12 strongly induced the DNA strand breakage condition. When compounds 1-12 were assayed for their ability to inhibit processing by glucosidases at the cellular level, no effects on glycoprotein processing were observed.

Key words: α -glucosidase, inhibitor, DNA cleavage, ER stress

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Hama, M.*1, Tanaka, M.*1, Yoshida, Y.*1, Demizu, Y.*1, Kurihara, M., Suemune, H.*1: **Synthesis of optically active cyclic α, α -disubstituted amino acids by enzymatic kinetic resolution and conformational analysis of their peptides.**

Peptide Science 2006, 39 (2006)

Optically active α, α -disubstituted amino acid is useful for control of the secondary structure of peptides. We have previously reported the synthesis of chiral cyclic α, α -disubstituted amino acid; (3R,4R)-1-amino-3,4-dimethoxycyclopentanecarboxylic acid; (R,R)-Ac₅c^{diOM}, starting from dimethyl L-(+)-tartrate as a chiral resource. Herein we studied the syntheses of optically active cyclic α, α -disubstituted amino acids by enzymatic kinetic resolution as a key step, and conformational analysis of their peptides. Starting from dimethyl malonate, we prepared cycloalkene diesters by bisalkylation, and subsequent olefin metathesis reaction using Grubbs-catalyst. Epoxidation of olefin, followed by acidic hydrolysis gave racemic cycloalkane-1,2-diols bearing diester. Kinetic resolution using lipase-catalyzed transesterification in organic solvent afforded optically active cycloalkane-1,2-diols. The optically active cycloalkane-1,2-diols could be converted into the chiral cyclic α, α -disubstituted amino acids by protection of alcohol, hydrolysis of monoester, and subsequent Curtius rearrangement.

Key words: peptide, α, α -disubstituted amino acid, conformational analysis

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Kurihara, M., Sato, Y., Hakamata, W., Okuda, H., Nagano, M.*1, Hama, M.*1, Demizu, Y.*1, Doi, M.*2, Tanaka, M.*1, Suemune, H.*1: **Computational study on helical structures of oligopeptides containing**

chiral cyclic α, α -disubstituted α -amino acids.

Peptide Science 2006, 88 (2006)

Prediction of the conformation of peptides using computational simulation is an interesting challenge for the design of functionalized and bioactive peptides. We have shown the Monte Carlo conformational search using *MacroModel* is useful for conformational study of oligopeptides prepared from α, α -disubstituted α -amino acids. Moreover, we have studied conformational analysis of oligopeptides containing chiral α, α -disubstituted α -amino acids to predict the helical screw sense of helical structures (α -helix, 3_{10} -helix). Here we report computational study on conformation of oligopeptides containing cyclic α, α -disubstituted α -amino acids with side-chain chiral centers.

Key words: peptide, computational study, conformational search, helical structure

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Tanaka, M.*1, Nagano, M.*1, Hama, M.*1, Kawabe, N.*1, Demizu, Y.*1, Kurihara, M., Doi, M.*2, Suemune, H.*1: **Controlling helical secondary structures by cyclic α, α -disubstituted amino acids having side-chain chiral centers.**

Peptide Science 2006, 135 (2006)

Helical-screw handedness in proteins is believed to be controlled by asymmetric centers at the α -position of L- α -amino acids. Recently, we have reported that the side-chain chiral centers of chiral cyclic α, α -disubstituted amino acid (S,S)-Ac5cdOM affected the helical secondary structure of its peptides, and the helical-screw direction could be controlled without a chiral center at the α -carbon atom. Also, we have reported that hexapeptides composed of chiral bicyclic α, α -disubstituted amino acid (R,R)-Ab (5,6) c formed both diastereomeric right-handed (P) and left-handed (M) helices. In the hexapeptide, there are twelve chiral centers at the side chain, but the helical-screw handedness can not be controlled. These results indicated that the side-chain chiral environments are important for control of the helical-screw sense of peptides. Thus, we planned to synthesize various chiral cyclic α, α -disubstituted amino acids, and study the secondary structures of their peptides. The presentation will include new results, which have not been published.

Key words: peptide, α, α -disubstituted amino acid, conformational analysis

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Soyama, A., Saito, Y., Kubo, T., Miyajima, A., Ohno, Y., Komamura, K.^{*1}, Ueno, K.^{*2}, Kamakura, S.^{*1}, Kitakaze, M.^{*1}, Tomoike, H.^{*1}, Ozawa, S. and Sawada, J.: **Sequence-based analysis of the *CYP2D6*^{*36}-*CYP2D6*^{*10} tandem-type arrangement, a major *CYP2D6*^{*10} haplotype in the Japanese population.**

Drug Metab. Pharmacokinet., **21**, 208-216 (2006)

The frequency of the *CYP2D6*^{*10} allele (100C>T) in the Japanese is relatively high (0.3-0.4), and the two ^{*10}-related genes, Ch1 (currently ^{*10B}) and Ch2 (^{*36}), and their tandem arrangement Ch (2) - Ch (1) (^{*36}-^{*10B}) have been reported. Although the tandem form of ^{*36}-^{*10} is assumed to be a major form, no detailed information has been reported for its intervening and flanking regions. Thus in this study, the tandem-type ^{*36}-^{*10B} and the single-type ^{*10} were analyzed by long-range PCR and sequencing of the subsequent nested PCR products. The sequence of the entire ^{*36}-^{*10} region confirmed the recombination of *CYP2D6*^{*10} with *CYP2D7P*. Also, we found that most of the ^{*10B}-harboring haplotypes have the upstream ^{*36} gene and that the majority of the remaining haplotypes are the single-type ^{*10B}. Haplotype frequencies of the single-type ^{*10} and ^{*36}-^{*10B} were 0.06 and 0.30, respectively, in the subjects analyzed. Additionally, several novel single nucleotide polymorphisms (SNPs) were found in the ^{*36} region and several ^{*36} haplotypes were identified. This sequence information is an important addition to the *CYP2D6* sequence data that was obtained by the human genome project.

Keywords: geneic polymorphism, *CYP2D6*, Japanese

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Fukushima-Uesaka, H., Saito, Y., Maekawa, K., Saeki, M., Kamatani, N.^{*1}, Kajio, H.^{*2}, Kuzuya, N.^{*2}, Yasuda, K.^{*2} and Sawada, J.: **Novel genetic variations and haplotypes of hepatocyte nuclear factor 4 α (*HNF4A*) found in Japanese type II diabetic patients.**

Drug Metab. Pharmacokinet., **21**, 337-346 (2006)

Thirty-nine single nucleotide variations, including 16 novel ones, were found in the 5' promoter region, all of the exons and their surrounding introns of *HNF4A* in 74 Japanese type II diabetic patients. The following novel variations were identified (based on the amino acid numbering of

splicing variant 2) : -208G>C in the 5' -promoter region; 1154C>T (A385V) and 1193T>C (M398T) in the coding exons; 1580G>A, 1852G>T, 2180C>T, 2190G>A, and 2362_2380del-19base in the 3' -untranslated region, and IVS1+231G>A, IVS2-83C>T, IVS3+50C>T, IVS3-54delC, IVS5+173_176delTTAG, IVS5-181_-180delAT, IVS8-106A>G, and IVS9-151A>C in the introns. The allele frequencies were 0.311 for 2362_2380del-19base, 0.054 for 1580G>A, 0.047 for 1852G>T, 0.020 for IVS1+231G>A, 0.014 for IVS9-151A>C, and 0.007 for the other 11 variations. In addition, one known nonsynonymous single nucleotide polymorphism, 416C>T (T139I), was detected at a 0.007 frequency. Based on the linkage disequilibrium profiles, the region analyzed was divided into three blocks. Haplotype analysis determined/inferred 10, 16, and 12 haplotypes for block 1, 2, and 3, respectively. Our results on *HNF4A* variations and haplotypes would be useful for pharmacogenetic studies in Japanese.

Keywords: geneic polymorphism, *HNF4A*, haplotype

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Kim, S.R., Saito, Y., Maekawa, K., Sugiyama, E., Kaniwa, N., Ueno, H.^{*1}, Okusaka, T.^{*1}, Morizane, C.^{*1}, Yamamoto, N.^{*1}, Ikeda, M.^{*1}, Yoshida, T.^{*1}, Minami, H.^{*1}, Furuse, J.^{*1}, Ishii, H.^{*1}, Saijo, N.^{*1}, Kamatani, N.^{*2}, Ozawa S. and Sawada J.: **Thirty novel genetic variations in the *SLC29A1* gene encoding human equilibrative nucleoside transporter 1 (hENT1).**

Drug Metab. Pharmacokinet. **21**, 248-256 (2006)

Thirty-nine genetic variations, including thirty novel ones, were found in the human *SLC29A1* gene, which encodes equilibrative nucleoside transporter 1, from 256 Japanese cancer patients administered gemcitabine. The found novel variations included -8166G>A, -8110A>G, -7947G>A, -7789T>C, -5595G>A, -3803_-3783delTCG-GGGAGGTGGCAGTGGGCG, -3548G>C, -3414G>A, -1355T>C, -34C>G, IVS1+141G>A, IVS1+260C>T, IVS1-82C>T, 177C>G, IVS3-6C>T, 564C>T, IVS8+44T>C, IVS8+90T>C, IVS8+97T>C, IVS8+131C>T, IVS8+169G>A, 933T>C, 954C>T, IVS11-52G>C, IVS11-46G>A, 1,288G>A, 1,641C>G, 1,703_1,704delIGT, 1812C>T, and 1861C>T. The frequencies were 0.051 for IVS8+169G>A, 0.012 for -7,947G>A, 0.006 for IVS1+141G>A and 1,703_1,704delIGT, 0.004 for -8,166G>A, -8,110A>G, -3,548G>C, -1,355T>C, -34C>G, IVS8+44T>C, and 1,812C>T, and 0.002 for the other 19

variations. Among them, 177C>G and 1,288G>A resulted in amino acid substitutions Asp59Glu and Ala430Thr, respectively. Using the detected polymorphisms, linkage disequilibrium analysis was performed, and 28 haplotypes were identified or inferred. Our findings would provide fundamental and useful information for genotyping SLC29A1 in the Japanese and probably other Asian populations.

Keywords: genetic polymorphism, SLC29A1/ hENT1, haplotype

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Soyama, A., Saito, Y., Ohno, Y., Komamura, K.^{*}, Kamakura, S.^{*}, Kitakaze, M.^{*}, Tomoike, H.^{*}, Ozawa, S. and Sawada J.: **Diverse structures of chimeric CYP-REP7/6-containing CYP2D6 and a novel defective CYP2D6 haplotype harboring single-type *36 and CYP-REP7/6 in Japanese.**

Drug Metab. Pharmacokinet., **21**, 395-405 (2006)

Chimeric REP7/6 has been used as a marker of CYP2D6 deletion, such as for CYP2D6*5. However, the CYP2D6*10D (*10D) haplotype found in a Japanese population consist of CYP2D6*10B, CYP2D7P-derived 3'-flanking region, and a chimeric repetitive sequence, CYP-REP7/6 (REP7/6). From our analysis, REP7/6 was found in 26 out of 254 Japanese subjects. Thus, the REP7/6-containing CYP2D6 genes (2D6-REP7/6) were analyzed in detail. In order to specifically detect the 2D6-REP7/6 structure, primers were designed in CYP2D6 intron 6 and the REP7/6 3'-flanking region. Among 26 subjects analyzed by PCR, 5 had 2D6-REP7/6. The other 21 subjects were confirmed to have *5 by another *5-specific primer set. Three out of five subjects with 2D6-REP7/6 had the *10D structure. However, further analysis by PCR and sequencing revealed that their haplotypes were further divided into tandem-type *36*10D (n=2) and single-type *10D (n=1). The remaining two subjects had a novel type of a *36-containing defective structure that consists of CYP2D6*36 and 3'-flanking REP7/6 (single-type *36-REP7/6). Then, REP7/6 sequences in *5, *10D, *36*10D, and single-type *36 were determined and classified into 5 types: types A to D for *5, type E for *10D and *36*10D, and type F for *36. These findings could be useful for accurate determination of *5 and REP7/6-harboring aberrant CYP2D6 haplotypes.

Keywords: genetic polymorphism, CYP2D6, Japanese

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Kim, S.R., Ozawa, S., Saito, Y., Kurose, K., Kaniwa, N., Kamatani, N.^{*1}, Hamaguchi, T.^{*2}, Shirao, K.^{*2}, Muto, M.^{*2}, Ohtsu, A.^{*2}, Yoshida, T.^{*2}, Matsumura, Y.^{*2}, Saijo, N.^{*2} and Sawada, J.: **Fourteen novel genetic variations and haplotype structures of the TYMS gene encoding human thymidylate synthase (TS).**

Drug Metab. Pharmacokinet., **21**, 509-516 (2006)

Forty genetic variations including 14 novel ones were found in the human TYMS gene, which encodes thymidylate synthase, in 263 Japanese cancer patients who received 5-fluorouracil (FU)-based chemotherapy. Three novel variations were located within the 28-bp tandem repeat sequence in the 5'-untranslated region (UTR) and were designated 5Rc, 3Rc-ins and 4Rc. Allele frequencies were 0.021 for 5Rc, 0.006 for 3Rc-ins and 0.002 for 4Rc. Other novel variations included -133G>C and -125G>C in the 5'-UTR; IVS1-278G>A, IVS2-68C>T, IVS2-23T>C, IVS4-141G>A, IVS4+122_+123insATTG, IVS5-100A>T and IVS6-111G>A in the introns; and 1244A>G and 1264G>A in the 3'-UTR. The allele frequencies were 0.34 for IVS4+122_+123insATTG, 0.042 for -133G>C, 0.011 for IVS4-141G>A, 0.006 for -125G>C, 0.004 for IVS1-278G>A, IVS2-68C>T, 1244A>G and 1264G>A, and 0.002 for IVS2-23T>C, IVS5-100A>T and IVS6-111G>A. Using the detected polymorphisms, linkage disequilibrium (LD) analysis was performed, which divided the TYMS gene into three LD blocks. The 28-bp tandem repeat sequence in the 5'-UTR was assigned as Block 2 with a total of 7 alleles. In Blocks 1 and 3, 7 and 19 haplotypes were determined/inferred, respectively. Our findings provide fundamental and useful information for genotyping TYMS in the Japanese and probably other Asian populations.

Keywords: genetic polymorphism, TYMS, haplotype

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Maekawa, K., Fukushima-Uesaka, H., Tohkin, M., Hasegawa, R., Kajio, H.^{*1}, Kuzuya, N.^{*1}, Yasuda, K.^{*1}, Kawamoto, M.^{*2}, Kamatani, N.^{*2}, Suzuki, K.^{*3}, Yanagawa, T.^{*3}, Saito, Y. and Sawada, J.: **Four novel defective alleles and comprehensive haplotype analysis of CYP2C9 in Japanese.**

Pharmacogenet. Genomics, **16**, 497-514 (2006)

Genetic variations in cytochrome P450 2C9 (CYP2C9) are known to contribute to interindividual and interethnic variability in response to clinical drugs such as warfarin. In this study, CYP2C9 from 263 Japanese sub-

jects was resequenced, resulting in the discovery of 62 variations including 32 novel ones. In addition to the 2 known nonsynonymous single nucleotide polymorphisms (SNPs), Ile359Leu (*3; allele frequency = 0.030) and Leu90Pro (*13; 0.002), seven novel nonsynonymous SNPs, Leu17Ile (0.002), Lys118ArgfsX9 (*25; 0.002), Thr130Arg (*26; 0.002), Arg150Leu (*27; 0.004), Gln214Leu (*28; 0.002), Pro279Thr (*29; 0.002), and Ala477Thr (*30; 0.002), were found. In vitro functional characterization of novel alleles using a mammalian cell expression system revealed that *25 was a null allele and that *26, *28, and *30 were defective alleles.

Linkage disequilibrium and haplotype analyses were performed using the detected variations. Although the haplotype structure of CYP2C9 was rather simple in Japanese, the haplotype distribution was quite different from those previously reported in Caucasians and Africans. Taken together, novel defective alleles and detailed haplotype structures would be useful for determining metabolic phenotypes of CYP2C9 substrate drugs in Japanese and probably Asians.

Keywords: genetic polymorphism, CYP2C9, function

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Saeki, M., Saito, Y., Sai, K., Maekawa, K., Kaniwa, N., Sawada, J., Kawamoto, M.^{*}, Saito, A.^{*} and Kamatani, N.^{*}: **A combinatorial haplotype of the UDP-glucuronosyltransferase 1A1 gene (#60-#1B) increases total bilirubin concentrations in Japanese volunteers.**

Clin. Chem., **53**, 356-358 (2007)

This study shows that either #60 or #1B alone has a slight effect on total bilirubin concentrations. The presence of both #60 and #1B on the same DNA strand (#60-#1B), however, significantly increased bilirubin concentrations when present with #6-#1A on the other chromosome. Thus, at least in the Japanese population, #60 and #1B marker variations should also be incorporated into the UGT1A1 genotyping in addition to #6 and #28 markers.

Keywords: UGT1A1, bilirubin, haplotype

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Fukushima-Uesaka, H., Saito, Y., Tohkin, M., Maekawa, K., Hasegawa, R., Kawamoto, M.^{*1}, Kamatani, N.^{*1}, Suzuki, K.^{*2}, Yanagawa, T.^{*2}, Kajio, H.^{*3}, Kuzuya, N.^{*3}, Yasuda, K.^{*3} and Sawada, J.: **Genetic variations and**

haplotype structures of the ABC transporter gene *ABCC1* in a Japanese population.

Drug Metab. Pharmacokinet., **22**, 48-60 (2007)

Multidrug resistance-related protein 1 (MRP1), an ATP-binding cassette transporter encoded by the *ABCC1* gene, functions as an efflux transporter for conjugated as well as unconjugated substrates. In this study, the 31 exons and their flanking introns of *ABCC1* were comprehensively screened for genetic variations in 153 Japanese subjects to elucidate the linkage disequilibrium (LD) profiles and haplotype structures of *ABCC1* that is necessary for pharmacogenetic studies of the substrate drugs. Eighty-six genetic variations including 31 novel ones were found. Of these, eight novel nonsynonymous variations, 726G>T (Trp-242Cys), 1199T>C (Ile400Thr), 1967G>C (Ser656Thr), 2530G>A (Gly844Ser), 3490G>A (Val1164Ile), 3550G>A (Glu1184Lys), 3901C>T (Arg1301Cys), and 4502A>G (Asp1501Gly), were detected with an allele frequency of 0.003. Based on the LD profiles, the analyzed regions of the gene were divided into five LD blocks. The multiallelic repeat polymorphism in the 5' -UTR was defined as Block -1. For Blocks 1, 2, 3 and 4, 32, 23, 23 and 13 haplotypes were inferred. This study would provide fundamental and useful information for the pharmacogenetic studies of MRP1-dependently effluxed drugs in Japanese.

Keywords: genetic polymorphism, *ABCC1*, haplotype

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Hanioka, N.^{*}, Tsuneto, Y.^{*}, Saito, Y., Sumada, T.^{*}, Maekawa, K., Saito, K.^{*}, Sawada, J. and Narimatsu, S.^{*}: **Functional characterization of two novel CYP2C19 variants (*CYP2C19*^{*18} and *CYP2C19*^{*19}) found in a Japanese population.**

Xenobiotica, **37**, 342-355 (2007)

Cytochrome P450 2C19 (*CYP2C19*) plays an important role in the metabolism of a wide range of therapeutic drugs and exhibits genetic polymorphism with interindividual differences in metabolic activity. We have previously described two *CYP2C19* allelic variants, namely *CYP2C19*^{*18} and *CYP2C19*^{*19} with Arg329His/Ile331Val and Ser51Gly/Ile331Val substitutions, respectively. In order to investigate precisely the effect of amino acid substitutions on *CYP2C19* function, *CYP2C19* proteins of the wild-type (*CYP2C19.1B* having Ile331Val) and variants (*CYP2C19.18* and *CYP2C19.19*) were heterologously

expressed in yeast cells, and their S-mephenytoin 4'-hydroxylation activities were determined. The Km value of CYP2C19.19 for S-mephenytoin 4'-hydroxylation was significantly higher (3.0-fold) than that of CYP2C19.1B. Although no significant differences in Vmax values on the basis of microsomal and functional CYP protein levels were observed between CYP2C19.1B and CYP2C19.19, the Vmax / Km values of CYP2C19.19 were significantly reduced to 29-47% of CYP2C19.1B. By contrast, the Km, Vmax or Vmax / Km values of CYP2C19.18 were similar to those of CYP2C19.1B. These results suggest that Ser-51Gly substitution in CYP2C19.19 decreases the affinity toward S-mephenytoin of CYP2C19 enzyme, and imply that the genetic polymorphism of *CYP2C19^S19* also causes variations in the clinical response to drugs metabolized by CYP2C19.

Keywords: genetic polymorphism, CYP2C19, function

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Fukushima-Uesaka, H., Saito, Y., Maekawa, K., Hasegawa, R., Suzuki, K.^{*1}, Yanagawa, T.^{*1}, Kajio, H.^{*2}, Kuzuya, N.^{*2}, Noda, M.^{*2}, Yasuda, K.^{*2}, Tohkin, M. and Sawada, J.: **Genetic variations of the ABC transporter gene *ABCC3* in a Japanese population.**

Drug Metab. Pharmacokinet., **22**, 129-135 (2007)

An ATP-binding cassette transporter, multidrug resistance-related protein 3 (MRP3), is encoded by the *ABCC3* gene. The MRP3 protein functions as an efflux transporter for conjugated as well as unconjugated substrates. In this study, the 31 *ABCC3* exons and their flanking introns were comprehensively screened for genetic variations in 89 Japanese subjects. Forty-six genetic variations, including 21 novel ones, were found: 8 were located in the 5'-flanking region, 14 in the coding exons, and 24 in the introns. Of these 46 variations, five novel nonsynonymous variations, 2221C>T (Gln741Stop), 2395G>A (Val799Met), 2798_2799delAG (Gln933ArgfsX64), 3657C>A (Ser1219Arg), and 4217C>T (Thr1406Met), were found as heterozygous variations. The allele frequencies were 0.011 for Ser1219Arg and 0.006 for the other four variations. Gln741Stop induces a stop codon at codon 741. Gln933ArgfsX64 causes a frame-shift at codon 933, resulting in early termination at codon 997. Both variations result in loss of 6 transmembrane helices (from the 12th to 17th helices) in the C-terminus and all regions of nucleotide binding domain 2. Thus, both variant proteins are assumed to be inactive. These data provide fundamen-

tal and useful information for pharmacogenetic studies on MRP3-transported drugs in Japanese.

Keywords: genetic polymorphism, *ABCC3*, Japanese

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Nakamura, R.: **Chimeric EGF Receptor That Detects IgE-binding/crosslinking.**

J. Allergy Clin. Immunol., **119**, S97 (2007)

RATIONALE: The crosslinking of high-affinity receptors for IgE (FcεRI) with IgE and allergen is an essential step for activating mast cells. We have generated a chimeric receptor that can detect binding and crosslinking of IgE with high sensitivity.

METHODS: The cDNAs of human FcεRI alpha and epidermal growth factor receptor (EGFR) were cloned. The fragments encoding the extracellular domain of FcεRI alpha and the intracellular domain of EGFR were fused. The chimeric receptor was expressed on HLR-Elk1 cells, which are the HeLa cell-derived cell line stably expressing luciferase reporter gene that is transactivated by Elk-1. The binding and crosslinking of human IgE was measured by confocal fluorescence microscopy and luciferase assay, respectively.

RESULTS: The chimeric receptors were efficiently expressed on the plasma membrane of HLR-Elk1 cells. Confocal microscopic analysis revealed that the cells bound human IgE. Addition of human IgE and anti-IgE dramatically increased expression of the luciferase reporter gene.

CONCLUSIONS: The cells expressing FcεRI-EGFR chimeric receptors could be a useful diagnostic tool that detects binding and crosslinking of IgE.

Keywords: IgE, IgE receptor, allergen

Nakajima, O., Teshima, R., Takagi, K., Okunuki, H. and Sawada, J.: **ELISA method for monitoring human serum IgE specific for Cry1Ab introduced into genetically modified corn.**

Regul. Toxicol. Pharmacol., **47**, 90-95 (2007)

Enzyme-linked immunosorbent assay (ELISA) is the most convenient method of monitoring the occurrence of IgE antibodies specific for novel proteins in genetically modified (GM) foods. The levels of IgE specific for a recombinant protein, Cry1Ab, were determined using an ELISA method. A soluble form of the Cry1Ab protein purified from pCold1 vector-transformed *Escherichia coli* pTf16/BL21 was used as the ELISA coating antigen, and 1M NaCl was used as the washing buffer to remove IgE

non-specifically bound to the coated antigen. Sera from 44 patients allergic to major food allergens were obtained, diluted 20-fold, tested, and found no identifiable IgE above background levels. We also tested sera from patients with corn allergy against whole extracts of non-GM and GM-corn (MON810) using immunoblotting. The staining patterns were similar for the two types of corn. These results indicate that significant levels of IgE antibodies specific to Cry1Ab were not found in the sera of Japanese patients with food allergies.

Keywords: human IgE, ELISA, Cry1Ab

Teshima, R., Amano, F.^{*1}, Nakamura, R., Tanaka, Y.^{*2} and Sawada, J.: **Effects of polyunsaturated fatty acids on calcium response and degranulation from RBL-2H3 cells.**

Int. Immunopharmacol., **7**, 205-210 (2007)

To investigate the biological activity of various polyunsaturated fatty acids (PUFAs) on the allergic reaction, we examined the effects of six PUFAs and two saturated fatty acids on calcium response and degranulation from rat basophilic leukemia (RBL-2H3) cells. Between 20 and 40 microM of six PUFAs (omega-6 series: arachidonic acid [AA, C20:4], gamma-linolenic acid [gamma-LN, C18:3] and linoleic acid [LA, C18:2]; omega-3 series: alpha-linolenic acids [alpha-LN, C18:3] and eicosapentaenoic acid [EPA, C20:5]; and omega-9 series: oleic acid [OLE, C18:1]), or two saturated fatty acids (stearic acid [STA, C18:0] and arachidic acid [AD, C20:0]) were used to examine the effects on calcium response and degranulation from RBL-2H3 cells. Calcium response was monitored using the fluorescent calcium indicator fura-2, while degranulation was monitored by measuring histamine release from the cells. Three omega-6 PUFAs (AA, gamma-LN and LA) dose-dependently increased the cytosolic free-calcium concentration and histamine release from RBL-2H3 cells. This phenomenon was specific to the omega-6 PUFAs, the omega-3 PUFAs (alpha-LN and EPA), omega-9 PUFA (OLE) and the saturated fatty acids (STA and AD) had no effect. The increase in the cytosolic free-calcium concentration caused by the omega-6 PUFAs depended on the existence of external calcium, cell viability and the cellular IP₃ levels remained unchanged throughout the experiment. These results suggest that omega-6 PUFAs work as direct mediators of calcium signaling pathways in RBL-2H3 cells.

Keywords: polyunsaturated fatty acid, degranulation, RBL-2H3 cells

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Chikamatsu S.^{*1}, Furuno, T.^{*2}, Kinoshita, Y.^{*1}, Inoh, Y.^{*2}, Hirashima, N.^{*1}, Teshima, R. and Nakanishi, M.^{*1}: **Effect of cot expression on the nuclear translocation of NF- κ B in RBL-2H3 cells.**

Mol. Immunol., **44**, 1490-1497 (2007)

Cot is a serine/threonine protein kinase and is classified as a mitogen-activated protein (MAP) kinase kinase kinase. Overexpression of this protein has been shown to activate the extracellular signal-regulated kinase, the c-Jun N-terminal kinase, and the p38 MAP kinase pathways and to stimulate NF-AT and NF-kappaB-dependent transcription. Here we have shown that Cot kinase activity is intimately involved in the high affinity receptor for IgE (Fc ϵ RI)-mediated nuclear translocation of NF-kappaB1 independent of NF-kappaB-inducing kinase (NIK) in rat basophilic leukemia (RBL-2H3) cells. A transfected green fluorescent protein-tagged NF-kappaB1 (GFP-NF-kappaB1) resided in the cytoplasm in RBL-2H3 cells and it remained in the cytoplasm even when Cot tagged with red fluorescent protein (Cot-RFP) was co-expressed. Western blotting analysis showed that IkappaB kinases (IKKs) were expressed in RBL-2H3 cells but NIK was not. GFP-NF-kappaB1 translocated from the cytoplasm to the nucleus after the aggregation of Fc ϵ RI in Cot-transfected cells but not in kinase-deficient Cot-transfected cells. This finding gives a new insight into the role of Cot in the Fc ϵ RI-mediated NF-kappaB activation in mast cells.

Keywords: Cot kinase, NF-kappaB, RBL-2H3 cells

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Hirayama, A., Adachi, R., Otani, S., Kasahara, T.^{*}, Suzuki, K.: **Cofilin plays a critical role in IL-8-dependent chemotaxis of neutrophilic HL-60 cells through changes in phosphorylation.**

J. Leukocyte Biol. **81**: 720-728 (2007)

Cofilin is a ubiquitous actin-binding protein. Only unphosphorylated cofilin binds actin and severs or depolymerizes filamentous actin (F-actin), and the inactive form of cofilin is phosphorylated at Ser 3. We recently reported that cofilin plays a regulatory role in superoxide production and phagocytosis by leukocytes, and in the present study we investigated the role of cofilin in the chemotaxis of neutrophilic HL-60 cells. Interleukin 8 (IL-8) is a potent physio-

logical chemokine, and it triggers a rapid, transient increase in F-actin beneath the plasma membrane and rapid dephosphorylation and subsequent rephosphorylation of cofilin. In this study cofilin phosphorylation was found to be inhibited by S3-R peptide, which consists of a peptide corresponding to part of the phosphorylation site of cofilin and a membrane-permeable arginine polymer. When S3-R peptide was introduced into the neutrophilic cells, their chemotactic activity was enhanced, whereas a control peptide that contained an inverted sequence of the phosphorylation site of cofilin had no enhancing effect. Cofilin small interfering RNA (cofilin siRNA) decreased cofilin expression by about half and inhibited chemotaxis. In IL-8-stimulated cells unphosphorylated cofilin accumulated around F-actin, and colocalization of F-actin and phosphorylated cofilin was observed, but these changes in cofilin localization were less prominent in cofilin siRNA-treated cells. The inhibitors of PI3 kinase wortmannin and LY294002 inhibited the chemotaxis and suppressed both IL-8-evoked dephosphorylation and rephosphorylation of cofilin. These results suggested that unphosphorylated cofilin plays a critical role in leukocytes chemotaxis and that PI3-kinase is involved in the control of the phosphorylation/dephosphorylation cycle of cofilin.

Keywords: chemotaxis, neutrophil, CXCL8

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Sai, K., Itoda, M., Saito, Y., Kurose, K., Katori, N., Kaniwa, N., Komamura, K.^{*1}, Kotake, T.^{*1}, Morishita, H.^{*1}, Tomoike, H.^{*1}, Kamakura, S.^{*1}, Kitakaze, M.^{*1}, Tamura, T.^{*2}, Yamamoto, N.^{*2}, Kunitoh, H.^{*2}, Yamada, Y.^{*2}, Ohe, Y.^{*2}, Shimada, Y.^{*2}, Shirao, K.^{*2}, Minami, H.^{*3}, Ohtsu, A.^{*3}, Yoshida, T.^{*4}, Saijo, N.^{*3}, Kamatani, N.^{*5}, Ozawa S. and Sawada, J: **Genetic variations and haplotype structures of the *ABCBI* gene in a Japanese population: an expanded haplotype block covering the distal promoter region, and associated ethnic differences.**

Ann. Hum. Genet. **70**: 605-622 (2006)

As functional *ABCBI* haplotypes were recently reported in the promoter region of the gene, we resequenced the *ABCBI* distal promoter region, along with other regions (the enhancer and proximal promoter regions, and all 28 exons) , in a total of 533 Japanese subjects. Linkage disequilibrium (LD) analysis based on 92 genetic variations revealed 4 LD blocks with the same make up as previously described (Blocks -1, 1, 2 and 3) , except that Block 1

was expanded to include the distal promoter region, and that a new linkage between polymorphisms -1789G>A in the distal promoter region and IVS5 + 123A>G in intron 5 was identified. We re-assigned Block 1 haplotypes, and added novel haplotypes to the other 3 blocks. The reported promoter haplotypes were further classified into several types according to tagging variations within Block 1 coding or intronic regions. Our current data reconfirm the haplotype profiles of the other three blocks, add more detailed information on functionally-important haplotypes in Block 1 and 2 in the Japanese population, and identified differences in haplotype profiles between ethnic groups. Our updated analysis of *ABCBI* haplotype blocks will assist pharmacogenetic and disease-association studies carried out using Asian subjects.

Keywords: *ABCBI*, haplotype, irinotecan

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Mutoh, K.^{*1}, Mitsuhashi, J.^{*1,2}, Kimura, Y.^{*3}, Tsukahara, S.^{*2}, Ishikawa, E.^{*2}, Sai, K., Ozawa, S., Sawada, J., Ueda, K.^{*3}, Katayama, K.^{*1} and Sugimoto, Y.^{*1,2}: **AT3587G germ-line mutation of the *MDR1* gene encodes a nonfunctional P-glycoprotein.**

Mol. Cancer Ther. **5**: 877-884 (2006)

The human multidrug resistance gene 1 (*MDR1*) encodes a plasma membrane P-glycoprotein (P-gp) that functions as an efflux pump for various structurally unrelated anticancer agents. We have identified two nonsynonymous germ-line mutations of the *MDR1* gene, C3583T *MDR1* and T3587G *MDR1*, in peripheral blood cell samples from Japanese cancer patients. Two patients carried the C3583T *MDR1* allele that encodes H1195Y P-gp, whereas a further two carried T3587G *MDR1* that encodes I1196S P-gp. Murine NIH3T3 cells were transfected with pCAL-MDR-IRES-ZEO constructs carrying either wild-type (WT) , C3583T, or T3587G *MDR1* cDNA and selected with zeocin. The resulting zeocin-resistant mixed populations of transfected cells were designated as 3T3/WT, 3T3/H1195Y, and 3T3/I1196S, respectively. The cell surface expression of I1196S P-gp in 3T3/I1196S cells could not be detected by fluorescence-activated cell sorting, although low expression of I1196S P-gp was found by Western blotting. H1195Y P-gp expression levels in 3T3/H1195Y cells were

slightly lower than the corresponding WT P-gp levels in 3T3/WT cells. By immunoblotting analysis, both WT P-gp and H1195Y P-gp were detectable as a 145-kDa protein, whereas I1196S P-gp was visualized as a 140-kDa protein. 3T3/I1196S cells did not show any drug resistance unlike 3T3/H1195Y cells. Moreover, a vanadate-trap assay showed that the I1196S P-gp species lacks ATP-binding activity. Taken together, we conclude from these data that T3587G MDR1 expresses a nonfunctional P-gp and this is therefore the first description of such a germ-line mutation. We contend that the T3587G MDR1 mutation may affect the pharmacokinetics of MDR1-related anticancer agents in patients carrying this allele.

Keywords: ABCB1, MDR1, P-glycoprotein

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Hajime Toyofuku: **Harmonization of international risk assessment protocol.**

Marine Pollution Bulletin, **53**, 579–590 (2006)

For over centuries developments in food production and new food safety management systems in most developed countries have been perceived by many to be efficient in the prevention of food-borne disease. Nevertheless a number of problems remain dominant, one of these being the high level of food-borne microbiological disease which seems, for some pathogens, to have increased over the last decades.

The development of an interdisciplinary approach with direct interaction between surveillance and risk analysis systems is described as a potential basis for improved prevention of food-borne disease. Quantitative microbiological risk assessment is a relatively new scientific approach, able to link data from food within the entire food chain and the various data on human disease to provide a clear estimation of risk. Today food safety is one of the WHO's top eleven priorities; the Organization calls for more systematic and aggressive steps to be taken to reduce significantly the risk of microbiological food-borne diseases. Dealing with this challenge is one of the major challenges for the 21st century in regard to food safety, implying a significant re-direction of food microbiology efforts in many parts of the world.

Keywords: Microbiological risk assessment, risk analysis

Hajime Toyofuku: **Joint FAO/WHO/IOC activities**

to provide scientific advice on marine biotoxins (research report) .

Marine Pollution Bulletin, **52**, 1735–1745 (2006)

The Joint FAO/WHO/IOC ad hoc Expert Consultation on Biotoxins in Molluscan Bivalves performed risk assessments for a number of biotoxins present in bivalve molluscs. For performing risk assessments, the Expert Consultation categorized the biotoxins into eight distinct groups based on chemical structure. The Expert Consultation established LOAELs for the azaspiracid (AZA), okadaic acid (OA), saxitoxin (STX), and domoic acid (DA) toxin groups. The derived provisional acute RfDs for the AZA, OA, STX, and DA toxin groups were 0.04 lg/kg bw, 0.33 lg/kg bw, 0.7 lg/kg bw, and 100 lg/kg bw, respectively. For the yessotoxin (YTX) group, a NOAEL was established, based on animal studies. Applying a safety factor of 100, a provisional acute RfD of 50 lg/kg bw was suggested for the YTX group. The Expert Consultation considered that the database for cyclic imines, brevetoxins, and pectenotoxins was insufficient to establish provisional acute RfDs for these three toxin groups.

Keywords: marine biotoxin, provisional acute reference dose (RfD), azaspiracid (AZA), domoic acid (DA), okadaic acid (OA), saxitoxin (STX)

Tweats, DJ^{*1}, Blakey, D^{*2}, Heflich, RH^{*3}, Jacobs, A^{*4}, Jacobsen, SD^{*5}, Morita, T, Nohmi, T, O' Donovan, MR^{*6}, Sasaki, YF^{*7}, Sofuni, T and Tice, R^{*8}: **Report of the IWGT working group on strategies and interpretation of regulatory in vivo tests. I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards.**

Mutation Res., **627**, 78-91 (2007)

In vivo genotoxicity tests play a pivotal role in genotoxicity testing batteries. However, there is a growing body of evidence that compound-related disturbances in the physiology of the rodents used in these assays can result in increases in micronucleated cells in the bone marrow that are not related to the intrinsic genotoxicity of the compound under test. For rodent bone marrow or peripheral blood micronucleus tests, these disturbances include changes in core body temperature (hypothermia and hyperthermia) and increases in erythropoiesis following prior toxicity to erythroblasts or by direct stimulation of cell division in these cells. This paper reviews relevant data from the literature and also previously unpublished data obtained from a questionnaire devised by the IWGT working group.

Keywords: IWGT, micronucleus, false positive

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 *2 Health Canada
 *3 National Center for Toxicological Research, US FDA
 *4 Center for Drug Evaluation and Research, US FDA
 *5 Novo Nordisk A/S
 *6 AstraZeneca R&D
 *7 Hachinohe National College of Technology
 *8 National Institute of Environmental Health Sciences

Tweats, DJ^{*1}, Blakey, D^{*2}, Heflich, RH^{*3}, Jacobs, A^{*4}, Jacobsen, SD^{*5}, Morita, T, Nohmi, T, O' Donovan, MR^{*6}, Sasaki, YF^{*7}, Sofuni, T and Tice, R^{*8}: **Report of the IWGT working group on strategy/interpretation for regulatory in vivo tests; II. Identification of in vivo-only positive compounds in the bone marrow micronucleus test.**

Mutation Res., **627**, 92-105 (2007)

A survey conducted as part of an International Workshop on Genotoxicity Testing (IWGT) has identified a number of compounds that appear to be more readily detected in vivo than in vitro. The reasons for this property varies from compound to compound and includes metabolic differences; the influence of gut flora; higher exposures in vivo compared to in vitro; effects on pharmacology, in particular folate depletion or receptor kinase inhibition. A decision tree is outlined as a guide for the evaluation of compounds that appear to be genotoxic agents in vivo but not in vitro. Keywords: IWGT, micronucleus test, in vivo-only positive compounds

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 *2 Health Canada
 *3 National Center for Toxicological Research, US FDA
 *4 Center for Drug Evaluation and Research, US FDA
 *5 Novo Nordisk A/S
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Marilyn J. Aardema^{*1}, Ronald D. Snyder^{*2}, Carol Spicer^{*3}, Katyayani Divi^{*4}, Takeshi Morita, Robert J. Mauthe^{*5}, David P. Gibson^{*1}, Sandra Soelster^{*6}, Patrick T. Curry^{*7}, Veronique Thybaud^{*8}, Giocondo Lorenzon^{*9}, Daniel Marzin^{*10}, Elisabeth Lorge^{*11}: **SFTG international collaborative study on in vitro micronucleus test, III. Using CHO cells.**

Mutation Res., **607**, 61-87 (2006)

In this report, results are presented from an international

study of the in vitro micronucleus assay using Chinese hamster ovary cells. No differences were seen in the sensitivity or accuracy of the responses in the presence of absence of cytochalasin B. Overall, these results demonstrate the suitability of Chinese hamster ovary cells for the in vitro micronucleus assay.

Keywords: in vitro micronucleus assay, CHO cells, cytochalasin B

- *1 Procter & Gamble Co.
 *2 Schering-Plough
 *3 Covance Laboratories Inc.
 *4 Bioreliance
 *5 Pfizer Inc.
 *6 Midwest BioResearch
 *7 IIT Research Institute
 *8 Sanofi Aventis
 *9 Proskelia Pharmaceuticals
 *10 Institut Pasteur
 *11 Servier Group

Fukuzawa, K.^{*1}, Mochizuki, Y.^{*2}, Tanaka, S.^{*3}, Kitaura, K.^{*4} and T. Nakano: **Molecular Interactions between Estrogen Receptor and Its Ligand Studied by the ab Initio Fragment Molecular Orbital Method.**

J. Phys. Chem. B, **110**, 16102-16110 (2006)

エストロゲン受容体とリガンドの相互作用を、FMO-MP2法を用いて解析を行った。

Keywords: estrogen receptor, FMO, MP2, molecular interaction

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 *2 アドバンスソフト株式会社
 *3 神戸大学
 *4 産業技術総合研究所

Ishikawa, T.^{*1}, Mochizuki, Y.^{*2}, Nakano, T., Amari, S.^{*3}, Mori, H.^{*4}, Honda, H.^{*4}, Fujita, T.^{*5}, Tokiwa, H.^{*1}, Tanaka, S.^{*5}, Komeiji, Y.^{*6}, Fukuzawa, K.^{*7}, Tanaka, K.^{*2} and Miyoshi, E.^{*4}: Fragment molecular orbital calculations on large scale systems containing heavy metal atom.

Chem. Phys. Lett., **427**, 159-165 (2006)

フラグメント分子軌道 (FMO) 法にモデルコアポテンシャル (MCP) 法を導入し、重原子を含む系のFMO計算を可能にした。実証計算として、シスプラチン-DNA複合体のFMO-MP2計算を行った。

Keywords: FMO, MCP, cisplatin, DNA, MP2

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*3 東京大学

*4 九州大学

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*6 産業技術総合研究所

*7 みずほ情報総研株式会社

Ishikawa, T.^{*1}, Mochizuki, Y.^{*1}, Imamura, K.^{*1}, Nakano, T., Mori, H.^{*2}, Tokiwa, H.^{*1}, Tanaka, K.^{*3}, Miyoshi, E.^{*2}, Tanaka, S.^{*4}: **Application of fragment molecular orbital scheme to silicon-containing systems.**

Chem. Phys. Lett., **430**, 361-366 (2006) .

フラグメント分子軌道 (FMO) 法のケイ素を含む系への拡張を行った。

Keywords: FMO, silicon

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Mochizuki, Y.^{*1}, Tanaka, K.^{*2}, Yamashita, K.^{*3}, Ishikawa, T.^{*1}, Nakano, T., Amari, S.^{*4}, Segawa, K., Murase, T.^{*3}, Tokiwa, H.^{*1} and Sakurai, M.^{*5}: **Parallelized integral-direct CIS (D) calculations with multilayer fragment molecular orbital scheme.**

Theor. Chem. Acc., **117**, 541-553 (2007)

CIS (D) 法と多層フラグメント分子軌道 (MLFMO) 法に基づいた励起状態計算法の開発を行った。

Keywords: CIS (D) , MLFMO, excited state

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*2 アドバンスソフト株式会社

*3 NECソフト株式会社

*4 東京大学

*5 東京工業大学

Mochizuki, Y.^{*1}, Nakano, T., Amari, S.^{*2}, Ishikawa, T.^{*1}, Tanaka, K.^{*3}, Sakurai, M.^{*4} and Tanaka, S.^{*5}: **Fragment molecular orbital calculations on red fluorescent protein (DsRed) .**

Chem. Phys. Lett., **433**, 360-367 (2007)

MLFMO-CIS (D) 法を用いた赤色蛍光タンパク質 (DsRed) の励起状態計算を行った。

Keywords: CIS (D) , MLFMO, excited state, DsRed

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*4 東京工業大学

*5 神戸大学

Mochizuki, Y.^{*1}, Komeiji, Y.^{*2}, Ishikawa, T.^{*1}, Nakano, T. and Yamataka, H.^{*1}: **A fully quantum mechanical simulation study on the lowest n- π^* state of hydrated formaldehyde.**

Chem. Phys. Lett., **437**, 66-72 (2007) .

FMO-MD法及びMLFMO-CIS (D) 法を用いた, 水和ホルムアルデヒドのソルバトクロミズムのシミュレーションを行った。

Keywords: FMO-MD, MLFMO-CIS (D) , solvatochromism, hydrated formaldehyde

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*2 産業技術総合研究所

Hirata-Koizumi, M., Saito M., Miyake S., Hasegawa R.: **Adverse events caused by drug interactions involving glucuroconjugates of zidovudine, valproic acid and lamotrigine, and analysis of how such potential events are discussed in package inserts of Japan, UK and USA.**

J. Clin. Pharm. Ther., **32**, 177-185 (2007)

Background and objective: As pharmacokinetic drug interactions frequently cause adverse events, it is important that the relevant information is given in package inserts (PIs). We previously analysed the provision of PIs for HMG-CoA reductase inhibitors and Ca antagonists, for which metabolism by cytochrome P450 could be a major interaction mechanism. In this article, we focus on interactions involving glucuroconjugates because many drugs and their metabolites undergo this conjugation.

Methods: We reviewed clinical drug interactions related to glucuroconjugates, focusing on reports of adverse events. Then, we picked out three important drugs (zidovudine, valproic acid and lamotrigine) , and examined how the literature information is reflected in the relevant PIs in Japan, UK and USA.

Results and discussion: Pharmacokinetic interactions related to glucuroconjugates were found with 33 drug combinations. Of these, five combinations induced clear adverse events: (i) severe anaemia due to zidovudine and caused by interaction with valproic acid, (ii) recurrence/increased frequency of seizure or increased manic states from a reduction in therapeutic effects of valproic acid caused by panipenem, (iii) meropenem or (iv) ritonavir and (v) of lamotrigine caused by oral contraceptives. Analysis of PIs showed a lack of description of the interaction of zido-

vudine with valproic acid in the Japanese PI. The UK PI mentioned this interaction without quantitative data, whereas full information was given in the US PI. A lack of description was also present on the interaction between valproic acid with ritonavir, reported in 2006, in the PIs of all three countries. For the interactions involving valproic acid and panipenem or meropenem, even though marked reduction of blood valproic acid level has been reported, no quantitative data were provided in any of the PIs.

Conclusion: Five combinations were identified to cause severe adverse events because of interactions related to glucuronconjugates. This information, including quantitative data, is not always properly provided in the relevant PIs in Japan, UK or USA. PIs should be improved to better inform healthcare providers and thereby help them and their patients.

Key words: adverse event, drug interaction, glucuronidation

Hasegawa R., Hirata-Koizumi M., Dourson M.^{*1}, Parker A.^{*1}, Hirose A., Nakai S.^{*2}, Kamata E., Ema M.: **Pediatric Susceptibility to 18 Industrial Chemicals, A Comparative Analysis of Newborn with Young Animals.**

Regul. Toxicol. Pharmacol., **47**, 296-307 (2007)

We comprehensively re-analyzed the toxicity data for 18 industrial chemicals from repeated oral exposures in newborn and young rats, which were previously published. Two new toxicity endpoints specific to this comparative analysis were identified, the first, the presumed no observed adverse effect level (pNOAEL) was estimated based on results of both main and dose-finding studies, and the second, the presumed unequivocally toxic level (pUETL) was defined as a clear toxic dose giving similar severity in both newborn and young rats. Based on the analyses of both pNOAEL and pUETL ratios between the different ages, newborn rats demonstrated greater susceptibility (at most 8-fold) to nearly two thirds of these 18 chemicals (mostly phenolic substances), and less or nearly equal sensitivity to the other chemicals. Exceptionally one chemical only showed toxicity in newborn rats. In addition, Benchmark Dose Lower Bound (BMDL) estimates were calculated as an alternative endpoint. Most BMDLs were comparable to their corresponding pNOAELs and the overall correlation coefficient was 0.904. We discussed how our results can be incorporated into chemical risk assessment approaches to protect pediatric health from direct oral exposure to chemicals.

Key words: pediatric susceptibility, industrial chemicals, phenols, newborn rats, childhood exposure, uncertainty factors, ADI, TDI, benchmark dose

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Emiko Sugiyama, Nahoko Kaniwa, Su-Ryang Kim, Ruri Kikura-Hanajiri, Ryuichi Hasegawa, Keiko Maekawa, Yoshiro Saito, Shogo Ozawa, Jun-ichi Sawada, Naoyuki Kamatani^{*1}, Junji Furuse^{*2}, Hiroshi Ishii^{*2}, Teruhiko Yoshida^{*3}, Hideki Ueno^{*4}, Takuji Okusaka^{*4}, and Nagahiro Saijo^{*2}: **Pharmacokinetics of Gemcitabine in Japanese Cancer Patients: The Impact of a Cytidine Deaminase Polymorphism.**

J. Clin. Oncol. **25**, 32-42 (2007)

Purpose: Gemcitabine is rapidly metabolized to its inactive metabolite, 2', 2'-difluorodeoxyuridine (dFdU), by cytidine deaminase (CDA). We previously reported that a patient with homozygous 208A alleles of CDA showed severe adverse reactions with an increase in gemcitabine plasma level. This study extended the investigation of the effects of CDA genetic polymorphisms on gemcitabine pharmacokinetics and toxicities.

Patients and Methods: Genotyping of CDA was performed by a direct sequencing of DNA obtained from the peripheral blood of Japanese gemcitabine-naive cancer patients (n=256). The patients recruited to the association study received a 30-minute intravenous infusion of gemcitabine at a dose of either 800 or 1,000 mg/m², and eight blood samples were periodically collected (n=250). Plasma levels of gemcitabine and dFdU were measured by high-performance liquid chromatography. Plasma CDA activities toward cytidine and gemcitabine were also measured (n=121).

Results: Twenty-six genetic variations, including 14 novel ones and two known nonsynonymous single nucleotide polymorphisms (SNPs), were detected. Haplotypes harboring the nonsynonymous SNPs 79A>C (Lys27Gln) and 208G>A (Ala70Thr) were designated *2 and *3, respectively. The allelic frequencies of the two SNPs were 0.207 and 0.037, respectively. Pharmacokinetic parameters of gemcitabine and plasma CDA activities significantly depended on the number of haplotype *3. Haplotype *3 was also associated with increased incidences of grade 3 or higher neutropenia in the patients who were coadministered fluorouracil, cisplatin, or carboplatin. Haplotype *2 showed no significant effect on gemcitabine pharmacokinetics.

Conclusion: Haplotype *3 harboring a nonsynonymous SNP,

208G>A (Ala70Thr) , decreased clearance of gemcitabine, and increased incidences of neutropenia when patients were coadministered platinum-containing drugs or fluorouracil.

Keywords: gemcitabine, cytidine deaminase, genetic polymorphism

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齋藤充生, 平田睦子, 浦野 勉, 三宅真二, 長谷川隆一.:
現行の添付文書に対する病院薬剤師の意識調査.

医療薬学, **33**, 442-450 (2007)

医療用医薬品の添付文書は, 医療従事者の重要な情報源である.

我が国の添付文書の記載内容は, 簡潔で分かりやすいことを目的として, 平成9年に大幅に改訂された.

我々は, 現在の添付文書の問題点及び使用方法を把握するため, (社)日本病院薬剤師会の協力を得て, 320施設を対象に, アンケート調査を実施した. 質問では, 添付文書に関わる全般的な事項と, 相互作用・薬物動態に関する情報についての調査を行った.

アンケートでは, 266施設 (83.1%) より回答が得られた. 添付文書の記載順序については, 殆どの回答者が支持していた (94.2%). 全ての回答者が使用上の注意の改訂時には, 何らかの根拠情報を求めていた. 相互作用・薬物動態に関する事項としては, 相互作用の表形式記載について, 現行形式を支持するとの意見が大多数 (99.2%) であったが, 相互作用欄において, 個別医薬品名, 定量的な薬物動態の変化率, 代謝酵素分子種等の記載は不十分との意見が多かった. 臨床における有害事象の発生の十分な情報がない相互作用については, 健常人の薬物動態試験で変化がある場合に記載すべきとの回答が多かった (67.7%). 今回実施したアンケート調査により, 相互作用欄の表形式を含め, 現行の添付文書の記載方法については, 概ね, 受け入れられているものの, 薬物間相互作用については, より具体的な情報を求めていることが判明した. 適正な薬物治療の選択および薬物相互作用の防止のためには, 現在の添付文書の利点である, 分かりやすさを損なうことなく, 医薬品の相互作用に関する重要な定量的データなどの必要な情報を取り入れる必要があると考えられる.

Key words: package insert, adequate drug use, drug interaction

Nakamura, Y.*1, Suzuki, T.*1, Igarashi, K., Kanno, J., Furukawa, T.*2, Tazawa, C.*1, Fujishima, F.*1, Miura, I.*1,

Ando, T., Moriyama, N., Moriya, T.*1, Saito, H.*4, Yamada, S.*3 and Sasano, H.*1: **PTOV1: a novel testosterone-induced atherogenic gene in human aorta.** *J Pathol.*, **209**, 522-531 (2006)

There are gender differences in the development of atherosclerosis, possibly owing to differences in sex steroid hormone action and/or metabolism. One of the atherogenic effects of testosterone is thought to be androgen receptor (AR) -mediated vascular smooth muscle cell (VSMC) proliferation. However, the detailed mechanism of this effect, particularly the identity of the genes associated with VSMC proliferation, remains largely unknown. Therefore, we first employed microarray analysis and, subsequently, quantitative RT-PCR to analyse RNA expression in AR-positive human VSMCs treated with testosterone in order to detect testosterone-induced genes associated with cell proliferation. We further examined whether the genes identified were involved in cell proliferation using small interfering RNA (siRNA) transfection. Expression of the gene products was then evaluated in human aorta with various degrees of atherosclerosis in order to evaluate the clinical relevance of the findings. Both microarray and quantitative RT-PCR analyses demonstrated marked induction of the human prostate overexpressed protein 1 (PTOV1) gene by testosterone in the cell lines: this gene was recently identified as a novel androgen-induced gene involved in prostate tumour cell proliferation. Inhibition of PTOV1 by transfection of its corresponding siRNA suppressed testosterone-induced cell proliferation. In human aorta, PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundantly detected in male aorta with mild atherosclerotic changes compared with female aorta or male aorta with severe atherosclerotic changes. These findings indicate that the PTOV1 gene is androgen-responsive in VSMCs and that it may play an important role in androgen-related atherogenesis in the human aorta, particularly early atherosclerosis in the male aorta, through regulating proliferation of neointimal VSMCs.

Key words: atherosclerosis, DNA microarray, PTOV1

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Arima, K.^{*1}, Chubacha, R.^{*1}, Gardiner, DM.^{*1}, Kanno, J., Iguchi, T.^{*2} and Blumberg, B.^{*1}: **Endocrine disrupting organotin compounds are potent inducers of adipogenesis in vertebrates.**

Mol Endocrinol., **20**, 2141-2155 (2006)

Dietary and xenobiotic compounds can disrupt endocrine signaling, particularly of steroid receptors and sexual differentiation. Evidence is also mounting that implicates environmental agents in the growing epidemic of obesity. Despite a long-standing interest in such compounds, their identity has remained elusive. Here we show that the persistent and ubiquitous environmental contaminant, tributyltin chloride (TBT), induces the differentiation of adipocytes in vitro and increases adipose mass in vivo. TBT is a dual, nanomolar affinity ligand for both the retinoid X receptor (RXR) and the peroxisome proliferators-activated receptor gamma (PPARgamma). TBT promotes adipogenesis in the murine 3T3-L1 cell model and perturbs key regulators of adipogenesis and lipogenic pathways in vivo. Moreover, in utero exposure to TBT leads to strikingly elevated lipid accumulation in adipose depots, liver, and testis of neonate mice and results in increased epididymal adipose mass in adults. In the amphibian *Xenopus laevis*, ectopic adipocytes form in and around gonadal tissues after organotin, RXR, or PPARgamma ligand exposure. TBT represents, to our knowledge, the first example of an environmental endocrine disrupter that promotes adipogenesis through RXR and PPARgamma activation. Developmental or chronic lifetime exposure to organotins may therefore act as a chemical stressor for obesity and related disorders.

Key words: Tributyltin chloride, adipogenesis, retinoid X receptor

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Watanabe, Y.^{*1}, Kokubo, H.^{*1,2}, Miyagawa-Tomita, S.^{*3}, Endo, M.^{*1}, Igarashi, K., Aisaki, KI., Kanno, J. and Saga, Y.^{*1,2}: **Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse.**

Development, **133**, 1625-1634 (2006)

Notch signaling is implicated in many developmental processes. In our current study, we have employed a transgenic strategy to investigate the role of Notch signaling

during cardiac development in the mouse. Cre recombinase-mediated Notch1 (NICD1) activation in the mesodermal cell lineage leads to abnormal heart morphogenesis, which is characterized by deformities of the ventricles and atrioventricular (AV) canal. The major defects observed include impaired ventricular myocardial differentiation, the ectopic appearance of cell masses in the AV cushion, the right-shifted interventricular septum (IVS) and impaired myocardium of the AV canal. However, the fates of the endocardium and myocardium were not disrupted in NICD1-activated hearts. One of the Notch target genes, *Hes1*, was found to be strongly induced in both the ventricle and the AV canal of NICD1-activated hearts. However, a knockout of the *Hes1* gene from NICD-activated hearts rescues only the abnormality of the AV myocardium. We searched for additional possible targets of NICD1 activation by GeneChip analysis and found that *Wnt2*, *Bmp6*, *jagged 1* and *Tnni2* are strongly upregulated in NICD1-activated hearts, and that the activation of these genes was also observed in the absence of *Hes1*. Our present study thus indicates that the Notch1 signaling pathway plays a suppressive role both in AV myocardial differentiation and the maturation of the ventricular myocardium.

Key words: Notch signaling, Heart formation, GeneChip analysis

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Miki, Y.^{*}, Suzuki, T.^{*}, Hatori, M.^{*}, Igarashi, K., Aisaki, KI., Kanno, J., Nakamura, Y.^{*}, Uzuki, M.^{*}, Sawai, T.^{*} and Sasano, H.^{*}: **Effects of aromatase inhibitors on human osteoblast and osteoblast-like cells: A possible androgenic bone protective effects induced by exemestane.**

Bone, **40**, 876-887 (2007)

Effects of aromatase inhibitors (AIs) on the human skeletal system due to systemic estrogen depletion are becoming clinically important due to their increasing use as an adjuvant therapy in postmenopausal women with breast cancer. However, possible effects of AIs on human bone cells have remained largely unknown. We therefore studied effects of AIs including the steroidal AI, exemestane (EXE), and non-steroidal AIs, Aromatase Inhibitor I (AI-I)

and aminoglutethimide (AGM), on a human osteoblast. We employed a human osteoblast cell line, hFOB, which maintains relatively physiological status of estrogen and androgen pathways of human osteoblasts, i.e., expression of aromatase, androgen receptor (AR), and estrogen receptor (ER) beta. We also employed osteoblast-like cell lines, Saos-2 and MG-63 which expressed aromatase, AR, and ERalpha/beta in order to further evaluate the mechanisms of effects of AIs on osteoblasts. There was a significant increment in the number of the cells following 72 h treatment with EXE in hFOB and Saos-2 but not in MG-63, in which the level of AR mRNA was lower than that in hFOB and Saos-2. Alkaline phosphatase activity was also increased by EXE treatment in hFOB and Saos-2. Pretreatment with the AR blocker, flutamide, partially inhibited the effect of EXE. AI-I exerted no effects on osteoblast cell proliferation and AGM diminished the number of the cells. hFOB converted androstenedione into E2 and testosterone (TST). Both EXE and AI-I decreased E2 level and increased TST level. In a microarray analysis, gene profile patterns following treatment with EXE demonstrated similar patterns as with DHT but not with E2 treatment. The genes induced by EXE treatment were related to cell proliferation, differentiation which includes genes encoding cytoskeleton proteins. We also examined the expression levels of these genes using quantitative RT-PCR in hFOB and Saos-2 treated with EXE and DHT and with/without flutamide. HOXD11 gene known as bone morphogenesis factor and osteoblast growth-related genes were induced by EXE treatment as well as DHT treatment in both hFOB and Saos-2. These results indicated that the steroidal aromatase inhibitor, EXE, stimulated hFOB cell proliferation via both AR dependent and independent pathways.

Keywords: Aromatase inhibitor, Human osteoblast, cell proliferation

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Morimoto, M. ^{*1}, Sakim, N. ^{*1}, Oginuma, M. ^{*2}, Kiso, M. ^{*1}, Igarashi, K., Aizaki, K., Kanno, J. and Saga, Y. ^{*1,2}: **The negative regulation of Mesp2 by mouse Ripply2 is required to establish the rostro-caudal patterning within a somite.**

Development, **134**, 1561-1569 (2007)

The Mesp2 transcription factor plays essential roles in segmental border formation and in the establishment of rostro-caudal patterning within a somite. A possible Mesp2

target gene, Ripply2, was identified by microarray as being downregulated in the Mesp2-null mouse. Ripply2 encodes a putative transcriptional co-repressor containing a WRPW motif. We find that Mesp2 binds to the Ripply2 gene enhancer, indicating that Ripply2 is a direct target of Mesp2. We then examined whether Ripply2 is responsible for the repression of genes under the control of Mesp2 by generating a Ripply2-knockout mouse. Unexpectedly, Ripply2-null embryos show a rostralized phenotype, in contrast to Mesp2-null mice. Gene expression studies together with genetic analyses further revealed that Ripply2 is a negative regulator of Mesp2 and that the loss of the Ripply2 gene results in the prolonged expression of Mesp2, leading to a rostralized phenotype via the suppression of Notch signaling. Our study demonstrates that a Ripply2-Mesp2 negative-feedback loop is essential for the periodic generation of the rostro-caudal polarity within a somite.

Keywords: Somite, Gene expression study, Ripply2-Mesp2 negative-feedback loop

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Takahashi, Y., Yasuhiko, Y., Kitajima, S., Kanno, J. and Saga, Y. ^{*}: **Appropriate suppression of Notch signaling by Mesp factors is essential for stripe pattern formation leading to segment boundary formation.**

Developmental Biology, **304**, 593-603 (2007)

Mesp1 and Mesp2 are homologous transcription factors that are co-expressed in the anterior presomitic mesoderm (PSM) during mouse somitogenesis. The loss of Mesp2 alone in our conventional Mesp2-null mice results in the complete disruption of somitogenesis, including segment border formation, rostro-caudal patterning and epithelialization of somitic mesoderm. This has led us to interpret that Mesp2 is solely responsible for somitogenesis. Our novel Mesp2 knock-in alleles, however, exhibit a remarkable upregulation of Mesp1. Removal of the pgk-neo cassette from the new allele leads to localization of Mesp1 and several gene expression, and somite formation in the tail region. Moreover, a reduction in the gene dosage of Mesp1 by one copy disrupts somite formation, confirming the involvement of Mesp1 in the rescue events. Furthermore, we find that activated Notch1 knock-in significantly upregulates Mesp1 expression, even in the absence of a Notch signal mediator, Psen1. This indicates that the Psen1-independent effects of

activated Notch1 are mostly attributable to the induction of Mesp1. However, we have also confirmed that Mesp2 enhances the expression of the Notch1 receptor in the anterior PSM. The activation and subsequent suppression of Notch signaling might thus be a crucial event for both stripe pattern formation and boundary formation.

Key words: somitogenesis, Mesp2, Mesp1

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Takahashi, Y., Takagi, A., Hiraoka, S.^{*1}, Koseki, H.^{*1}, Kanno, J., Rawls, A.^{*2} and Saga, Y.^{*3}: **Transcription factors Mesp2 and Paraxis have critical roles in axial musculoskeletal formation.**

Developmental Dynamics, in press. (2007)

Mesp2 and Paraxis are basic HLH-type transcription factors co-expressed in the presomitic mesoderm and are required for normal somite formation. Here we show that Mesp2/Paraxis double-null mice exhibit a distinct phenotype unexpected from either Mesp2 or Paraxis single-null mice. In the posterior region of the body, most of the skeletal components of both the vertebral body and neural arches are severely reduced and only a rudimental lamina and ribs remain, indicating a strong genetic interaction in the sclerotomal cell lineage. However, yeast two-hybrid analyses revealed no direct interaction between Mesp2 and Paraxis. The Mesp2/Paraxis double-null embryo has caudalized somites, revealed by expanded Uncx4.1 expression pattern observed in the Mesp2-null embryo, but the expression level of Uncx4.1 was significantly decreased in mature somites, indicative of hypoplasia of lateral sclerotome derivatives. By focusing on vertebral column formation we found that expressions of Pax1, Nkx3.1, and Bapx1 are regulated by Paraxis and that Pax9 expression was severely affected in the Mesp2/Paraxis double-null embryo. Furthermore, the expression of Pax3, a crucial factor for hypaxial muscle differentiation, is regulated by both Mesp2 and Paraxis in the anteriormost PSM and nascent somite region. The present data strongly suggest that patterning events by bHLH-type transcription factors have deep impacts on regional chondrogenic and myogenic differentiation of somitic cells, mainly via control of Pax genes.

Key words: Mesp2, Paraxis, somitogenesis, vertebra

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Matsugami, T.R.^{*1}, Tanemura, K., Mieda, M.^{*1}, Na-

katomi, R.^{*2}, Yamada, K.^{*3}, Kondo, T.^{*2}, Ogawa, M.^{*2}, Obata, K.^{*2}, Watanabe, M., Hashikawa, T.^{*2} and Tanaka, K.^{*1}: **Indispensability of the glutamate transporters GLAST and GLT1 to brain development.**

Proc Natl Acad Sci USA., **103** (32), 12161-12166 (2006)

Previous in vitro studies have shown that the neurotransmitter glutamate is important in brain development. Paradoxically, loss-of-function mouse models of glutamatergic signaling that are generated by genetic deletion of glutamate receptors or glutamate release show normal brain assembly. We examined the direct consequences on brain development of extracellular glutamate buildup due to the depletion of the glutamate transporters GLAST and GLT1. GLAST/GLT1 double knockout mice show multiple brain defects, including cortical, hippocampal, and olfactory bulb disorganization with perinatal mortality. Here, we report abnormal formation of the neocortex in GLAST/GLT1 mutants. Several essential aspects of neuronal development, such as stem cell proliferation, radial migration, neuronal differentiation, and survival of SP neurons, were impaired. These results provide direct in vivo evidence that GLAST and GLT1 are necessary for brain development through regulation of extracellular glutamate concentration and show that an important mechanism is likely to be maintenance of glutamate-mediated synaptic transmission.

Key words: glutamate, glutamate transporters, brain development

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Araya, R.^{*1}, Noguchi, T.^{*1}, Yuhki, M.^{*1}, Kitamura, N.^{*1}, Higuchi, M.^{*1}, Saido, T.C.^{*1}, Seki, K.^{*1}, Itoharu, S.^{*1}, Kawano, M.^{*1}, Tanemura, K., Takashima, A.^{*1}, Yamada, K.^{*1}, Kondoh, Y.^{*2}, Kanno, I.^{*2}, Wess, J.^{*3} and Yamada, M.^{*1}: **Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice.**

Neurobiol Dis., **24** (2), 334-344 (2006)

The M5 muscarinic acetylcholine receptor (M5R) has been shown to play a crucial role in mediating acetylcholine-dependent dilation of cerebral blood vessels. We show that male M5R^{-/-} mice displayed constitutive constriction of cerebral arteries using magnetic resonance angiography in vivo. Male M5R^{-/-} mice exhibited a significantly reduced cerebral blood flow (CBF) in the cerebral cortex,

hippocampus, basal ganglia, and thalamus. Cortical and hippocampal pyramidal neurons from M5R^{-/-} mice showed neuronal atrophy. Hippocampus-dependent spatial and nonspatial memory was also impaired in M5R^{-/-} mice. In M5R^{-/-} mice, CA3 pyramidal cells displayed a significantly attenuated frequency of the spontaneous postsynaptic current and long-term potentiation was significantly impaired at the mossy fiber-CA3 synapse. Our findings suggest that impaired M5R signaling may play a role in the pathophysiology of cerebrovascular deficits. The M5 receptor may represent an attractive novel therapeutic target to ameliorate memory deficits caused by impaired cerebrovascular function.

Key words: muscarinic acetylcholine receptor, reduced cerebral blood flow (CBF), memory

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Yamada, M.^{*1}, Tanemura, K., Okada, S.^{*2}, Iwanami, A.^{*2}, Nakamura, M.^{*2}, Mizuno, H.^{*1}, Ozawa, M.^{*1}, Ohyama-Goto, R.^{*1}, Kitamura, N.^{*1}, Kawano, M.^{*1}, Tan-Takeuchi, K.^{*1}, Ohtsuka, C.^{*1}, Miyawaki, A.^{*1}, Takashima, A.^{*1}, Ogawa, M.^{*1}, Toyama, Y.^{*2}, Okano, H.^{*2} and Kondo, T.^{*1}: **Electrical stimulation modulates fate determination of differentiating embryonic stem cells.**

Stem Cells., **25** (3), 562-570 (2007)

A clear understanding of cell fate regulation during differentiation is key in successfully using stem cells for therapeutic applications. Here, we report that mild electrical stimulation strongly influences embryonic stem cells to assume a neuronal fate. Although the resulting neuronal cells showed no sign of specific terminal differentiation in culture, they showed potential to differentiate into various types of neurons in vivo, and, in adult mice, contributed to the injured spinal cord as neuronal cells. Induction of calcium ion influx is significant in this differentiation system. This phenomenon opens up possibilities for understanding novel mechanisms underlying cellular differentiation and early development, and, perhaps more importantly, suggests possibilities for treatments in medical contexts.

Key words: electrical stimulation, embryonic stem cells, neuronal fate

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Hirabayashi, Y. and Inoue, T.: **Implications of hemopoietic progenitor cell kinetics and experimental leukemogenesis: Relevance to Gompertzian mortality as possible hematotoxicological endpoint.**

Exp Hematol., **35** (4 Suppl 1), 125-33 (2007)

Objective. The aim of this study is to investigate a possible implication in cell kinetics of the hemopoietic progenitors to the experimental leukemogenesis to elucidate the relevance of various leukemic mode of action to Gompertzian survival curves, a new parameter based on the lifespan. Materials and Methods. Mice, C3H/He, and C57BL/6 strain, male and female, with or without genetic modifications, e.g., p53-deficiency or thioredoxin overexpression were used in the present hemopoietic stem/progenitor research, radiation- or benzene-induced leukemogenesis followed by histopathological examination. A lethal dose of radiation for bone marrow transplantation, and a graded increased dose up to 5 Gy of x-rays for induction of hemopoietic malignancies were given. For caloric restriction studies, 77 kcal/week was maintained in accordance to different restriction-timing. For assays of hemopoietic colonization, colonyforming unit spleen and colony-forming unit granulocyte macrophage were evaluated. Hemopoietic progenitor cell-specific kinetics were studied by continuous labeling of bromodeoxyuridine for cycling cells, followed by ultraviolet (UV) exposure and hemopoietic colonization (bromodeoxyuridine UV [BUUV] method). Various experimental survival curves were applied to a mathematical analysis by Gompertz-Makeham law of mortality. Results. Referring current authors' studies on leukemogenesis induced by ionizing radiation and benzene exposure, implications of hemopoietic progenitor cell kinetics to the experimental leukemogenesis were evaluated by means of a novel experimental tool, the BUUV method. Comparative studies to elucidate relevancies of these data, including two prevention studies, one on caloric restriction and the other on antioxidative thioredoxin overexpression, to those Gompertzian survival curves of experimental animals were analyzed. Conclusion. The Gompertzian expression may elucidate an appropriate toxicological endpoint for evaluating the effect of radiation and/or benzene-exposure on the lifespan and its modification by various experimental preventive measures.

Key words: Gompertzian law of mortality, radiation-induced leukemia, benzene-induced leukemia

Minami, A.^{*}, Tsuboi, I.^{*}, Harada, T.^{*}, Fukumoto, T.^{*}, Hi-

ramoto, M.* , Koshinaga, M.* , Hirabayashi, Y., Kanno, J., Inoue, T. and Aizawa, S.*: **Inflammatory biomarker, neopterin, suppresses B lymphopoiesis for possible facilitation of granulocyte responses, which is severely altered in age-related stromal-cell-impaired mice, SCI/SAM.**

Exp Biol Med (Maywood) , **232** (1) , 134-45 (2007)

Neopterin is produced by monocytes and is a useful biomarker of inflammatory activation. We found that neopterin enhanced in vivo and in vitro granulopoiesis triggered by the stromal-cell production of cytokines in mice. The effects of neopterin on B lymphopoiesis during the enhancement of granulopoiesis were determined using the mouse model of senescent stromal-cell impairment (SCI) , a sub-line of senescence-accelerated mice (SAM) . In non-SCI mice (a less senescent stage of SCI mice) , treatment with neopterin decreased the number of colonies, on a semisolid medium, of colony-forming units of pre-B-cell progenitors (CFU-preB) from unfractionated bone marrow (BM) cells, but not that from a population rich in pro-B and pre-B cells without stromal cells. Neopterin upregulated the expression of genes for the negative regulators of B lymphopoiesis such as tumor necrosis factor- (TNF-) , interleukin-6 (IL-6) , and transforming growth factor-β (TGF-β) in cultured stromal cells, implying that neopterin suppressed the CFU-preB colony formation by inducing negative regulators from stromal cells. The intraperitoneal injection of neopterin into non-SCI mice resulted in a marked decrease in the number of femoral CFU-preB within 1 day, along with increases in TNF- and IL-6 expression levels. However, in SCI mice, in vivo and in vitro responses to B lymphopoiesis and the upregulation of cytokines after neopterin treatment were less marked than those in non-SCI mice. These results suggest that neopterin predominantly suppressed lymphopoiesis by inducing the production of negative regulators of B lymphopoiesis by stromal cells, resulting in the selective suppression of in vivo B lymphopoiesis. These results also suggest that neopterin facilitated granulopoiesis in BM by suppressing B lymphopoiesis, thereby contributing to the potentiation of the inflammatory process; interestingly, such neopterin function became impaired during senescence because of attenuated stromal-cell function, resulting in the downmodulation of the host-defense mechanism in the aged.

Keywords: neopterin, B lymphopoiesis, stromal-cell-impaired mouse

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Li, G.X., Hirabayashi, Y., Yoon, B.I., Kawasaki, Y., Tsuboi, I., Kodama, Y., Kurokawa, Y., Yodoi, J.* , Kanno, J. and Inoue, T.: **Thioredoxin overexpression in mice, model of attenuation of oxidative stress, prevents benzene-induced hemato-lymphoid toxicity and thymic lymphoma.**

Exp Hematol., **34** (12) , 1687-97 (2006)

Objective. Reactive oxygen species (ROS) , generated following benzene exposure, are considered to trigger the development of hematopoietic neoplasms, although little supporting evidence has been found. In this study, we examined whether the experimental elimination of ROS generated following benzene exposure prevents the development of benzene-induced hematopoietic disorders to clarify the mechanism underlying the development of benzene-induced hematopoietic disorders. Methods. C57BL/6 mice, overexpressing human thioredoxin (h-Trx-Tg) , were used to examine the possible nullification of ROS induction following benzene exposure. The experimental group was exposed to 300 ppm benzene 6 hours/day, 5 days/week, for 26 weeks, and lifetime observation followed by molecular and histopathological examinations were carried out. Results. The present study using h-Trx-Tg mice showed a complete suppression of the development of thymic lymphoma induced by benzene inhalation (0% in h-Trx-Tg vs 30% in wildtype (Wt) mice) . This was associated with a 48% decrease in the incidence of clastogenic micronucleated reticulocyte induction in the h-Trx-Tg mice compared with the Wt control after 2 weeks of inhalation. As underlying mechanisms, the attenuation of oxidative stress was accompanied by a complete abrogation of hemato-lymphoid toxicity, as shown by the upregulation of the activity of superoxide-dismutase, and a consequently stable ROS level, as determined by cell sorting using 20, 70-dichlorodihydrofluorescein diacetate, along with a significant attenuation of the overexpression of a cell cycle-dependent kinase inhibitor, p21. Conclusion. The attenuation of benzene-induced oxidative stress and that of the consequent lymphomagenesis were observed for the first time, and these indicate a role of oxidative stress in benzene-induced clastogenesis and lymphomagenesis. (These attenuations were not seen in nonthymic lymphomas, and no leukemias developed in C57BL/6 used in this study.) During the constitutive overexpression of h-Trx, the expression of aryl-hydrocarbon receptor in h-Trx-Tg mice was downregulated, which may also contribute to the at-

tenuation.

Keywords: Thioredoxin, Benzene-induced leukemia, oxidative stress

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Minehata, K.^{*1,4}, Takeuchi, M.^{*1}, Hirabayashi, Y., Inoue, T., Donovan, P.J.^{*2}, Tanaka, M.^{*1,3} and Miyajima, A.^{*1,3}: **Oncostatin m maintains the hematopoietic microenvironment and retains hematopoietic progenitors in the bone marrow.**

Int J Hematol., **84** (4) , 319-327 (2006)

Bone marrow (BM) functions as the primary hematopoietic tissue throughout adult life by providing a microenvironment for the proliferation, differentiation, and retention of hematopoietic stem cells and progenitors. We describe novel roles for oncostatin M (OSM) in the BM hematopoietic microenvironment. Hematopoietic progenitor activity in OSM-deficient mice was reduced in BM but elevated in the spleen and peripheral blood. The level of circulating granulocyte colony-stimulating factor (G-CSF) was increased, whereas that of stromal cell-derived factor 1 (SDF-1) was decreased in OSM-deficient mice. Moreover, the ability of OSM-deficient BM stromal cells to support hematopoiesis in vitro was significantly reduced. These results indicate that OSM plays a unique role in hematopoiesis by maintaining the proper microenvironment for BM hematopoiesis; it also retains hematopoietic progenitors in BM by regulating G-CSF and SDF-1 levels.

Keywords: Oncostatin M, Hematopoiesis, Stromal cell-derived factor 1

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Fukumoto T*, Tsuboi I*, Harada T*, Hiramoto M*, Minami A*, Koshinaga M*, Hirabayashi Y, Kanno J, Inoue T, Aizawa S*. **Inflammatory biomarker, neopterin, enlarges splenic mast-cell-progenitor pool: prominent impairment of responses in age-related stromal cell-impairment mouse SCI/SAM.**

Int Immunopharmacol., **6** (12), 1847-58 (2006)

Neopterin is produced by monocytes and is a useful biomarker of inflammatory responses. We found that neopterin enhances granulopoiesis, but suppresses B-lymphopoiesis triggered by the positive and negative regulations of cytokines produced by stromal cells in mice. In this study, neopterin was found to regulate mast cell development, which was confirmed in the mouse model of senescent stromal-cell impairment (SCI). In non-SCI mice (=less senescent stage of SCI mice), neopterin decreased the number of colonies of IL-3-dependent mast-cell progenitor cells (CFU-mast) from unfractionated bone-marrow cells, but not that from the lineage-negative bone-marrow cell population without stromal cells in a semisolid in vitro system. Neopterin increased the gene expression and protein production of TGF-beta, a negative regulator of CFU-mast, in cultured stromal cells, indicating that neopterin suppressed CFU-mast colony formation by inducing TGF-beta in stromal cells. In contrast to this in vitro study, in vivo treatment with neopterin did not significantly up-regulate TGF-beta. The intravenous injection of neopterin into mice decreased the number of femoral CFU-mast and the expression level of the gene for stem cell factor (SCF), a positive regulator of CFU-mast, whereas the number of splenic CFU-mast and SCF gene expression level increased. In SCI mice, the in vivo and in vitro responses of mast cell development and cytokine gene expression level to neopterin treatment were less marked than those in non-SCI mice. These results suggest that, firstly, neopterin augments the splenic pool of CFU-mast by the production of SCF, and secondly, such neopterin function becomes impaired during senescence because of an impaired stromal-cell function, resulting in the down-modulation of host-defense mechanisms.

Keywords: neopterin, mast cell, Senescence-accelerated mice (SAM)

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Kii, I.^{*1}, Amizuka, N.^{*2}, Minqi, L.^{*2}, Kitajima, S., Saga, Y.^{*3} and Kudo, A.^{*1}: **Periostin is an extracellular matrix protein required for eruption of incisors in mice.**

Biochem Biophys Res Commun., **342**, 766-772 (2006)

A characteristic tooth of rodents, the incisor continuously grows throughout life by the constant formation of dentin and enamel. Continuous eruption of the incisor is accompanied with formation of shear zone, in which the periodontal ligament is remodeled. Although the shear zone plays a

role in the remodeling, its molecular biological aspect is barely understood. Here, we show that periostin is essential for formation of the shear zone. Periostin *-/-* mice showed an eruption disturbance of incisors. Histological observation revealed that deletion of periostin led to disappearance of the shear zone. Electron microscopy revealed that the disappearance of the shear zone resulted from a failure in digestion of collagen fibers in the periostin *-/-* mice. Furthermore, immunohistochemical analysis using anti-periostin antibodies demonstrated the restricted localization of periostin protein in the shear zone. Periostin is an extracellular matrix protein, and immunoelectron microscopy showed a close association of periostin with collagen fibrils *in vivo*. These results suggest that periostin functions in the remodeling of collagen matrix in the shear zone.

Keyword: Periostin, Eruption, Tooth

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Kojima S*, Uchida K. *, Sasaki K. *, Sunagawa M. *, Ohno Y., Kamikawa Y. *: **The suppressant effect of GEA3162 on spontaneous serotonin release from human colonic mucosa *in vitro*.**

Eur. J. Pharmacol., **550**, 162-165 (2006)

The effect of a lipophilic nitric oxide (NO) -releasing compound 5-amino-3- (3,4-dichlorophenyl) 1,2,3,4-oxatriazolium (GEA3162) on the spontaneous release of 5-hydroxytryptamine (5-HT) from human colonic mucosa was investigated *in vitro*. In the presence of tetrodotoxin, spontaneous outflow of 5-HT from the human colonic mucosa was measured by high-performance liquid chromatography with electrochemical detection. GEA3162 concentration-dependently suppressed the 5-HT outflow, but neither the NO-activated soluble guanylate cyclase inhibitor 1H-(1,2,4)-oxadiazolo (4,3-a) quinoxalin-1-one (ODQ) nor peroxynitrite scavenger ebselen affected the suppressant effect of GEA3162. Moreover, neither the L-type calcium channel blocker nifedipine, NO synthase inhibitor L-NG-nitroarginine methyl ester nor guanylate cyclase activator and nifedipine-insensitive 5-HT release, and that GEA3162 can suppress the 5-HT release via an action on colonic mucosa through mechanism independent of ODQ-sensitive cyclic GMP system or peroxynitrite generation.

Key words: human colone, serotonin, GEA3162

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Shinozaki, Y, Koizumi, S., Ohno, Y., Nagao, T., and Inoue, K*.: **Extracellular ATP counteracts the ERK1/2-mediated death-promoting signaling cascades in astrocytes.**

Glia, **54**, 606-618 (2006)

Oxidative stress is the main cause of the neuronal death in pathological conditions. Hydrogen peroxide (H₂O₂) , one of the reactive oxygen species (ROS) , activates many intracellular signaling cascades including src family and mitogen-activated protein kinases (MAPK) such as stress-activated protein kinase (SAPK, JNK) p38 MAPK and ERK1/2, some of which are highly involved in the induction of cellular damage. We previously showed that H₂O₂ induced cell death in astrocytes and ATP, acting on P2Y₁ receptors, protected against it. Here we show that ERK1/2 and src tyrosine kinase are involved in the H₂O₂-evoked death promoting signals in astrocytes and that activation of P2Y₁ receptors counteracts such signals by increasing gene expression and activity of protein tyrosine phosphatase (PTP) . Although H₂O₂ activated three MAPKs including ERK1/2, p38 and JNK, only the activation of ERK1/2 participated in the H₂O₂-evoked cell death. H₂O₂ induced sustained activation of ERK1/2, which was seen mainly in the nucleus region. The MEK1/2 blockers reduced the H₂O₂-evoked cell death as well as the phosphorylated ERK1/2 (P-ERK1/2) signals and their accumulation in the nucleus region. Moreover, we found that H₂O₂ activated src tyrosine kinase family, which was an upstream signal for ERK1/2. Pretreatment of the cells with ATP and 2Me-SADP strongly inhibited the H₂O₂-evoked activation of src tyrosine kinase, resulting in the inhibition of the P-ERK1/2 accumulation in the nucleus. Activation of P2Y₁ receptors enhanced the gene expression and activity of PTP, which was responsible for the inhibition of src tyrosine kinase. Taken together, ATP, acting on P2Y₁ receptors, upregulates the PTP expression and its activity, which counteracts the H₂O₂-promoted death signaling cascades including ERK1/2 and its upstream signal src tyrosine kinase in astrocytes.

Keywords: P2Y₁, src, ERK1/2

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Koizumi, S., Fujishita, K., Tsuda, M*, Inoue, K*.: **The extracellular ATP-mediated epidermal keratinocyte-to-sensory neuron communication; an involvement of keratinocytic ATP in induction of pain.**

Pain research, **21**, 133-139 (2006)

ATP acts as an intercellular messenger in a variety of cells. Here we characterized the Ca^{2+} wave propagation mediated by extracellular ATP in cultured normal human epidermal keratinocytes (NHEKs) also co-cultured with mouse dorsal root ganglion (DRG) neurons. We also asked about physiological consequence of the ATP-mediated communication in relation to pain by behavioral analysis. Pharmacological characterization showed that NHEKs express functional metabotropic P2Y2 receptors. When a cell was gently stimulated with a glass pipette, an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was observed, followed by propagating Ca^{2+} waves in neighboring cells in an extracellular ATP-dependent fashion. Using an ATP-imaging technique, the release and diffusion of ATP among NHEKs were confirmed. DRG neurons are known to innervate the epidermis that is mainly composed of keratinocytes. In the co-culture of NHEKs and DRG neurons, mechanical stimulation-evoked Ca^{2+} waves in NHEKs evoked the $[\text{Ca}^{2+}]_i$ elevation in adjacent DRG neurons, which was also dependent on extracellular ATP and the activation of P2Y2 receptors. Extracellular ATP is a dominant messenger that forms intercellular Ca^{2+} waves in NHEKs. In addition, Ca^{2+} waves in NHEKs could produce a $[\text{Ca}^{2+}]_i$ elevation in DRG neurons, suggesting dynamic cross talk between skin and sensory neurons mediated by extracellular ATP. Next we investigated a physiological consequence of the ATP-mediated communications. Injection of the P2Y2 and P2Y4 receptor agonist uridine 5'-triphosphate (UTP) into plantar surface in rats produced the mechanical allodynia in a concentration-dependent manner. The UTP-induced mechanical allodynia was inhibited by the P2 receptor antagonist PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate) or antisense oligonucleotide for P2Y2 receptors. Taken together, ATP is a key molecule that mediates pain signaling from skin to sensory neurons.

Keywords: Pain, P2Y, UTP

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Koizumi, S., Shigemoto-Mogami, Y., Nasu-Tada, K., Shinozaki, Y., Ohsawa, K.^{*1}, Tsuda, M.^{*2}, Joshi, B.V.^{*3}, Jacobson, K.A.^{*3}, Kohsaka, S.^{*1} and Inoue, K.^{*2}: **UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis.**

Nature, **446**, 1091-1095 (2007)

Microglia, brain immune cells, engage in the clearance of dead cells or dangerous debris, which is crucial to the maintenance of brain functions. When a neighbouring cell

is injured, microglia move rapidly towards it or extend a process to engulf the injured cell. Because cells release or leak ATP when they are stimulated or injured, extracellular nucleotides are thought to be involved in these events. In fact, ATP triggers a dynamic change in the motility of microglia *in vitro* and *in vivo*, a previously unrecognized mechanism underlying microglial chemotaxis; in contrast, microglial phagocytosis has received only limited attention. Here we show that microglia express the metabotropic P2Y₆ receptor, whose activation by endogenous agonist uridine 5'-diphosphate (UDP) triggers microglial phagocytosis. UDP facilitated the uptake of microspheres in a P2Y₆ receptor-dependent manner, which was mimicked by the leakage of endogenous UDP when hippocampal neurons were damaged by kainic acid *in vivo* and *in vitro*. In addition, systemic administration of kainic acid in rats resulted in neuronal cell death in the hippocampal CA1 and CA3 regions, where increases in mRNA encoding P2Y₆ receptors that colocalized with activated microglia were observed. Thus, the P2Y₆ receptor is upregulated when neurons are damaged, and could function as a sensor for phagocytosis by sensing diffusible UDP signals, which is a previously unknown pathophysiological function of P2 receptors in microglia.

Keywords: microglia, P2Y₆, phagocytosis

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Yoshida, H.^{*}, Kobayashi, D.^{*}, Ohkubo, S., Nakahata, N.^{*}: **ATP stimulates interleukin-6 production via P2Y receptors in human HaCaT keratinocytes.**

Eur. J. Pharmacol., **540**, 1-9 (2006)

We evaluated the role of ATP in functions of human HaCaT keratinocytes. ATP was released from HaCaT cells by changing the culture medium. Reverse transcription-polymerase chain reaction analysis revealed that HaCaT cells expressed multiple P2 purinergic receptor mRNAs. UTP was the most potent agonist to increase the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). UTP and ATP caused the accumulation of $[\text{3H}]$ inositol phosphates, suggesting that UTP binds to the Gq/11-coupled P2Y receptor. UTP increased IL-6 mRNA and protein levels, and the increases were inhibited by a P2 purinergic receptor antagonist (suramin, 300 microM). While a protein kinase C inhibitor (GF109203X, 10 microM) was without effect, an intracellular free Ca^{2+} chelator (BAPTA-AM, 50 microM) suppressed UTP-mediated

IL-6 induction. These results suggest that 1) ATP is released from HaCaT cells upon physical stimulation and may act as an autocrine molecule, and 2) the stimulation of P2Y receptors causes IL-6 production via mRNA expression through $[Ca^{2+}]_i$ elevation.

Keywords: P2Y receptor, HaCaT cell, Interleukin-6

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Honma, S.^{*1}, Saika, M.^{*1}, Ohkubo, S., Kurose, H.^{*2}, Nakahata, N.^{*1}: **Thromboxane A₂ receptor-mediated G12/13-dependent glial morphological change.** *Eur. J. Pharmacol.*, **545**, 100-108 (2006)

Glial cells express thromboxane A₂ receptor, but its physiological role remains unknown. The present study was performed to examine thromboxane A₂ receptor-mediated morphological change in 1321N1 human astrocytoma cells. Thromboxane A₂ receptor agonists U46619 and STA2 caused a rapid morphological change to spindle shape from stellate form of the cells pretreated with dibutyl cyclic AMP, but neither carbachol nor histamine caused the change, suggesting that Gq pathway may not mainly contribute to the change. Rho kinase inhibitor Y-27632 inhibited U46619-induced morphological change, and U46619 increased the GTP-bound form of RhoA accompanied with actin stress fiber formation. These responses were reduced by expression of p115-RGS that inhibits G12/13 signaling pathway. U46619 also caused the phosphorylation of extracellular signal-regulated kinase (ERK) and $[^3H]$ thymidine incorporation mainly through G12/13-Rho pathway. These results suggest that stimulation of thromboxane A₂ receptor causes the morphological change with proliferation mainly through G12/13 activation in glial cells.

Keywords: Thromboxane A₂ receptor, G12 family G protein, Rho

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Kobayashi, D.^{*}, Ohkubo, S., Nakahata, N.^{*}: **Contribution of extracellular signal-regulated kinase to UTP-induced interleukin-6 biosynthesis in HaCaT keratinocytes.**

J. Pharmacol., Sci., **102**, 368-376 (2006)

UTP causes interleukin (IL) -6 production via mRNA expression through P2Y₂/P2Y₄ receptors in human HaCaT keratinocytes. In the present study, we analyzed the mechanism of UTP-induced IL-6 production in these cells. UTP, an agonist of P2Y₂/P2Y₄ receptors, induced phosphoryla-

tion of extracellular signal-regulated kinase (ERK) in a concentration- and time-dependent manner. PD98059, a MEK (mitogen-activated protein kinase kinase) inhibitor, and BAPTA-AM [O,O'-bis (2-aminophenyl) ethyleneglycol-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester], an intracellular Ca²⁺ chelator, reduced UTP-induced ERK phosphorylation and IL-6 mRNA expression. 2-APB [(2-aminoethoxy)diphenylborane], an inositol 1,4,5-trisphosphate (IP₃)-receptor antagonist, inhibited UTP-induced IL-6 mRNA expression; and the action of A23187, a Ca²⁺ ionophore, resembled the action of UTP. In contrast, protein kinase C (PKC) downregulation and pertussis toxin did not affect UTP-induced IL-6 mRNA expression, suggesting that PKC and Gi are not involved in the UTP-induced IL-6 production. However, AG1478, an epidermal growth factor (EGF)-receptor inhibitor, partially decreased UTP-induced ERK phosphorylation and IL-6 expression. These results suggest that UTP-induced IL-6 production is in part mediated via phosphorylation of ERK through Gq/11/IP₃/ $[Ca^{2+}]_i$ and transactivation of the EGF receptor.

Keywords: interleukin-6, extracellular signal-regulated kinase (ERK), HaCaT

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Yoshida, M.^{*1, *2}, Sato, Y.^{*1}, Shimura, T.^{*1}, Ohkubo, S., Honma, S.^{*2}, Tanaka, T.^{*3}, Kurimoto, T.^{*3}, Nakahata, N.^{*1}: **Distinct effect of Z-335, a new thromboxane A₂ receptor antagonist, on rabbit platelets and aortic smooth muscle.**

Pharmacology, **79**, 50-56 (2007)

The effect of a novel thromboxane A₂ receptor (TP) antagonist, (+/-)-sodium[2-(4-chlorophenylsulfonylaminoethyl)-indan-5-yl]acetate monohydrate (Z-335), on the U46619-induced responses was compared between rabbit platelets and aorta. Z-335 inhibited platelet shape change induced by U46619 with higher efficacy than SQ29548, a common TP antagonist. The U46619-induced platelet aggregation was inhibited by Z-335 in a noncompetitive manner, while it was competitively inhibited by SQ29548. Z-335 inhibited U46619-induced vasoconstriction of rabbit aorta with higher efficacy than SQ29548. The pA₂ value of Z-335 in aortic vasoconstriction was significantly higher than in platelet shape change. The competitive binding study showed the higher pK_i value of Z-335 against $[^3H]$ -SQ29548 binding in rabbit aortic smooth muscle cells than in platelets. These data suggest that Z-335 has useful characteristics of TP antagonism.

Keywords: Thromboxane A₂, Platelets, Aortic smooth muscle cells

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Masaki Saito*, Megumi Hori*, Yutaro Obara*, Yasushi Ohizumi*, Satoko Ohkubo and Norimichi Nakahata*: **Neurotrophic factor production in human astrocytoma cells by 2,5,6-tribromogamine via activation of epsilon isoform of protein kinase C.**

Eur. J. Pharm. Sci., **28**, 263-271 (2006)

It is known that astrocytes secrete several neurotrophic factors to promote the survival of neurons. For the treatment of neuronal disorders, low molecular weight compounds inducing neurotrophic factor synthesis are useful, because neurotrophic factors are polypeptides which cannot cross the blood brain barrier. When rat pheochromocytoma (PC-12) cells were cultivated in the medium of human astrocytoma cells (1321N1) treated with 2,5,6-tribromogamine, they differentiated to neuron-like cells possessing neurites, indicating that 2,5,6-tribromogamine released neurotrophic factors from 1321N1 cells. In fact, 2,5,6-tribromogamine increased nerve growth factor (NGF) protein synthesis and secretion through mRNA expression. 2,5,6-Tribromogamine inhibited carbachol-induced phosphoinositide hydrolysis as well as phorbol 12,13-myristate acetate did. The inhibition was recovered by bisindolylmaleimide I (GF109203X), a specific protein kinase C (PKC) inhibitor, indicating that 2,5,6-tribromogamine may activate PKC. The morphological differentiation of PC-12 cells by the medium treated with 2,5,6-tribromogamine was also reduced by GF109203X. 2,5,6-Tribromogamine translocated PKC- ϵ but not PKC- α or PKC- ζ , to membrane fraction from cytosol fraction. These results indicate that 2,5,6-tribromogamine promotes the synthesis and secretion of neurotrophic factors including NGF in 1321N1 cells via an activation of PKC ϵ .

Keywords: 2,5,6-Tribromogamine, 1321N1 human astrocytoma cells, Nerve growth factor

* 東北大学・薬

Sasaki, M.*, Miyosawa, K.*, Ohkubo, S., Nakahata, N.*: **Physiological significance of thromboxane A₂ receptor dimerization.**

J. Pharmacol., Sci., **100**, 263-270 (2006)

The thromboxane A₂ receptor (TP), one of the G pro-

tein-coupled receptors (GPCRs), consists of two splicing variants, TP α and TP β , which differ in their C-terminal regions. In the present study, we investigated whether TP α and TP β formed homo- or hetero-dimers and whether the dimerization changed the function of TP. The immunofluorescent analysis using human embryonic kidney (HEK) 293 cells expressing either FLAG-tagged TP α or TP β showed that TP α is mainly distributed on plasma membranes and TP β existed on plasma membranes and within the cells. Co-immunoprecipitation analysis using HEK293 cells expressing both TP α and TP β showed that TP α and TP β formed homo- and hetero-dimers. U46619, a TP agonist, caused phosphoinositide hydrolysis and elevation of [Ca²⁺]_i in a concentration-dependent manner in Chinese hamster ovary (CHO) cells expressing TP α or TP β . The responses were observed to a greater extent in the cells expressing TP α than TP β . In the cells expressing both TP α and TP β , U46619-induced responses were observed to a lesser extent than in the cells expressing TP α alone. Furthermore, [³H]SQ29548 binding showed that the level of the cell surface expression of TP was the following order: the cells expressing TP α > TP α and TP β > TP β . These results indicate that TP α and TP β formed homo- and hetero-dimers, and TP-mediated signaling may be regulated by the hetero-dimer.

Keywords: thromboxane A₂ receptor (TP) α , TP β , dimerization

* 東北大学・薬

Miyosawa, K.*, Sasaki, M.*, Ohkubo, S., Nakahata, N.*: **Different Pathways for Activation of Extracellular Signal-Regulated Kinase through Thromboxane A₂ Receptor Isoforms.**

Biol. Pharm. Bull., **29**, 719-724 (2006)

Thromboxane A₂ receptor (TP) consists of two alternatively spliced isoforms, TP α and TP β , which differ in their cytoplasmic tails. In the present study, we examined the difference in signal transduction of TP α and TP β , using stably expressing cells of TP α and TP β . The cells expressing TP α (TP α -SC2) and TP β (TP β -SC15) were selected based on the similar binding sites of [³H]-SQ29548, a TP antagonist. U46619, a TP agonist, elicited phosphoinositide hydrolysis in TP α -SC2 and TP β -SC15 cells with a similar concentration-dependency. U46619 also caused the phosphorylation of extracellular signal-regulated kinase (ERK1/2) in both TP α -SC2 and TP β -SC15 cells. While

the peak of the phosphorylation of ERK1/2 was observed 5 min after addition of U46619 in TP α -SC2 cells, the long lasting phosphorylation up to 60 min was in TP β -SC15 cells. U46619-induced phosphorylation of ERK1/2 at 5 min was inhibited by pertussis toxin in both cells, suggesting that Gi is involved in the phosphorylation mediated via both TP isoforms. Interfering G12/13 activity by overexpression of p115-RGS reduced U46619-induced ERK1/2 phosphorylation in TP β -SC15 cells, but not in TP α -SC2 cells. H89, an inhibitor of protein kinase A (PKA), reduced U46619-induced ERK1/2 phosphorylation in TP α -SC2 cells, but not in TP β -SC15 cells. These results indicate that Gi may be involved in TP-mediated ERK1/2 phosphorylation in both isoforms. In addition, H89-sensitive kinase and G12/13 may be involved in TP-mediated ERK1/2 phosphorylation in TP α and TP β , respectively.

Keywords: thromboxane A2 receptor- α (TP α), thromboxane A2 receptor- β (TP β), extracellular signal-regulated kinase (ERK1/2)

* 東北大学・薬

Sato, K., Akaishi, T.^{*1}, Matsuki, N.^{*2}, Ohno, Y., Nakazawa, K.: **β -Estradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells.**

Brain Res., **1150**, 108-120 (2007)

培養海馬切片を用いて海馬のシナプス形成に対する β -エストラジオール (E2) の作用を検討した。E2 は後シナプス部マーカーである PSD95 発現を CA3 野 stratum lucidum (CA3SL) で上昇させた。E2 は CA3SL の樹状突起起始部のスパイン密度も上昇させ PSD95 はスパイン頭部に密集していた。E2 の作用は培養一日目に歯状回 (DG) を切除すると消失した。アンモン核神経細胞, DG 神経細胞, およびこれらの混合細胞からなる海馬小領域培養系を用いた FM1-43 解析では, E2 は DG 神経細胞を含む小領域培養において前シナプス部の数を増加させた。脳由来神経栄養因子 (BDNF) 受容体の強力な阻害薬である K252a および BDNF 機能中和抗体は海馬切片培養と小領域培養で見受けられた E2 の作用を完全に阻害したが, 核内エストロゲン受容体 (nER) の強力な阻害薬である ICI182,780 (ICI) は阻害しなかった。DG 神経細胞での BDNF 発現量はアンモン核神経細胞より顕著に高く, E2 は発現レベルに影響を与えなかった。E2 は DG 神経細胞からの BDNF 放出を有意に促進した。PKA の選択的阻害薬である KT5720 と cAMP の非水解性ジエステレオマーであり強力な PKA 阻害薬

である Rp-cAMP は E2 による BDNF 放出促進を完全に抑制したが, ICI と MEK 阻害剤である U0126 は阻害しなかった。これらの結果は E2 は DG 神経細胞からの BDNF 放出を nER 非依存的かつ PKA 依存的に促進することにより苔状線維—CA3 神経細胞間のシナプス形成を誘導していることを示唆している。

Keywords: estrogen, hippocampus, synaptogenesis

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Kurebayashi H, Ohno Y.: **Metabolism of acrylamide to glycidamide and their cytotoxicity in isolated rat hepatocytes: protective effects of GSH precursors.**

Arch Toxicol. **80**, 820-828 (2006)

Acrylamide (AA) is a widely studied industrial chemical that is neurotoxic, mutagenic to somatic and germ cells, and carcinogenic in rodents. The recent discovery of AA at ppm levels in a wide variety of commonly consumed foods has energized research efforts worldwide to define toxicity and prevention. Metabolism and cytotoxicity of AA and its epoxide glycidamide (GA) were studied in the hepatocytes freshly isolated from male Sprague-Dawley rats. The isolated hepatocytes metabolized AA to GA. The formation of GA followed Michaelis-Menten kinetic parameters yielded apparent $K_m = 0.477 \pm 0.100$ and 0.263 ± 0.016 mM, $V_{max} = 6.5 \pm 2.1$ and 26.4 ± 3.0 nmol/h/ 10^6 cells, and $CL_{int} = 14 \pm 5$ and 100 ± 12 μ l/h/ 10^6 cells for the hepatocytes from untreated and acetone-treated rats, respectively. There were lower K_m and marked increases in V_{max} (fourfold) and in CL_{int} (sevenfold) in acetone-treated rat hepatocytes. The data suggest that CYP2E1 played a major role in metabolizing AA to more toxic GA. Both AA and GA induced a concentration- and time-dependent glutathione (GSH) depletion of the hepatocytes. From decreasing rates of GSH contents in hepatocytes, the parameters of glutathione S-transferase (GST) in hepatocytes to AA and GA were calculated to be $K_m = 1.4$ and 1.5 mM, $V_{max} = 21$ and 33 nmol/h/ 10^6 cells, and $CL_{int} = 15$ and 23 μ l/h/ 10^6 cells, respectively. GA 1.5-times more readily depleted GSH content than AA. GA decreased the viability of hepatocytes at 3 mM, but AA did not. These data indicate that GA is more toxic than AA as assessed by intracellular GSH depletion and loss of viability of hepatocytes. GSH precursors such as N-acetylcysteine and methionine provided significant anti-cytotoxic effects on the decrease of GSH content and cell viability of hepatocytes induced by

GA and AA.

Keywords: Isolated hepatocytes, Acrylamide, Glycidamide

Usami, M., Mitsunaga, K.* and Nakazawa, K.: **Two-dimensional electrophoresis of protein from cultured postimplantation rat embryos for developmental toxicity studies.**

Toxicol. In Vitro, **21**, 521-526 (2007)

A simple method for two-dimensional electrophoresis (2-DE) of rat embryonic protein was described. Rat embryos cultured for 24h from day 10.5 of gestation were used as protein samples. When an embryo was used as a protein sample, about 800 protein spots were detected by silver staining in a 2-DE gel of the standard format. Eighty-one protein spots were identified by mass spectrometry for a primary 2-DE map. The same method could be applied to yolk sac membranes from the cultured embryos. The present method was considered to be suitable for a concomitant 2-DE analysis in in vitro developmental toxicity studies.

Keywords: two-dimensional electrophoresis, rat embryo, yolk sac

*東邦大学薬学部

Kojima, H., Ishii, I.*, Nakata, S.* and Konishi, H.*: **Dose-response evaluation using an Epidermal model, an alternative to skin irritation testing.**

Altern. Animal Test. Experiment **11**, 177-184 (2006)

Using 3-dimensional cultured human skin and epidermal models, an alternative to skin irritation testing have been developed. In the ECVAM (European Centre for the Validation of Alternative Methods) validation protocol, hazardous chemicals have been identified using these models. We considered it likely that these models would also be useful for examining the dose-response of the toxicity of a chemical. Therefore, we used the epidermal model (LabCyteTM, Japan Tissue Engineering Co. Ltd.) to evaluate the dose-response of cytotoxicity and compared the findings with those for irritancy by human patch testing of 4 chemicals: sodium lauryl sulfate, benzethonium chloride, nonanoic acid and propylene glycol.

The cytotoxicity of the chemicals was found to be stronger than the irritancy shown by human patch data. Difference ratios ranging from 1.4 to 52.7 were found between irritancy and EC50 (concentration causing a 50% reduction in the MTT assay compared to the untreated control value) in LabCyteTM for 4 chemicals and solvents.

We consider that these models are useful for evaluating

the dose-response of skin irritancy, and could be used to establish a database for risk assessment of chemicals.

Key words: skin irritation, alternative, human patch test

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Ishii, Y.*, Umemura, T., Kanki, K., Kuroiwa, Y., Nishikawa, A., Ito, R.*, Saito, K.*, Nakazawa, H.* and Hirose, M.: **Possible involvement of NO-mediated oxidative stress in induction of rat forestomach damage and cell proliferation by combined treatment with catechol and sodium nitrite.**

Arch. Biochem. Biophys., **447**, 127-135 (2006)

To clarify the mechanisms underlying forestomach carcinogenesis in rats by co-treatment with catechol and sodium nitrite (NaNO_2), we investigated the involvement of oxidative stress resulting from reaction of the two compounds. Since generation of semiquinone radical, hydroxyl radical ($\cdot\text{OH}$), and peroxynitrite ($\text{ONOO}\cdot$) arose through the reaction of catechol with NO, we proposed that superoxide resulting from catechol oxidation reacted with excess NO, consequently yielding $\cdot\text{OH}$ via $\text{ONOO}\cdot$. Male F344 rats were co-treated with 0.2% catechol in the diet and 0.8% NaNO_2 in the drinking water for 2 weeks. Prior to occurrence of histological evidence indicating epithelial injury and hyperplasia, 8-hydroxydeoxyguanosine levels in forestomach epithelium significantly increased from 12 h together with appearance of immunohistochemically nitrotyrosine-positive epithelial cells. There were no remarkable changes in rats given each chemical alone. We conclude that oxidative stress due to NO plays an important role in induction of forestomach epithelial damage, cell proliferation, and thus presumably forestomach carcinogenesis.

Keywords: sodium nitrite, nitric oxide, hydroxyl radical

*Department of Analytical Chemistry, Hoshi University

Ishii, Y.*, Umemura, T., Nishikawa, A., Iwasaki, Y.*, Ito, R.*, Saito, K.*, Hirose, M. and Nakazawa, H.*: **Determination of nitrotyrosine and tyrosine by high-performance liquid chromatography with tandem mass spectrometry and immunohistochemical analysis in livers of mice administered acetaminophen.**

J. Pharm. Biomed. Anal., **41**, 1325-1331 (2006)

Nitrotyrosine (NTYR) is used as a biomarker of nitrate pathology caused by peroxynitrite ($\text{ONOO}\cdot$) formation. NTYR measurement in biological materials usually employs such methodologies as immunohistochemistry staining,

high-performance liquid chromatography and gas chromatography. In this study, we developed a method for the determination of tyrosine (TYR) and NTYR, using liquid chromatography with tandem mass spectrometry (LC-MS/MS). In order to confirm the applicability of our method to an *in vivo* system, we measured protein-bound NTYR levels using by LC-MS/MS method and immunohistochemical analysis in liver of B6C3F1 mice at 2 h, 4 h and 8 h after administration of 300 mg/kg acetaminophen (APAP). A mass spectrometer equipped with an electrospray ionization source using a crossflow counter electrode and ran in the positive ion mode (ESI⁺) was set up for multiple reaction monitoring (MRM), which monitored the transitions 182.2>136.2, 227.1>181.2, 191.3>144.4 and 236.3>189.5, for TYR, NTYR, [¹³C9]-TYR and [¹³C9]-NTYR, respectively. NTYR was detected all liver samples of mice by the proposed LC-MS/MS method. The concentration range of NTYR per milligram protein in samples was 0.17-0.3 pmol/mg protein. And the level reached a maximum at 4 h. These data were well correlated with the result obtained by an immunohistochemical reaction with anti-NTYR antibody.

Keywords: nitrotyrosine, peroxyxynitrite, LC-MS/MS

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Umemura, T., Kanki, K., Kuroiwa, Y., Ishii, Y.^{*}, Okano, K.^{*}, Nohmi, T., Nishikawa, A., and Hirose, M.: ***In vivo* mutagenicity and initiation following oxidative DNA lesion in the kidneys of rats given potassium bromate.**

Cancer Sci., **97**, 829-835 (2006)

To clarify the role of 8-OHdG formation as a starting point for carcinogenesis, we examined the dose-dependence and time-course of changes of OGG1 mRNA expression, 8-OHdG levels and *in vivo* mutations in the kidneys of gpt delta rats given KBrO₃ in their drinking water for 13 weeks. There were no remarkable changes in OGG1 mRNA in spite of some increments being statistically significant. Increases of 8-OHdG occurred after 1 week at 500 p.p.m. and after 13 weeks at 250 p.p.m. Elevation of Spi⁻ mutant frequency, suggestive of deletion mutations, occurred after 9 weeks at 500 p.p.m. In a two-stage experiment, F344 rats were given KBrO₃ for 13 weeks then, after a 2-week recovery, treated with 1% NTA in the diet for 39 weeks. The incidence and multiplicity of renal preneoplastic lesions in rats given KBrO₃ at 500 p.p.m. followed by NTA treatment were significantly higher than in rats treated with

NTA alone. Results suggest that a certain period of time might be required for 8-OHdG to cause permanent mutations. The two-step experiment shows that cells exposed to the alteration of the intranuclear status by oxidative stress including 8-OHdG formation might be able to form tumors with appropriate promotion.

Keywords: potassium bromate, 8-hydroxydeoxyguanosine, OGG1

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Umemura, T., Kuroiwa, Y., Kitamura, Y., Ishii, Y.^{*1}, Kanki, K., Kodama, Y., Itoh, K.^{*2}, Yamamoto, M.^{*2}, Nishikawa, A. and Hirose, M.: **A crucial role of Nrf2 in *in vivo* defense against oxidative damage by an environmental pollutant, pentachlorophenol.**

Toxicol. Sci., **90**, 111-119 (2006)

We examined oxidative stress and cell proliferation, along with other hepatotoxicological parameters, in the livers of Nrf2-deficient (wild:+/+, heterozygous:+/-, homozygous:-/-) animals fed PCP in their diet at doses of 0, 150, 300, 600, or 1200 ppm for 4 weeks. For measurement of methoxyresorufin-O-demethylase (CYP 1A2), NAD(P):quinone oxidoreductase 1 (NQO1), and UDP-glucuronosyltransferase (UDP-GT), an additional study was performed with all but the 150-ppm dose. Significant elevation of 8-OHdG levels in the liver DNA was observed only in -/- mice treated with PCP at 1200 ppm. Levels of TBARS were also raised significantly compared to those of the relevant +/+ mice. Bromodeoxyuridine labeling indices (BrdU-LIs) of hepatocytes in -/- mice were significantly higher at all doses than those in the relevant +/+ mice. Relative liver weights were unchanged in mice lacking Nrf2, whereas liver weight in +/+ and +/- mice was increased. Histopathologically, centrilobular hepatocyte necrosis was severe in the -/- mice that received 600 ppm. Although CYP 1A2 activity was elevated in all treated mice, increases in NQO1 levels and UDP-GT activities did not occur only in -/- mice. These data suggest that Nrf2 plays a key role in prevention of PCP-induced oxidative stress and cell proliferation.

Keywords: pentachlorophenol, Nrf2, 8-OH-dG

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Umemura, T., Kodama, Y., Nishikawa, A., Hioki, K.^{*1}, Nomura, T.^{*1}, Kanki, K., Kuroiwa, Y., Ishii, Y.^{*2},

Kurokawa, Y.^{*3} and Hirose, M.: **Nine-week detection of six genotoxic lung carcinogens using the rasH2/BHT mouse model.**

Cancer Lett., **231**, 314-318 (2006)

A 9-week in vivo rasH2/butylhydroxytoluene (BHT) model for the detection of genotoxic lung carcinogens was validated, using six potent positive test compounds, dimethylnitrosamine (DMN; 15 mg/kg, i.p.), diethylnitrosamine (DEN; 100 mg/kg, i.p.), ethylnitrosourea (ENU; 120 mg/kg, i.p.), 3-methylcholanthrene (MC; 100 mg/kg, i.p.), 7,12-dimethylbenz (a) anthracene (DMBA; 5 mg/kg, i.g.) and benzo (a) pyrene (B (a) P; 80 mg/kg, i.p.), each given to rasH2 mice of both genders by single administration for initiation followed by promoter BHT treatment. Statistically significant increase in the incidence and multiplicity of lung tumors was observed in rasH2 mice treated with BHT following exposure to all of the carcinogens tested. The data overall suggest the rasH2/BHT model to be a powerful screening tool for genotoxic lung carcinogens.

Keywords: rasH2mouse, butylhydroxytoluene, lung carcinogen

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Umemura, T. and Kurokawa, Y. *: **Etiology of bromate-induced cancer and possible modes of action-studies in Japan.**

Toxicology, **221**, 154-157 (2006)

Renal cell tumors were significantly increased in male and female rats given potassium bromate at 250 and 500 mg/L in drinking water. In at least one other study renal cell tumors were produced in male rats at 125 mg/L. Among male mice given 750 mg/L of potassium bromate, there were no significant differences in renal cell tumors between treated and control groups after 88 weeks on test. In oxidative DNA damage tests 8-oxodeoxyguanosine (8-oxodG also referred to as 8-OH-dG) was induced in DNA in the male rat kidney in 1 week, and in females after 3 weeks at 500 mg/L, and also in both male and female rats at 250 mg/L, but not at 125 mg/L. DNA adducts are considered to be an initial step in the carcinogenesis process, however, the administered doses are not always sufficient to cause mutations, possibly due to DNA repair. In the two-step rat renal carcinogenesis model using *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN) as initiator, promotion activity by potassium bromate was measured

using the BrdU labeling index. The promoting activity of bromate in male rats was much greater and extended to doses as low as 60 mg/L in male rats, whereas in females the response was limited to 250 and 500 mg/L. Therefore, it was concluded that the mechanisms contributing to cancer in the male rat were more complex than in the female rat. The accumulation of alpha2u-globulin in the kidneys of male rats exposed to potassium bromate probably accounts for the greater labeling index in the male rat relative to the female rat.

Keywords: potassium bromate, 8-hydroxydeoxyguanosine, *N*-ethyl-*N*-hydroxyethylnitrosamine

* Sasaki Foundation

Okazaki, K., Ishii, Y.*, Kitamura, Y., Maruyama, S.*, Umemura, T., Miyauchi, M., Yamagishi, M., Imazawa, T., Nishikawa, A., Yoshimura, Y.*, Nakazawa, H.* and Hirose, M.: **Dose-dependent promotion of rat forestomach carcinogenesis by combined treatment with sodium nitrite and ascorbic acid after initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine: possible contribution of nitric oxide-associated oxidative DNA damage.**

Cancer Sci., **97**, 175-182 (2006)

Dose-dependent promotion effects of combined treatment with NaNO₂ and ascorbic acid (AsA) on gastric carcinogenesis were examined in rats pretreated with MNNG. In the forestomach, the incidence of hyperplasia was increased dose dependently by the treatment with NaNO₂ alone. Incidences of neoplastic lesions were dramatically increased by the combined treatment with NaNO₂ and AsA in a dose-dependent manner, but AsA itself had no effect. In a second short-term experiment conducted for sequential observation, necrosis and strong inflammation were found in the forestomach epithelium shortly after commencing combined treatment with 1.0% AsA and 0.2% NaNO₂, followed by hyperplasia, whereas there were no obvious effects in the glandular stomach. In addition, after a 4 h treatment with 1.0% AsA and 0.2% NaNO₂, a slight increase in the 8-OHdG levels in the forestomach epithelium was observed by LC-ECD system, albeit without statistical significance. In vitro, ESR demonstrated nitric oxide formation during incubation with NaNO₂ and AsA under acidic conditions. Thus, NaNO₂ was demonstrated to exert promoter action in the forestomach, with AsA acting as a strong copromoter through cytotoxicity and regenerative cell proliferation, possibly mediated by oxidative DNA damage, but the combined treatment with

NaNO₂ and AsA had little influence on glandular stomach carcinogenesis.

Keywords: sodium nitrite, ascorbic acid, 8-OHdG

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Kitamura, Y., Umemura, T., Kanki, K., Ishii, Y., Kuroiwa, Y., Masegi, T.^{*}, Nishikawa, A. and Hirose, M.: **Lung as a new target in rats of 2-amino-3-methylimidazo[4,5-*f*]quinoline carcinogenesis: results of a two-stage model initiated with *N*-bis(2-hydroxypropyl) nitrosamine.**

Cancer Sci., **97**, 368-373 (2006)

The effects of IQ on the promotion stage of DHPN-induced lung carcinogenesis and contributions of oxidative stress were investigated in rats. Groups of 20 male 6-week-old F344 rats were given 0.1% DHPN in their drinking water for 2 weeks for initiation. From the age of 9 weeks, they were treated with 0, 150 and 300 p.p.m. of IQ in the diet for 27 weeks. Control rats were similarly fed 300 p.p.m. IQ or basal diet alone without the preceding initiation. IQ clearly ($P < 0.01$) enhanced the multiplicity of lung tumors in a dose-dependent manner (DHPN alone, 3.63 +/- 1.80; DHPN +150 p.p.m. IQ, 11.50 +/- 5.04; DHPN +300 p.p.m. IQ, 18.83 +/- 4.58 [no./rat]). In addition, the incidence of lung tumors in the 300 p.p.m. IQ alone group (25%) was significantly ($P < 0.05$) higher than that in the non-treatment group (0%). In a second experiment, male rats were given IQ at doses of 0 and 300 p.p.m. in the diet for one week in order to analyze 8-OHdG formation, levels of TBARS and BrdU-LI in the lungs. There were no changes in 8-OHdG or TBARS levels, but significant elevation of BrdU-LI occurred in the IQ administration group. The overall data clearly indicate that IQ is a potent lung carcinogen in rats, in which oxidative stress may not be involved in lung carcinogenesis.

Keywords: IQ, 8-hydroxydeoxyguanosine, TBARS

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Kitamura, Y., Umemura, T., Okazaki, K., Kanki, K., Imazawa, T., Masegi, T.^{*}, Nishikawa, A. and Hirose M.: **Enhancing effects of simultaneous treatment with sodium nitrite on 2-amino-3-methylimidazo[4,5-*f*]quinoline-induced rat liver, colon and Zymbal' s gland carcinogenesis after initiation with diethylnitrosamine and 1,2-dimethylhydrazine.**

Int. J. Cancer, **118**, 2399-2404 (2006)

Combined effects of sodium nitrite (NaNO₂) and 2-amino-

no-3-methylimidazo[4,5-*f*]quinoline (IQ) on liver, colon and Zymbal' s gland carcinogenesis were assessed using a rat two-stage carcinogenesis model, with a focus on involvement of oxidative stress. Male 6-week-old F344 rats were given a single intraperitoneal injection of 200 mg/kg of diethylnitrosamine and 4 subcutaneous injections of 40 mg/kg of 1,2-dimethylhydrazine for initiation. Then, they were administered 0 or 300 ppm IQ in the diet or 0, 0.1 or 0.2% NaNO₂ in their drinking water for 27 weeks. The treatment with NaNO₂+IQ significantly enhanced colon and Zymbal' s gland carcinogenesis and tended to enhance hepatocarcinogenesis. The incidence of lung tumors in the IQ-treated groups was significantly increased as compared with the initiation alone group. In a second experiment, male rats were given IQ or NaNO₂ under the same conditions as before for 1 week, and at sacrifice, their liver and colon tissue or mucosa were collected for analysis of 8-OHdG, thiobarbituric acid reactive substances (TBARS), acrolein-modified protein and the bromodeoxyuridine-labeling index (BrdU-LI) (in the colon). In the colon, 8-OHdG, acrolein-modified protein levels and BrdU-LI were significantly increased by the combined treatment. These results indicate that the treatment with NaNO₂ enhances IQ-induced colon and Zymbal' s gland carcinogenesis in rats and that oxidative DNA damage and lipid peroxidation may partly be involved, especially in the colon.

Keywords: sodium nitrite, IQ, 8-OHdG

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Kitamura, Y., Yamagishi, M., Okazaki K., Umemura T., Imazawa T, Nishikawa A., Matsumoto, W.^{*} and Hirose, M.: **Lack of chemopreventive effects of alpha-eleostearic acid on 7,12-dimethylbenz[*a*]anthracene (DMBA) and 1,2-dimethylhydrazine (DMH)-induced mammary and colon carcinogenesis in female Sprague-Dawley rats.**

Food Chem. Toxicol., **44**, 271-277 (2006)

alpha-Eleostearic acid is one of the conjugated linolenic acids from tung oil, which is obtained from the seeds of *Aleurites fordii*. The effects of dietary alpha-eleostearic acid (18:3, n-5) on the post-initiation period of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 1,2-dimethylhydrazine (DMH)-induced mammary and colon carcinogenesis were examined using female Sprague-Dawley (SD) rats. For initiation, rats were given subcutaneous injections of 40 mg/kg body weight (5 times) and 20 mg/kg body weight (3 times) of DMH during the age of 6-8 weeks

and a single intragastric administration of 50 mg/kg body weight of DMBA at 9 weeks. Then, the animals were treated with 0%, 0.01%, 0.1% or 1.0% alpha-eleostearic acid for 34 weeks. Control rats received the basal diet alone or 1.0% alpha-eleostearic acid without prior initiation treatment. All surviving animals were killed at week 37 of the experiment. There were no statistically significant alterations in any of the parameters for either mammary or colon tumors. These results thus indicate that alpha-eleostearic acid does not exert clear modification effects on DMBA and DMH-induced mammary and colon carcinogenesis, at least under the present experimental conditions.

Keywords: alpha-eleostearic acid, DMBA, DMH

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Kuroiwa Y., Nishikawa A., Imazawa T., Kitamura Y., Kanki K., Ishii Y., Umemura T. and Hirose M.: **A sub-chronic toxicity study of dunaliella carotene in F344 rats.**

Food Chem. Toxicol., **44**, 138-145 (2006)

Dunaliella carotene, extracted from dunaliella alga (*Dunaliella bardawil* or *Dunaliella salina*), for use as a food-coloring agent, has beta-carotene as its mainly constituent. As there have been no reports of toxicological evaluation, a 90-day subchronic toxicity study was here performed in F344 rats at dose levels of 0 (control), 0.63%, 1.25%, 2.5% and 5% in powdered basal diet. The average daily intakes of dunaliella carotene were 352, 696, 1420 and 2750 mg/kg/day, respectively, for males, and 370, 748, 1444 and 2879 mg/kg/day for females. No mortality or treatment-related clinical signs were observed throughout the experimental period in any of the groups. Body weight gain was slightly but significantly ($p < 0.05$) reduced from week 5 to the end of the experiment in 2.5% and 5% males. Increased PLT were observed in 1.25% and 5% males, and 2.5% and 5% females. Significant elevations or tendencies for increase in serum T. Cho and Ca were observed in all treated males and females, with clear dose-dependence in males. Organ weight measurement and histopathological observation revealed no toxicological changes. Based on growth suppression, no-observed-adverse-effect-levels (NOAELs) were estimated to be 1.25% (696 mg/kg/day) for males and 5% (2879 mg/kg/day) for females. As increases in serum Ca were observed in the lowest group in both sexes, a no-observed-effect level (NOEL) could not be determined in this study.

Keywords: dunaliella carotene, F344 rats

Kuroiwa, Y., Nishikawa, A., Kitamura, Y., Kanki, K., Ishii, Y., Umemura, T. and Hirose, M.: **Protective effects of benzyl isothiocyanate and sulforaphane but not resveratrol against initiation of pancreatic carcinogenesis in hamsters.**

Cancer Lett., **241**, 275-280 (2006)

Potential chemopreventive effects of naturally occurring agents were investigated using a new 16-week medium-term pancreatic carcinogenesis models in hamsters. Male 6-week-old Syrian hamsters were subcutaneously injected with 10 mg/kg body weight *N*-nitrosobis (2-oxopropyl) amine (BOP) four times within a week, and fed a diet supplemented with 80 ppm benzyl isothiocyanate (BITC), 80 ppm sulforaphane (SFN) or 10 ppm resveratrol (RES) during the initiation or post-initiation stages. For the initiation stage, each chemical was given for 3 weeks including 1 week before and after the BOP injections. With post-initiation exposure, the groups were changed from basal diet 1 week after the last BOP injection, and then fed each chemical for 14 weeks. All the animals were sacrificed after 16 weeks. The multiplicities of combined pancreatic lesions including atypical hyperplasias and adenocarcinomas were significantly decreased by BITC and SFN given in the initiation but not the post-initiation stage. On the other hand, RES, a naturally occurring inhibitor of cyclooxygenase-2 (COX-2) reported chemopreventive effects, failed to show significant effects on pancreatic carcinogenesis in either the initiation or post-initiation stages. Our data suggest that the naturally occurring isothiocyanates BITC and SFN can block BOP-initiation of hamster pancreatic carcinogenesis.

Keywords: benzyl isothiocyanate, sulforaphane, resveratrol

Nishikawa, A., Sai, K., Okazaki, K., Son, H.-Y., Kanki, K., Nakajima, M.^{*1}, Kinae, N.^{*1}, Nohmi, T., Trosko, J.E.^{*2}, Inoue, T. and Hirose, M.: **MX, a by-product of water chlorination, lacks *in vivo* genotoxicity in *gpt* delta mice but inhibits gap junctional intercellular communication in rat WB cells.**

Environ. Mol. Mutagen., **47**, 48-55 (2006)

3-Chloro-4-(dichloromethyl)-5-hydroxy-2 (5H)-furanone (MX), a by-product of water chlorination, is a potent bacterial mutagen and rat carcinogen. In the present study, the *in vivo* mutagenicity, cell proliferative activity, and carcinogenicity of MX were investigated in *gpt* delta mice. Groups of 5 male and female 7-week-old *gpt* delta C57BL/6J transgenic mice were given MX at doses of 0, 10, 30, or 100 ppm in their drinking water for 12

weeks, and then killed to assess *in vivo* mutagenicity using 6-thioguanine and Spi⁺ selection, and cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen (PCNA). Further groups of 10 male and female gpt delta mice were given 0 or 100 ppm MX for 78 weeks, and a full necropsy with histopathological examination of all organs was conducted to detect neoplastic lesions. The 12-week MX treatment did not result in mutagenicity in the livers or lungs or cell proliferative activity in several organs of the mice, and the 78-week treatment did not cause carcinogenicity. Additional investigations were conducted to evaluate the potential of MX to inhibit gap junctional intercellular communication (GJIC) in rat liver epithelial cells (WB cells) by the scrape loading/dye transfer method. Inhibition of GJIC was detected within 2 hr with a non-cytotoxic dose of MX (4 μ /ml), followed by partial restoration after 5 hr. A second phase of inhibition occurred after 10 hr and then the lowered level persisted for the 24 hr-incubation period. Dose-dependent inhibition was evident at both 2 hr and 24 hr, with much stronger effects at the former time. These findings indicate that MX is not mutagenic, mitogenic or carcinogenic in mice, and suggest that the compound exerts epigenetic actions leading to GJIC inhibition.

Keywords: MX, water, gpt delta mice

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Kuroiwa, K., Shibutani, M., Inoue, K., Lee, K-Y., Woo, G-H. and Hirose, M.: **Subchronic toxicity study of water pepper extract in F344 rats.**

Food Chem. Toxicol., **44**, 1236-1244 (2006)

A subchronic toxicity study of water pepper extract (WPE) from *Polygonum hydropiper L.* was conducted in groups of 10 male and 10 female F344 rats fed powdered diets containing 0, 62.5, 250, 1000 or 4000 ppm concentrations for 13 weeks. Suppression of body weight gain due to decreased food consumption was observed in both sexes at 4000 ppm, and at autopsy, increase of relative weights was observed for the brain, liver, spleen, kidneys, and testes in these animals, suggestive of the reflection of the reduced body weights. At this dose, slight increases of blood urea nitrogen in both sexes and serum alanine aminotransferase, Na and Cl in females, were observed, suggestive of weak hepatic and renal toxicity, at least in females. The same females also exhibited slight decrease of red blood cells and haematocrit, slight increase of mean corpuscular volume

and mean corpuscular haemoglobin, and minimal increase of splenic haemosiderin deposition, providing evidence of slight haemolytic anemia. On the other hand, enhanced accumulation of mast cells was observed in the mesenteric lymph nodes at 4000 ppm in males and 1000 and 4000 ppm in females. Considering the anti-anaphylactic properties of polygodial, a major constituent of WPE, the mast cell accumulation was concluded to be an adaptive change in response to the subchronic oral administration of WPE. Based on the present toxicity data, 1000 ppm was determined to be the no-observed-adverse-effect level, translating into 57.4 and 62.9 mg/kg/day for male and female rats, respectively.

Keywords: subchronic toxicity, water pepper extract, F344 rats

Fujimoto, H., Shibutani, M., Kuroiwa, K., Inoue, K., Woo, G-H., U, M. and Hirose, M.: **A case report of a spontaneous gastrointestinal stromal tumor (GIST) occurring in a F344 rat.**

Toxicol. Pathol., **34**, 164-167 (2006)

We report a case of gastrointestinal stromal tumor (GIST) that developed in a male F344 rat at week 101 of an experiment in a carcinogenicity study. Macroscopically, the primary tumor, which measured 1 cm in diameter, involved the submucosal tissue of the forestomach at the lesser curvature extending to the glandular stomach and esophagus. Histopathologically, the tumor was composed of neoplastic cells with small- to medium-sized spindle-shaped single nuclei and fibrillary cytoplasm lacking distinct cell borders. It invaded extensively into the tunica muscularis and subserosa, further extending to the lamina propria mucosa and serosal surface. A few densely proliferating portions showed a tendency to storiform pattern. Metastatic tumor nodules were found in the liver, spleen, and femur bone marrow, with multiple nodules, up to 1 cm in diameter, apparent in the liver. Immunohistochemically, diffuse, but weak cytoplasmic immunoreactivity for KIT was evident, and most neoplastic cells also exhibited strong immunoreactivity for α -smooth muscle actin and vimentin. Sparse nuclear S-100-immunoreactive cells were further observed, but none of neoplastic cells were immunoreactive for CD34, caldesmon, desmin, cytokeratin, or synaptophysin. Collectively, these features meet the criteria for a GIST, with limited potential for differentiation to smooth muscle and neural cells.

Keywords: gastrointestinal stromal tumor (GIST); KIT; interstitial cell of Cajal

Lee, K.-Y., Shibutani, M., Inoue, K., Kuroiwa, K., U, M., Woo, G.-H. and Hirose, M.: **Methacarn fixation--effects of tissue processing and storage conditions on detection of mRNAs and proteins in paraffin-embedded tissues.**

Anal. Biochem., **351**, 36-43 (2006)

In this study, we examined suitable conditions for tissue fixation with methacarn and ethanol dehydration and storage of paraffin-embedded tissues (PETs) on gene expression analysis. With fixation and dehydration of rat liver tissues for up to 16 h (overnight) and 1 week, respectively, at 4 degrees C, integrity of extracted total RNAs and polypeptides did not vary, the former integrity being constantly lower than that with unfixed frozen tissue, while protein yield was slightly reduced with increasing dehydration. Retained expression levels of mRNAs and proteins were mostly unaffected by the period of fixation but slightly fluctuated with the length of dehydration. When PETs were stored for up to 12 months, integrity of both total RNAs and polypeptides was retained at 4 degrees C but reduced at room temperature. Reduced expression levels of mRNAs and proteins were also noted by storage at room temperature after 12 and 3 months, respectively. However, neither tissue processing nor storage affected variability in either mRNA or protein levels among samples. Thus, the results suggest that, for gene expression analysis, tissues can be fixed with methacarn and dehydrated for at least 1 day and 1 week, respectively, and PETs can be stored for at least 12 months, but a temperature of 4 degrees C is preferable. Keywords: methacarn; molecular integrity; paraffin-embedded tissue

Ota, Y., Hasumura, M., Okamura, M.^{*1}, Takahashi, A.^{*1}, Ueda, M., Onodera, H.^{*2}, Imai, T., Mitsumori, K.^{*1} and Hirose, M.: **Chronic toxicity and carcinogenicity of dietary administered ammonium sulfate in F344 rats.**

Food Chem. Toxicol., **44**, 17-27 (2006)

Chronic toxicity and carcinogenicity studies of ammonium sulfate, used as a food additive in fermentation, were performed in male and female Fisher 344 rats at dietary concentrations of 0, 0.1, 0.6 and 3.0% in a 52-week toxicity study and 0, 1.5 and 3.0% in a 104-week carcinogenicity study. Treatment with ammonium sulfate caused significant increase in kidney and/or liver weights in males and females of the 3.0% diet group, but no effects were found on survival rate, body weights, and hematological, serum

biochemical or histopathological parameters at any dose levels in the chronic toxicity study. Regarding carcinogenicity, ammonium sulfate did not exert any significant influence on the incidences of tumors in any of the organs and tissues examined. It was concluded that the no observed adverse effect level (NOAEL) of ammonium sulfate was the 0.6% diet, which is equivalent to 256 and 284 mg/kg bw/day in males and females, respectively, and the compound is non-carcinogenic under the conditions of the study.

Keywords: ammonium sulfate; chronic toxicity; carcinogenicity

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Takizawa, T., Imai, T., Ueda, M., Onodera, H. and Hirose, M.: **Comparison of enhancing effects of different goitrogen treatments in combination with β -estradiol-3-benzoate for establishing a rat two-stage thyroid carcinogenesis model to detect modifying effects of estrogenic compounds.**

Cancer Sci., **97**, 25-31 (2006)

With the aim of establishing a sensitive model for the detection of weak effects of endocrine disrupting chemicals on thyroid carcinogenesis, thyrotrophic and tumor-promoting influences of β -estradiol-3-benzoate (EB) in combination with representative anti-thyroidal agents (goitrogens), sulfadimethoxine (SDM), propylthiouracil (PTU), potassium perchlorate (PPC), iopanoic acid (IOP) or iodine deficient diet were evaluated in a short-term (7-day) experiment without *N*-bis (2-hydroxypropyl) nitrosamine (DHPN)-initiation and a long-term (30-week) experiment with DHPN-initiation in ovariectomized F344 rats, respectively. In the short-term experiment, the most remarkable thyrotrophic effects were found in the PTU-treated group, followed by the SDM- and PPC-cases, while EB treatment alone caused slight increase in thyroidal weights but no apparent morphological changes. Concomitant treatment with EB and anti-thyroidal agents enhanced the changes of thyroid weights, histopathological findings and/or serum thyroid hormone levels in the SDM (30 and 100 ppm), PTU (5 and 30 ppm) and PPC (100 ppm), IOP (30 and 100 mg/kg) or iodine deficient diet groups. In the long-term experiment after DHPN-initiation, EB alone slightly increased small numbers of animals with follicular hyperplasias, adenomas and adenocarcinomas, while simultaneous treatment with anti-thyroidal chemicals was associated with increase in the incidences of focal hyperplasias, adenomas and/or

adenocarcinomas. The enhancement was most remarkable with PTU (5 ppm), followed by PTU (2 ppm), SDM (100ppm) and PPC (100 ppm). The results showed that EB has only a marginal promoting effect on DHPN-induced rat thyroid carcinogenesis and that anti-thyroidal chemicals, particularly PTU are effective as co-promoting agents.

Keywords: estradiol, goitrogen, thyroid gland

Onose, J., Imai, T., Hasumura, M., Cho, Y.M. and Hirose, M.: **A new medium-term rat colon bioassay applying neoplastic lesions as endpoints for detection of carcinogenesis modifiers – validation with known modifiers.**

Cancer Lett., **232**, 272-278 (2006)

We have established a medium-term colorectal carcinogenesis rat model initiated with 1,2-dimethylhydrazine (DMH) followed by dextran sodium sulfate (DSS) treatment, featuring induction of neoplastic lesions within 10 weeks. In the present study, we examined its ability to detect modification of colon lesion development with 10 or 20 week experimental periods. F344 male rats were given three subcutaneous injections of DMH (40 mg/kg b.w.) in a week followed by free access to drinking water containing 1% DSS for a week. One week after this regimen, basal diet alone, basal diet containing 0.04% nimesulide or 2% lactoferrin as known inhibitors, 0.3% deoxycholic acid (DCA) as a promoter or 1.5% 1-hydroxyanthraquinone (1-HA) as a carcinogen were supplied. At week 10, the incidence and multiplicity of combined adenomas and adenocarcinomas were significantly ($p < 0.05$ or 0.01) decreased by nimesulide and lactoferrin, and values for adenomas were significantly ($p < 0.01$) increased in the 1-HA group. There was no clear change in the DCA group. At week 20, multiplicity and volume of the tumors were significantly ($p < 0.01$ or 0.05) decreased by nimesulide, but no effect was now evident with lactoferrin. Multiplicity and volume of tumors were significantly ($p < 0.01$) increased in 1-HA group and a similar tendency was apparent ($p = 0.08$) with DCA. It is concluded that this system offers a useful tool for detection of colorectal carcinogenesis modifiers within 10-20 weeks, pending further studies for verification employing other model chemicals.

Keywords: colon, bioassay, dextran sodium sulfate

Imai, T., Onose, J., Hasumura, M., Takizawa, T. and Hirose, M.: **Indomethacin induces small intestinal damage and inhibits amitrole-associated thyroid**

carcinogenesis in rats initiated with *N*-bis (2-hydroxypropyl) nitrosamine.

Toxicol. Lett., **164**, 71-80 (2006)

Effects of intestinal damage on thyroid carcinogenesis due to amitrole (AT) were examined in F344 male rats initiated with *N*-bis (2-hydroxypropyl) nitrosamine (DHPN). In experiment 1, rats were provided with diet containing 0.03% AT for 20 weeks after a single subcutaneous injection of DHPN (2800 mg/kg body weight), and concomitantly received 0.01% indomethacin (IM) in the diet to cause small intestinal damage or 1% dextran sodium sulfate (DSS) in the drinking water for induction of colitis following a schedule of intermittent one-week administration and one-week withdrawal for a total of 10 times. Groups without AT- and/or IM or DSS-treatment were also included. Histopathological examination revealed significant reduction in the incidence and multiplicity of follicular cell adenomas and adenocarcinomas in the group concomitantly treated with IM, but no change in the DSS group, as compared with the AT alone group. In experiment 2, rats were similarly fed diet containing AT for 3 weeks with concomitant IM or DSS treatment after a DHPN-initiation, and serum thyroid stimulating hormone levels were found to be significantly elevated only in the IM case. The increase in thyroid follicular cell proliferation due to AT was also clearly suppressed in the group concomitantly treated with IM. From these findings, IM-induced intestinal damage may inhibit thyroid carcinogenesis in rats, although contributions of other factors, such as a direct inhibitory effect of IM to thyroid follicular cell proliferation cannot be ruled out.

Keywords: thyroid carcinogenesis; 3-aminotriazole; indomethacin

Cho, Y. M., Onodera, H., Ueda, M., Imai, T. and Hirose, M.: **A 13-week subchronic toxicity study of dietary administered morin in F344 rats.**

Food Chem. Toxicol., **44**, 891-897 (2006)

A subchronic toxicity study of a flavonoid morin was performed in both sexes of F344 rats with dietary administration at concentrations of 0%, 0.625%, 1.25%, 2.5% and 5% (w/w) for 13 weeks. No mortality or abnormal clinical signs were observed throughout the experimental period in any group. Although a slight tendency for increase in food intake was noted in both sexes of the 2.5% and 5.0% groups, slight non-significant body weight decrease was observed in 5.0% males. Significant increases in alanine transaminase (ALT; over 2.5%), alkaline phosphatase

(ALP; 1.25% and 5.0%) and relative liver weights (1.25% and 2.5%) in males and in gamma-glutamyl transpeptidase (gamma-GT), aspartate transaminase (AST), ALT, relative liver weights in the 2.5% and 5.0% females and ALP in 5.0% females were noted. Increased urea nitrogen and relative kidney weights at dose of 1.25% and above and creatinine at 5.0% were observed also in females. On histopathological observation, hepatocyte hypertrophy was detected in 3 of 10 5.0% females. Based on the above findings, the no-observed-adverse-effect level (NOAEL) for both sexes was estimated to be 0.625% (299 and 356 mg/kg b.w./day for males and females, respectively).

Keywords: morin, F344 rats, subchronic toxicity

Cho, Y. M., Imai, T., Hasumura, M. and Hirose, M. : **Lack of enhancement of susceptibility to mammary and thyroid carcinogenesis in rats exposed to DMBA and DHPN following prepubertal iodine deficiency.**

Cancer Sci., **97**, 1031-1036 (2006)

Epidemiologic and experimental studies suggest that iodine deficiency increases the risk of mammary as well as thyroid cancers, but susceptibility to tumor development when this occurs during the prepubertal stage is not completely understood. In the present study, we therefore evaluated this question in F344 rats. Dams during the lactation period and their weaned offspring until postnatal week 7 were fed an iodine-free diet. Female offspring were then given 7,12-dimethylbenz[*a*]anthracene (DMBA, 50 mg/kg body weight) by gavage for mammary tumor induction in week 7. Both the male and female rats were given free access to drinking water containing *N*-bis (2-hydroxypropyl) nitrosamine (DHPN), (0.1 and 0.2% for male and female rats, respectively) for wide spectrum tumor induction in organs, including the thyroid gland, from weeks 7-11. All offspring were killed at week 50 for histopathological examination. The iodine deficiency had no significant influence on incidences and/or multiplicities of mammary and thyroid tumors. Furthermore, tumor induction in the liver, kidney, lung, esophagus and urinary bladder was not affected in either sex. The present results thus indicate a lack of influence of iodine deficiency condition early in life on subsequent carcinogenic susceptibility.

Keywords: mammary gland, thyroid gland, iodine deficiency

Torous, D. ^{*1}, Asano, N. ^{*2}, Tometsko, C. ^{*1}, Sugunan, S., Dertinger, S. ^{*1}, Morita, T. and Hayashi, M. : **Per-**

formance of flow cytometric analysis for the micronucleus assay— a reconstruction model using serial dilutions of malaria infected cells with normal mouse peripheral blood.

Mutagenesis, **21**, 11-13 (2006)

Micronucleus induction was studied for the DNA target clastogens mitomycin C (MMC) and 1-β-D-arabinofuranosylcytosine (Ara-C), and also the non-DNA target aneugen colchicine (COL) in order to evaluate the dose-response relationship at very low dose levels. The acridine orange (AO) supravital staining method was used for microscopy and the anti-CD71-FITC based method was used for flow cytometric analysis. In the AO method, 2000 reticulocytes were analysed as commonly advised, but in the flow cytometric method, 2000, 20 000, 200 000 and 1 000 000 reticulocytes were analysed for each sample to increase the detecting power (i.e. sensitivity) of the assay. The present data show that increasing the number of cells scored increases the statistical power of the assay when the cell was considered as a statistical unit. Even so, statistically significant differences from respective vehicle controls were not observed at the lowest dose level for MMC and Ara-C, or the lower four dose levels for COL, even after one million cells were analysed. When the animal was considered as a statistical unit, only the top dose group for each chemical showed significant increase of micronucleated reticulocytes frequency. As non-linear dose-response curves were obtained for each of the three chemicals studied, these observations provide evidence for the existence of a practical threshold for the DNA target clastogens as well as the non-DNA target aneugen studied.

Keywords: micronuclei, flow cytometric analysis, threshold

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Asano, N. ^{*1}, Torous, D. ^{*2}, Tometsko, C. ^{*2}, Dertinger, S. ^{*2}, Morita, T. and Hayashi, M. : **Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C and colchicine.**

Mutagenesis, **21**, 15-20 (2006)

Micronucleus induction was studied for the DNA target clastogens mitomycin C (MMC) and 1-β-D-arabinofuranosylcytosine (Ara-C), and also the non-DNA target aneugen colchicine (COL) in order to evaluate the dose-response relationship at very low dose levels. The acridine orange (AO) supravital staining method was used for microscopy

and the anti-CD71-FITC based method was used for flow cytometric analysis. In the AO method, 2000 reticulocytes were analysed as commonly advised, but in the flow cytometric method, 2000, 20 000, 200 000 and 1 000 000 reticulocytes were analysed for each sample to increase the detecting power (i.e. sensitivity) of the assay. The present data show that increasing the number of cells scored increases the statistical power of the assay when the cell was considered as a statistical unit. Even so, statistically significant differences from respective vehicle controls were not observed at the lowest dose level for MMC and Ara-C, or the lower four dose levels for COL, even after one million cells were analysed. When the animal was considered as a statistical unit, only the top dose group for each chemical showed significant increase of micronucleated reticulocytes frequency. As non-linear dose-response curves were obtained for each of the three chemicals studied, these observations provide evidence for the existence of a practical threshold for the DNA target clastogens as well as the non-DNA target aneugen studied.

Keywords; micronuclei, flow cytometric analysis, threshold

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Kirkland, D. ^{*1}, Aardema, M. ^{*2}, Mueller, L^{*3}. and Hayashi M. : **Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens-II. Further analysis of mammalian cell results, repetitive predictivity and tumour profiles.**

Mutat. Res., **608**, 29-42 (2006)

One of the consequences of the low specificity of the in vitro mammalian cell genotoxicity assays reported in our previous paper is industry and regulatory agencies dealing with a large number of false-positive results during the safety assessment of new chemicals and drugs. Addressing positive results from in vitro genotoxicity assays to determine which are "false" requires extensive resources, including the conduct of additional animal studies. In order to reduce animal usage, and to conserve industry and regulatory agency resources, we thought it was important to raise the question as to whether the protocol requirements for a valid in vitro assay or the criteria for a positive result could be changed in order to increase specificity without a significant loss in sensitivity of these tests. We therefore analysed some results of the mouse lymphoma assay (MLA) and the chromosomal aberration (CA) test obtained for rodent car-

cinogens and non-carcinogens in more detail. For a number of chemicals that are positive only in either of these mammalian cell tests there was no correlation between rodent carcinogenicity and level of toxicity, magnitude of response or lowest effective positive concentration. On the basis of very limited in vitro and in vivo data, we could also find no correlation between the above parameters and formation of DNA adducts. Therefore, a change to the current criteria for required level of toxicity in the MLA, to limit positive calls to certain magnitudes of response, or to certain concentration ranges would not improve the specificity of the tests without significantly reducing the sensitivity.

Keywords; carcinogens, mouse lymphoma assay, chromosomal aberration

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MaCgregor, J.T. ^{*1}, Bishop, M.E. ^{*2}, McNamee, J.P. ^{*3}, Hayashi, M., Asano, N. ^{*4}, Wakata, A. ^{*5}, Nakajima, M. ^{*6}, Saito, J. ^{*7}, Aidoo, A. ^{*2}, Moore, M.M. ^{*2}, Dertinger, S.D. ^{*8} : **Flow Cytometric Analysis of Micronuclei in Peripheral Blood Reticulocytes: II. An Efficient Method of Monitoring Chromosomal Damage in the Rat.**

Toxicol Sci., **94**, 92-107 (2006)

We have evaluated a flow cytometric method that allows assessment of micronucleated reticulocytes (MN-RETs) in microliter quantities of peripheral blood and compared results using this assay with those of established microscopic methods of scoring bone marrow and peripheral blood from rats treated with well-characterized genotoxic agents. Young reticulocytes (RETs) are labeled with FITC-anti-CD71 (transferrin receptor) and micronuclei with propidium iodide (with RNase treatment). Red blood cells parasitized with Plasmodia serve as a calibration standard for DNA content. Microscopic scoring used acridine orange (AO) staining of methanol-fixed slides or supravital AO staining. The effect of the rat spleen on the parameters evaluated was determined by comparing age- and sex-matched normal and splenectomized rats treated with cyclophosphamide, cis-platin, or vinblastine under treatment conditions that established a steady-state frequency of MN-RETs in the bone marrow and peripheral blood compartments. The data demonstrate the sensitivity and reproducibility of the flow cytometric assay in the Sprague-Dawley rat, and comparative studies using identical blinded samples at multiple labora-

tories show that inter- and intra-laboratory reproducibility is much higher with the flow method than with the microscopic methods currently employed for regulatory studies. A significant effect of splenic selection against genotoxicant-induced MN-RETs was observed with each of the three scoring methodologies, despite the fact that the flow and supravital AO techniques restrict analysis to the youngest fraction of RETs. The high precision of flow-based measurements also demonstrated a slight but statistically significant level of selection against spontaneously arising MN-RET. Despite these spleen effects, assay sensitivity for blood-based analyses was maintained by the flow method as it was shown to have superior counting statistics, lower variability, and higher sensitivity than manual scoring. The data suggest that flow cytometric assessment of micronucleus induction can be integrated into routine toxicity testing, eliminating the need for a separate bioassay.

Keywords; micronuclei, flow cytometric analysis, chromosomal damage

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Dertinger, S.D. ^{*1}, Bishop, M.E. ^{*2}, McNamee, J.P. ^{*3}, Hayashi, M., Suzuki, T., Asano, N. ^{*4}, Nakajima, M. ^{*5}, Saito, J. ^{*6}, Moore, M. ^{*2}, Torous, D.K. ^{*1}, Macgregor, J.T. ^{*7} : **Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: I. Intra- and inter-laboratory comparison with microscopic scoring.** *Toxicol Sci.*, **94**, 83-91 (2006)

Accumulating evidence suggests that reticulocytes (RETs) in the peripheral blood of rats may represent a suitable cell population for use in the micronucleus assay, despite the ability of the rat spleen to selectively remove micronucleated erythrocytes from the peripheral circulation. To evaluate the analytical performance of a previously described flow cytometric method (Torous et al., 2003, *Toxicol. Sci.* **74**, 309-314) that may allow this assay to be conducted using peripheral blood in lieu of bone marrow sampling, we compared the sensitivity and performance characteristics of the

flow cytometric technique with two established microscopy-based scoring methods. Peripheral blood samples from single Sprague-Dawley rats treated for 6 days with either vehicle or cyclophosphamide were prepared in replicate for scoring by the three methods at different laboratories. These blood-based measurements were compared to those derived from bone marrow specimens from the same animals, stained with acridine orange, and scored by microscopy. Through the analysis of replicate specimens, inter- and intralaboratory variability were evaluated for each method. Scoring reproducibility over time was also evaluated. These data support the premise that rat RETs harvested from peripheral blood are a suitable cell population to assess genotoxicant-induced micronucleus formation. The interlaboratory comparison provides evidence of the general robustness of the micronucleus endpoint using different analytical approaches. Furthermore, data presented herein demonstrate a clear advantage of flow cytometry-based scoring over microscopy-significantly lower inter- and intralaboratory variation and higher statistical sensitivity.

Keywords; micronuclei, flow cytometric analysis, chromosomal damage

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Oka, H.^{*}, Yoshimura, H.^{*}, Ohuchida, A.^{*} and Honma, M.: **Relationship between p53 status and 5-fluorouracil sensitivity in 3 cell lines.**

Mutat. Res., **606**, 52-60 (2006)

Mouse lymphoma L5178Ytk+/- (MOLY) cells and human lymphoblastoid TK6, and WTK-1 cells are widely used to detect mutagens in vitro. MOLY and WTK-1 cells have a p53 mutation while TK6 cells do not, although TK6 cells were derived from the same parental line as WTK-1 cells. The p53 gene is associated with DNA repair and induction of apoptosis. In this study, we tested the clastogen 5-fluorouracil (5-FU), in the tk assay and the in vitro micronucleus (MN) in MOLY, TK6, and WTK-1 cells to clarify whether differential responses were related to p53 gene status. We also determined the effect of 5-FU on the ratio of apoptotic cells and on cell cycle distribution in each cell line. MOLY cells were more sensitive than

WTK-1 cells to 5-FU even though both have a mutated p53 gene, suggesting that the responses were attributable to other, perhaps levels of activated genes or enzymes.

Keywords: 5-fluorouracil, p53, Tk assay, in vitro micronucleus assay

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Umabayashi, Y. ^{*1}, Honma, M., Abe, T. ^{*1}, Ryuto, H. ^{*1}, Suzuki, H. ^{*2}, Shimazu, T. ^{*2}, Ishioka, N. ^{*3}, Iwaki, M. ^{*1}, Yatagai, F. ^{*1}: **Mutation induction after low-dose carbon-ion beam irradiation of frozen human cultured cells.**

Biological Sci. in Space, **19**, 237-241 (2006)

To study the genetic effects of low-dose and low-dose-rate ionizing radiation at the chromosomal level, frozen human lymphoblastoid TK6 cells were exposed to 10cGy dose delivered by a carbon-ion (24.5kv/um) beam. The relative increase in TK mutation frequency of the irradiated cells after the longer preservation at -80C is probably due to the lower cell-viability compared to the un-irradiated level. These results clearly demonstrated that this types of analysis can be used for the detection of low-dose ionizing radiation effects in frozen cells.

Keywords: loss of heterozygosity (LOH) , Thymidine kinase (TK) , frozen cells, carbon-ion beam

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Yamada, M., Nunoshiba, T. ^{*1}, Shimizu, M. ^{*2}, Gruz, P., Kamiya, H. ^{*3}, Harashima, H. ^{*3} and Nohmi, T.: **Involvement of Y-family DNA polymerases in mutagenesis caused by oxidized nucleotides in *Escherichia coli*.**

J. Bacteriol., **188**, 4992-4995 (2006)

Escherichia coli DNA polymerase IV incorporated 2-hydroxy-dATP opposite template guanine or thymine and 8-hydroxy-dGTP exclusively opposite adenine in vitro. Mutator phenotypes in *sod/fur* strains were substantially diminished by deletion of *dinB* and/or *umuDC*. DNA polymerases IV and V may be involved in mutagenesis caused by incorporation of the oxidized deoxynucleoside triphosphates.

Keywords: Y-family DNA polymerases, oxidized nucleotides, misincorporation

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Ikeda, M. ^{*1}, Masumura, K., Matsui, K., Kohno, H. ^{*2}, Sakuma, K. ^{*1}, Tanaka, T. ^{*2} and Nohmi, T.: **Chemopreventive effects of nobiletin against genotoxicity induced by 4- (methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK) in the lung of *gpt delta* transgenic mice.**

Genes and Environ., **28**, 84-91 (2006)

Nobiletin, a major component of citrus polymethoxyflavones, possesses anticancer, antiviral and anti-inflammatory activities. To evaluate the chemopreventive potential against lung cancer induced by cigarette smoke, we examined suppressive effects of nobiletin against genotoxicity induced by 4- (methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK) , the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt delta* transgenic mice. Nobiletin reduced the NNK-induced mutation frequencies by 25-45% in both sexes and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant (P<0.05) . Nobiletin as well as 8-methoxypsoralen, an inhibitor of CYP2A, reduced the genotoxicity of NNK by more than 50% in bacterial mutation assay. The results suggest that nobiletin may be chemopreventive against NNK-induced lung cancer and also that the chemopreventive efficacy may be due to inhibition of certain CYP enzymes involved in the metabolic activation of NNK.

Keywords: nobiletin, NNK, chemoprevention

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Jiang, L. ^{*}, Zhong, Y. ^{*}, Akatsuka, S. ^{*}, Liu, Y. ^{*}, Dutta, K.K. ^{*}, Lee, W. ^{*}, Onuki, J. ^{*}, Masumura, K., Nohmi, T. and Toyokuni, S. ^{*}: **Deletion and single nucleotide substitution at G:C in the kidney of *gpt delta* transgenic mice after ferric nitrilotriacetate treatment.**

Cancer Sci., **97**, 1159-11167 (2006)

An iron chelate, ferric nitrilotriacetate (Fe-NTA) , induces oxidative renal proximal tubular damage that subsequently leads to a high incidence of renal cell carcinoma in rodents, presenting an intriguing model of free radical-induced carcinogenesis. In the present study, we used *gpt delta* transgenic mice, which allow efficient detection of point mutations and deletions in vivo, to evaluate the mutation spectra, in association with the formation of 8-oxoguanine and acrolein-modified adenine during the first 3 weeks of carcinogenesis. The results demonstrate that the

iron-based Fenton reaction is mutagenic in vivo in the renal tubular cells and induces characteristic mutations.

Keywords: *gpt*-delta mice, ferric nitrilotriacetate, acrolein

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Takeiri, A.^{*}, Mishima, M.^{*}, Tanaka, K.^{*}, Shioda, A.^{*}, Harada, A.^{*}, Watanabe, K.^{*}, Masumura, K. and Nohmi, T.: **A newly established GDL1 cell line from *gpt* delta mice well reflects the in vivo mutation spectra induced by mitomycin C.**

Mutat. Res., **609**, 102-115 (2006)

In order to create a novel in vitro test system for detection of large deletions and point mutations, we developed an immortalized cell line. A SV40 large T antigen expression unit was introduced into fibroblasts derived from *gpt* delta mouse lung tissue and a selected clone was established as the *gpt* delta L1 (GDL1) cell line. The novel GDL1 cells were examined for mutant frequencies and for molecular characterization of mutations induced by mitomycin C (MMC). We compared the spectrum of MMC-induced mutations observed in vitro to that of in vivo using *gpt* delta mice, which we reported previously. Although a slight difference was observed in MMC-induced mutation spectra between in vitro and in vivo, the mutations detected in vitro included all of the types of mutations observed in vivo. The present study demonstrates that the newly established GDL1 cell line is a useful tool to detect and analyze various mutations including large deletions in mammalian cells.

Keywords: GDL-1 cell line, mitomycin C, mutation spectra

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Kirkland, D.J.^{*1}, Hayashi, M., Jacobson-Kram, D.^{*2}, Kasper, P.^{*3}, MacGregor, J.T.^{*4}, Müller, L.^{*5} and Uno, Y.^{*6}: **Summary of major conclusions from the 4th IWGT, San Francisco, 9–10 September, 2005.**

Mutat. Res., **627**, 5-9 (2007)

Seven individual working groups addressed either specific aspects of the strategy for genetic toxicology testing or the design of protocols for specific assays. Comprehensive summaries of the outcome of each Working Group (WG) are given in the individual WG reports. The following outline summarises the main points that either differ from existing published recommendations (as in the case of mouse lymphoma test and in vivo micronucleus assay) or are key features to be considered in the development of new guidelines for an improved testing strategy.

Keywords: genotoxicity testing, IWGT workshops, conclusions

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Hayashi, M., MacGregor, J.T.^{*1}, Gatehouse, D.G.^{*2}, Blakey, D.H.^{*3}, Dertinger, S.D.^{*4}, Abramsson-Zetterberg, L.^{*5}, Krishna, G.^{*6}, Morita, T., Russo, A.^{*7}, Asano, N.^{*8}, Suzuki, H.^{*9}, Ohshima, W.^{*10} and Gibson, D.^{*11}: **In vivo erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test.**

Mutat. Res., **627**, 10-30 (2007)

The in vivo micronucleus assay working group of the International Workshop on Genotoxicity Testing (IWGT) discussed new aspects in the in vivo micronucleus (MN) test, including the regulatory acceptance of data derived from automated scoring, especially with regard to the use of flow cytometry, the suitability of rat peripheral blood reticulocytes to serve as the principal cell population for analysis, the establishment of in vivo MN assays in tissues other than bone marrow and blood (for example liver, skin, colon, germ cells), and the biological relevance of the single-dose-level test. Our group members agreed that flow cytometric systems to detect induction of micronucleated immature erythrocytes have advantages based on the presented data, e.g., they give good reproducibility compared to manual scoring, are rapid, and require only small quantities of peripheral blood. The consensus of the group was that any system that meets the validation criteria recommended by the IWGT (2000) should be acceptable. Whichever method is chosen, it is desirable that each laboratory should determine the minimum sample size required to ensure that scoring error is maintained below the level of animal-to-animal variation. In the second IWGT, the potential to use rat peripheral blood reticulocytes as target cells for the micronucleus assay was discussed, but a consensus regarding acceptability for regulatory purposes could not be reached at that time. The working group reviewed

the results of micronucleus assays using target cells/tissues other than hematopoietic cells. We also discussed the relevance of the liver micronucleus assay using young rats, and the importance of understanding the maturation of enzyme systems involved in the processes of metabolic activation in the liver of young rats. Additional data obtained from colon and skin MN models have been integrated into the data bases, enhancing confidence in the utility of these models. A fourth topic discussed by the working group was the regulatory acceptance of the single-dose-level assay. There was no consensus regarding the acceptability of a single dose level protocol when dose-limiting toxicity occurs. A limit test at a single dose level is currently accepted when toxicity is not dose-limiting.

Keywords; micronucleus assay, automation, flow cytometry

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Thybaud, V. ^{*1}, Aardema, M. ^{*2}, Clements, J. ^{*3}, Dearfield, K. ^{*4}, Galloway, S. ^{*5}, Hayashi, M., Jacobson-Kram, D. ^{*6}, Kirkland, D. ^{*3}, Macgregor, J.T. ^{*7}, Marzin, D. ^{*8}, Ohyama, W. ^{*9}, Schuler, M. ^{*10}, Suzuki, H. ^{*11} and Zeiger, E. ^{*12} : **Strategy for genotoxicity testing: Hazard identification and risk assessment in relation to in vitro testing.**

Mutat. Res., **627**, 41-58 (2007)

This report summarizes the proceedings of the September 9-10, 2005 meeting of the Expert Working Group on Hazard Identification and Risk Assessment in Relation to In Vitro Testing, part of an initiative on genetic toxicology. The objective of the Working Group was to develop recommendations for interpretation of results from tests commonly included in regulatory genetic toxicology test batteries, and to propose an appropriate strategy for follow-up testing when positive in vitro results were obtained in these assays. The Group noted the high frequency of positive in vitro findings in the genotoxicity test batteries with agents found not to be carcinogenic and thought not to pose a carcino-

genic health hazard to humans. The Group agreed that a set of consensus principles for appropriate interpretation and follow-up testing when initial in vitro tests are positive was needed. Current differences in emphasis and policy among different regulatory agencies were recognized as a basis of this need. Using a consensus process among a balanced group of recognized international authorities from industry, government, and academia, it was agreed that a strategy based on these principles should include guidance on: (1) interpretation of initial results in the "core" test battery; (2) criteria for determining when follow-up testing is needed; (3) criteria for selecting appropriate follow-up tests; (4) definition of when the evidence is sufficient to define the mode of action and the relevance to human exposure; and (5) definition of approaches to evaluate the degree of health risk under conditions of exposure of the species of concern (generally the human) .

Keywords; genotoxicity, Hazard identification, risk assessment

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Kirkland, D. ^{*1}, Pfueller, S. ^{*2}, Tweats, D. ^{*3}, Aardema, M. ^{*4}, Corvi, R. ^{*5}, Darroudi, F. ^{*6}, Elhajouji, A. ^{*7}, Glatt, H.-R. ^{*8}, Hastwell, P. ^{*9}, Hayashi, M., Kasper, P. ^{*10}, Kirchner, S. ^{*11}, Lynch, A. ^{*9}, Marzin, D. ^{*12}, Maurici, D. ^{*5}, Meunier, J.-R. ^{*13}, Müller, L. ^{*11}, Nohynek, G. ^{*14}, Parry, J. ^{*3}, Parry, E. ^{*3}, Thybaud, V. ^{*15}, Tice, R. ^{*16}, Benthem, J.V. ^{*17}, Vanparys, P. ^{*18} and White, P. ^{*19} :

How to reduce false positive results with in vitro genotoxicity testing and avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop.

Mutat. Res., **628**, 31-55 (2007)

Workshop participants agreed that genotoxicity tests in

mammalian cells in vitro produce a remarkably high and unacceptable occurrence of irrelevant positive results (e.g. when compared with rodent carcinogenicity). As reported in several recent reviews, the rate of irrelevant positives (i.e. low specificity) for some studies using in vitro methods (when compared to this “gold standard”) means that an increased number of test articles are subjected to additional in vivo genotoxicity testing, in many cases before, e.g. the efficacy (in the case of pharmaceuticals) of the compound has been evaluated. If in vitro tests were more predictive for in vivo genotoxicity and carcinogenicity (i.e. fewer false positives) then there would be a significant reduction in the number of animals used. Beyond animal (or human) carcinogenicity as the “gold standard”, it is acknowledged that genotoxicity tests provide much information about cellular behaviour, cell division processes and cellular fate to a (geno) toxic insult. Since the disease impact of these effects is seldom known, and a verification of relevant toxicity is normally also the subject of (sub) chronic animal studies, the prediction of in vivo relevant results from in vitro genotoxicity tests is also important for aspects that may not have a direct impact on carcinogenesis as the ultimate endpoint of concern. In order to address the high rate of in vitro false positive results, a 2-day workshop was held at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy in April 2006. More than 20 genotoxicity experts from academia, government and industry were invited to review data from the currently available cell systems, to discuss whether there exist cells and test systems that have a reduced tendency to false positive results, to review potential modifications to existing protocols and cell systems that might result in improved specificity, and to review the performance of some new test systems that show promise of improved specificity without sacrificing sensitivity. It was concluded that better guidance on the likely mechanisms resulting in positive results that are not biologically relevant for human health, and how to obtain evidence for those mechanisms, is needed both for practitioners and regulatory reviewers.

Keywords; genotoxicity in vitro, false positive, animal tests

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*10 BfArM, Genetic Toxicology

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*12 Institut Pasteur de Lille

*13 L' Oréal

*14 L' Oréal, Worldwide Safety Department

*15 Sanofi-Aventis

*16 NICEATM, NIEHS

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Burlinson, B. ^{*1}, Tice, RR. ^{*2}, Speit, G. ^{*3}, Agurell, E. ^{*4}, Brendler-Schwaab, SY. ^{*5}, Collins, AR. ^{*6}, Escobar, P. ^{*7}, Honma, M., Kumaravel, TS. ^{*8}, Nakajima, M. ^{*9}, Sasaki, YF. ^{*10}, Thybaud, V. ^{*11}, Uno, Y. ^{*12}, Vasquez, M. ^{*13}, and Hartmann, A. ^{*14} : **Fourth International Workgroup on Genotoxicity testing: Results of the in vivo Comet assay workgroup.**

Mutat. Res., **627**, 31-35 (2007)

As part of the Fourth International Workshop on Genotoxicity Testing (IWGT), held 9-10 September 2005 in San Francisco, California, an expert working group on the Comet assay was convened to review and discuss some of the procedures and methods recommended in previous documents. We agreed that a minimum reporting standard would be developed which would be consistent with OECD in vivo genotoxicity test method guidelines.

Keywords: single cell gel assay, Comet assay, DNA damage, genotoxicity

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Moore, MM. ^{*1}, Honma, M., Clements, J. ^{*2}, Bolesfoldi, J. ^{*3}, Burlinson, B. ^{*4}, Cifone, M. ^{*5}, Clark, J. ^{*6}, Clay, P. ^{*7}, Doppalapudi, R. ^{*8}, Fellows, M. ^{*9}, Gollapudi, B. ^{*10}, Hou, S. ^{*2}, Jenkinson, J. ^{*11}, Muster, W. ^{*12}, Pant, K. ^{*6}, Kidd, DA. ^{*2}, Lorge, E. ^{*13}, Lloyd, M. ^{*2}, Myhr, B. ^{*14}, O' Donovan, M. ^{*9}, Riach, C. ^{*15}, Stankowski, Jr. LF. ^{*16}, Thakur, AK. ^{*5}, and Van Goethem, F. ^{*17}: **Mouse lymphoma thymidine kinase gene mutation assay: Meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment.**

Mutat. Res., **627**, 36-40 (2007)

The Mouse Lymphoma Assay (MLA) Workgroup of the International Workshop on Genotoxicity Testing (IWGT), comprised of experts from Japan, Europe and the United States, met on September 9, 2005, in San Francisco, CA, USA. This meeting of the MLA Workgroup was devoted to reaching a consensus on issues involved with 24-h treatment.

Keywords: mouse lymphoma assay, in vitro mutation, thymidine kinase

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Ku, WW. ^{*1}, Bigger, A. ^{*2}, Brambilla, G. ^{*3}, Glatt, H. ^{*4}, Gocke, E. ^{*5}, Guzzie, PJ. ^{*1}, Hakura, A. ^{*6}, Honma, M., Martus, H-J. ^{*7}, Obach, RS. ^{*1}, and Roberts, R. ^{*8}: **Strategy for genotoxicity testing—Metabolic considerations.**

Mutat. Res., **627**, 59-77 (2007)

The report from the 2002 International Workshop on Genotoxicity Tests (IWGT) Strategy Expert Group emphasized metabolic considerations as an important area to address in developing a common strategy for genotoxicity testing. A working group convened at the 2005 4th IWGT to discuss this area further and propose practical strategy recommendations.

Keywords: genotoxicity, metabolism, testing, strategy

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Wang, J. ^{*}, Chen, T. ^{*}, Honma, M., Chen, L. ^{*}, Moore, M. ^{*}: **3'-Azido-3'-deoxythymidine induces deletions in L5178Y mouse lymphoma cells.**

Environ. Mol. Mutagen., **48**, 248-257 (2007)

3'-Azido-3'-deoxythymidine (AZT), a nucleoside analogue used for the treatment of acquired immunodeficiency syndrome (AIDS), induced a significant dose-related increase in the thymidine kinase (Tk) mutant frequency (MF) in L5178Y/Tk (+/-) 3.7.2C mouse lymphoma cells. Treatment with 1 mg/ml (3,742 μM) AZT for 24 hr resulted in a MF of 407 x 10⁻⁶ compared to a control MF of 84 x 10⁻⁶. The mutation spectrum of mutants from AZT-treated cells was also significantly different from that of spontaneous mutants. More deletions and fewer intragenic mutations were observed in the mutants from the AZT-treated culture than independent mutants from the untreated control. Our data indicate that AZT primarily induced LOH mutations in L5178Y mouse lymphoma cells and a large number of LOH mutations resulted from deletions.

Keywords: 3-azido-3-deoxythymidine, mouse lymphoma assay, loss of heterozygosity, mutation spectrum

* NCTR, FDA, USA

Honma, M., Sakuraba, M., Koizumi, T., Takashima, Y., Sakamoto, H., and Hayashi, M.: **Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells.**

DNA Repair **6**, 781-788 (2007)

DNA double strand breaks (DSBs) are usually repaired

through either non-homologous end-joining (NHEJ) or homologous recombination (HR). To clarify the role of NHEJ, we investigated the genetic consequences of NHEJ repair of DSBs in human cells. Human lymphoblastoid cell lines TSCE5 and TSCE105 have, respectively, single and double I-SceI endonuclease sites in the endogenous thymidine kinase gene (TK) located on chromosome 17q. I-SceI expression generated DSBs at the TK gene. We found mutations involved in the DSBs in the TK gene in 3% of TSCE5 cells and 30% of TSCE105 cell clones. Most of the mutations in TSCE5 were small (1-30bp) deletions with a 0-4bp microhomology at the junction. Most of the mutations in TSCE105, on the other hand, were deletions that encompassed the two I-SceI sites generated by NHEJ at DSBs. Interestingly, some mutants formed a new I-SceI site by perfectly joining the two original I-SceI sites without deletion of the broken-ends. Thus, NHEJ must help maintain genomic integrity in mammalian cells by repairing DSBs as well as by preventing many deleterious alterations. Keywords: DNA double strand break (DSB), Non-homologous end-joining (NHEJ), Homologous recombination (HR), I-SceI, Deletion, Genomic integrity

Luan, Y.^{*1}, Suzuki, T., Palanisamy, R.^{*2}, Takashima, Y., Sakamoto, H., Sakuraba, M., Koizumi, T., Saito, M., Matsufuji, H.^{*3}, Yamagata, K.^{*3}, Yamaguchi, T., Hayashi, M., Honma, M.: **Potassium bromate treatment predominantly causes large deletions, but not GC>TA transversion in human cells.**

Mutat. Res., **619**, 113-123 (2007)

Potassium bromate (KBrO₃) is strongly carcinogenic in rodents and mutagenic in bacteria and mammalian cells in vitro. In this study, we investigated the in vitro genotoxicity of KBrO₃ in human lymphoblastoid TK6 cells using the comet (COM) assay, the micronucleus (MN) test, and the thymidine kinase (TK) gene mutation assay. After a 4 h treatment, the alkaline and neutral COM assay demonstrated that KBrO₃ directly yielded DNA damages including DNA double strand breaks (DSBs). KBrO₃ also induced MN and TK mutations concentration-dependently. Molecular analysis revealed that 90% of the induced mutations were large deletions that involved loss of heterozygosity (LOH) at the TK locus. These results indicate that the major genotoxicity of KBrO₃ may be due to DSBs that lead to large deletions rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed.

Keywords: Potassium bromate (KBrO₃), TK-mutation,

Loss of heterozygosity (LOH), 8-Hydroxydeoxyguanosine (8OHdG)

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Ikedo, M.^{*1}, Masumura, K., Sakamoto, Y., Wang, B.^{*2}, Neno, M.^{*2}, Sakuma, K.^{*1}, Hayata, I.^{*2} and Nohmi, T.: **Combined genotoxic effects of radiation and a tobacco-specific nitrosamine in the lung of *gpt* delta transgenic mice.**

Mutat. Res., **626**, 15-25 (2007)

It is important to evaluate the health effects of low-dose-rate or low-dose radiation in combination with chemicals as humans are exposed to a variety of chemical agents. Here, we examined combined genotoxic effects of low-dose-rate radiation and 4- (methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK), the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt* delta transgenic mice. Possible mechanisms underlying the combined genotoxicity of radiation and NNK are discussed, and the importance of evaluation of combined genotoxicity of more than one agent is emphasized.

Keywords: *gpt*-delta mice, NNK, low-dose-rate radiation

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Xu, A.^{*1,2}, Smilenov, L.B.^{*1}, He, P.^{*1}, Masumura, K., Nohmi, T., Yu, Z.^{*2} and Hei, T.K.^{*1}: **New insight into intrachromosomal deletions induced by chrysotile in *gpt* delta transgenic.**

Environ. Health Perspective, **115**, 87-92 (2007)

Considerable evidence has shown that exposure to asbestos fibers results in the generation of chromosomal aberrations and multilocus mutations using various in vitro approaches. In the present study, we investigated the mutant fractions and the patterns induced by chrysotile fibers in *gpt* delta transgenic mouse primary embryo fibroblasts MEFs and compared the results obtained with hydrogen peroxide H₂O₂ in an attempt to illustrate the role of oxyradicals in fiber mutagenesis. Our results provide novel information on the frequencies and types of mutations induced by asbestos fibers in the *gpt* delta transgenic mouse mutagenic assay, which shows great promise for evaluating fiber/particle mutagenicity in vivo.

Keywords: chrysotile asbestos, *gpt* delta mouse, kilobase-sized mutation

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De Felice, M.^{*}, Medagli, B.^{*}, Esposito, L.^{*}, De Falco, M.^{*}, Pucci, B.^{*}, Rossi, M.^{*}, Gruz, P., Nohmi, T. and Pisani, F.M.^{*}: **Biochemical evidence of a physical interaction between *Sulfolobus solfataricus* B-family and Y-family DNA polymerases.**

Extremophiles, **11**, 277-282 (2007)

The hyper-thermophilic archaeon *Sulfolobus solfataricus* possesses two functional DNA polymerases belonging to the B-family (Sso DNA pol B1) and to the Y-family (Sso DNA pol Y1). Herein we report evidence that Sso DNA pol B1 physically interacts with DNA pol Y1 by surface plasmon resonance measurements and immuno-precipitation experiments. The results have important implications for understanding the polymerase-switching mechanism on the damaged template strand during genome replication in *S. solfataricus*.

Keywords: *Sulfolobus solfataricus*, DNA polymerases, interaction

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Barone, F.^{*1}, McCullouch, S.D.^{*2}, McPherson, P.^{*3}, Maga, G.^{*4}, Yamada, M., Nohmi, T., Minoprio, A.^{*1}, Mazzei, F.^{*1}, Kunkel, T.A.^{*2}, Karran, P.^{*3} and Bignami, M.^{*1}: **Replication of 2-hydroxyadenine-containing DNA substrates and recognition by human MutS α .**

DNA Repair, **6**, 355-366 (2007)

2-Hydroxyadenine (2-OH-A), a product of DNA oxidation, is a potential source of mutations. We investigated how representative DNA polymerases from the A, B and Y families dealt with 2-OH-A in primer extension experiments. A template 2-OH-A reduced the rate of incorporation by DNA polymerase α and Klenow fragment. Replication of a template 2-OH-A by human DNA polymerase η and Archaea Dpo4 was mutagenic and caused base substitutions. Thermodynamic analysis showed that 2-OH-A forms stable base pairs with T, C and G, and to a lesser extent with A. Oligonucleotides containing 2-OH-A base pairs, including the preferred 2-OH-A:T, were recognized by the human MutS α .

Keywords: 2-Hydroxyadenine, DNA polymerase, MutS α

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Shimizu, M.^{*1}, Gruz, P., Kamiya, H.^{*2}, Masutani, C.^{*3}, Xu, Y.^{*4}, Usui, Y.^{*1}, Sugiyama, H.^{*4}, Harashima, H.^{*2}, Hanaoka, F.^{*3}, Nohmi, T.: **Efficient and Erroneous Incorporation of Oxidized DNA Precursors by Human DNA Polymerase η .**

Biochemistry, **46**, 5515-5522 (2007)

Oxidation of DNA precursors, i.e., dNTP pool, as well as DNA is a major source of mutagenesis and carcinogenesis. Here, we report the remarkable nature of human DNA polymerase η that incorporates oxidized dNTPs into a nascent DNA strand in an efficient and erroneous manner. The polymerase almost exclusively incorporated 8-hydroxy-dGTP opposite template adenine (A) at 60% efficiency of normal dTTP incorporation, and incorporated 2-hydroxy-dATP opposite template thymine, guanine, or cytosine at substantial rates. We propose that human DNA polymerase η may participate in oxidative mutagenesis through the efficient and erroneous incorporation of oxidized dNTPs during DNA synthesis.

Keywords: DNA polymerase η , oxidized dNTPs, erroneous incorporation

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Ema, M., Fujii, S.^{*1}, Ikka, T.^{*1}, Matsumoto, M., Hirose, A. and Kamata, E.: **Early pregnancy failure induced by dibutyltin dichloride in mice.**

Environ Toxicol, **22**, 44-52 (2007)

In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0-3 or days 4-7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0-3, the rate of nonpregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0-3 at 15.2 mg/kg and higher and on days 4-7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0-3 or days 4-7. A decline in

the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0-3 or days 4-7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure.

Keyword: dibutyltin dichloride, organotin, pregnancy failure, early embryonic loss, progesterone

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Ema, M., Fukunishi, K.*¹, Matsumoto, M., Hirose, A., Kamata, E. and Ihara, T. *¹:**Developmental toxicity of dibutyltin dichloride in cynomolgus monkeys.**

Reprod Toxicol., **23**, 12-19 (2007)

Dibutyltin dichloride (DBTCl) has been shown to be teratogenic in rats. The present study was conducted to determine the teratogenic potential of DBTCl given to pregnant monkeys during the entire period of organogenesis. Cynomolgus monkeys were dosed once daily by nasogastric intubation with DBTCl at 0, 2.5 or 3.8 mg/kg on days 20-50 of pregnancy, the whole period of organogenesis. The pregnancy outcome was determined on day 100 of pregnancy. In both DBTCl-treated groups, a significant increase in the incidence of pregnant females with soft stool and/or diarrhea, and with yellowish stool was observed. Maternal body weight gain at 3.8 mg/kg and food consumption at 2.5 and 3.8 mg/kg were decreased during the administration period. The survival rate of fetuses at terminal cesarean sectioning was decreased in the DBTCl-treated groups and significantly decreased at 2.5 mg/kg. There were no changes in the developmental parameters of surviving fetuses, including fetal body weight, crown-rump length, tail length, sex ratio, anogenital distance and placental weight, in the DBTCl-treated groups. No external, internal or skeletal malformations were found in the fetuses in any group. Although internal and skeletal variations were found, no difference in the incidence of fetal variation was noted between the control and DBTCl-treated groups. No effect on skeletal ossification was observed in fetuses in the DBTCl-treated groups. The data demonstrate that DBTCl is embryolethal but not teratogenic in cynomolgus monkeys.

Keyword: dibutyltin, organotin, teratogenicity, embryolethality, monkey

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Ema, M., Fujii, S. *¹, Matsumoto, M., Hirose, A. and Kamata, E.: **Prenatal developmental toxicity study of basic rubber accelerator, 1,3-di-o-tolylguanidine, in rats.**

Reprod. Toxicol., **22**, 672-678 (2006)

Pregnant rats were given 1,3-di-o-tolylguanidine (DTG) by gavage at 0, 10, 20 or 40 mg/kg bw/day on days 6-19 of pregnancy and the pregnancy outcome was determined on day 20 of pregnancy. At 40 mg/kg bw/day, deaths were observed in four out of 24 females. The incidences of females showing mydriasis at 20 and 40 mg/kg bw/day and showing decreased locomotor activity at 40 mg/kg bw/day were significantly increased. Alopecia, bradypnea, prone position and tremor were also observed at mg/kg bw/day. The maternal body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were significantly reduced. A significantly decreased weight of the gravid uterus, increased incidence of postimplantation loss, decreased number of live fetuses, and lowered weights of fetuses and placentae were found at 40 mg/kg bw/day. The incidences of the total number of fetuses with external malformations at 40 mg/kg bw/day and with skeletal malformations at 20 and 40 mg/kg bw/day were significantly increased. Significantly higher incidences of fetuses with brachydactyly and short tail and defects of caudal vertebrae, phalanges and metacarpals were observed at 40 mg/kg bw/day. Delayed ossification was also noted at 40 mg/kg bw/day. The data indicate that DTG is teratogenic at maternal toxic doses and the NOAELs of DTG for maternal and developmental toxicity are 10 mg/kg bw/day in rats.

Keyword: di-o-tolylguanidine, rubber accelerator, sigma ligand, prenatal developmental toxicity, teratogenicity, malformation, rat

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Ema, M., Kimura, E.*¹, Matsumoto, M., Hirose, A. and Kamata, E.: **Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-o-tolylguanidine, in rats.**

Reproductive Toxicology, **22**, 30-36 (2006)

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-o-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40-49 days beginning 14 days before mating to day 3 of lactation throughout the

mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and vi-

ability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.

Keyword: di-o-tolylguanidine, rubber accelerator, sigma ligand, reproductive and developmental toxicity, teratogenicity, malformation, rat

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