Pan-Pacific International Partnership Conference on Pharmaceutical and Life Sciences (The 4th US-Japan Joint Conference)

February 22 - 23, 2008
Nagoya, Japan

Abstracts

Hosted by Nagoya City University (NCU) Graduate School of Pharmaceutical Sciences
with Support by Innovative Drug Discovery and Design Project

Co-organized by
Kyoto University (KU)
Meijo University (MU)
Showa Pharmaceutical University (SPU)
The University of Sydney (USYD)
The University of Tokyo (UT, Tokyo)
Tokyo University of Pharmacy and Life Sciences (TUPLS)
University of Southern California (USC)
University of Toyama (UT, Toyama)

Pan-Pacific International Partnership Conference on Pharmaceutical and Life Sciences (The 4th US-Japan Joint Conference)

Date: February 22 (Fri) - 23 (Sat), 2008

Venue: Nagoya City University
Graduate School of Pharmaceutical Sciences (Mizuno Hall)
3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

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Organizing Committee (members from hosting NCU)

Satoshi Fujii	Mitsuharu Hattori
Hidetoshi Hayashi	Tsunehiko Higuchi
Naohide Hirashima	Kazuhiro Kondo
Susumu Ohya	Hiroaki Yuasa, Chair

Advisory Board (members from co-organizing partner universities)

Tatsuo Akitaya (MU)	Ronald Alkana (USC)
Kim-Hak Chan (USYD)	Ken-ichi Hosoya (UT, Toyama)
Hiroyuki Kusuhara (UT, Tokyo)	Yoshinobu Takakura (KU)
Hiro-o Toyoda (TUPLS)	Hiroshi Yamazaki (SPU)

Supports
Supported by a Grant-in-Aid for Research in Nagoya City University
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Adjunct meetings
Organizing Committee/Advisory Board Meeting (February 23)
Partnership Meeting (February 23)

Access to Nagoya City University

Centrair Airport, Nagoya: A new international airport located about 20 miles south to Nagoya; 28 minutes by train and 60 min by bus from Nagoya Station.

Nagoya Municipal Airport: A long existing airport located north to Nagoya, serving only for several domestic flights; 28 min by bus from Nagoya Station.

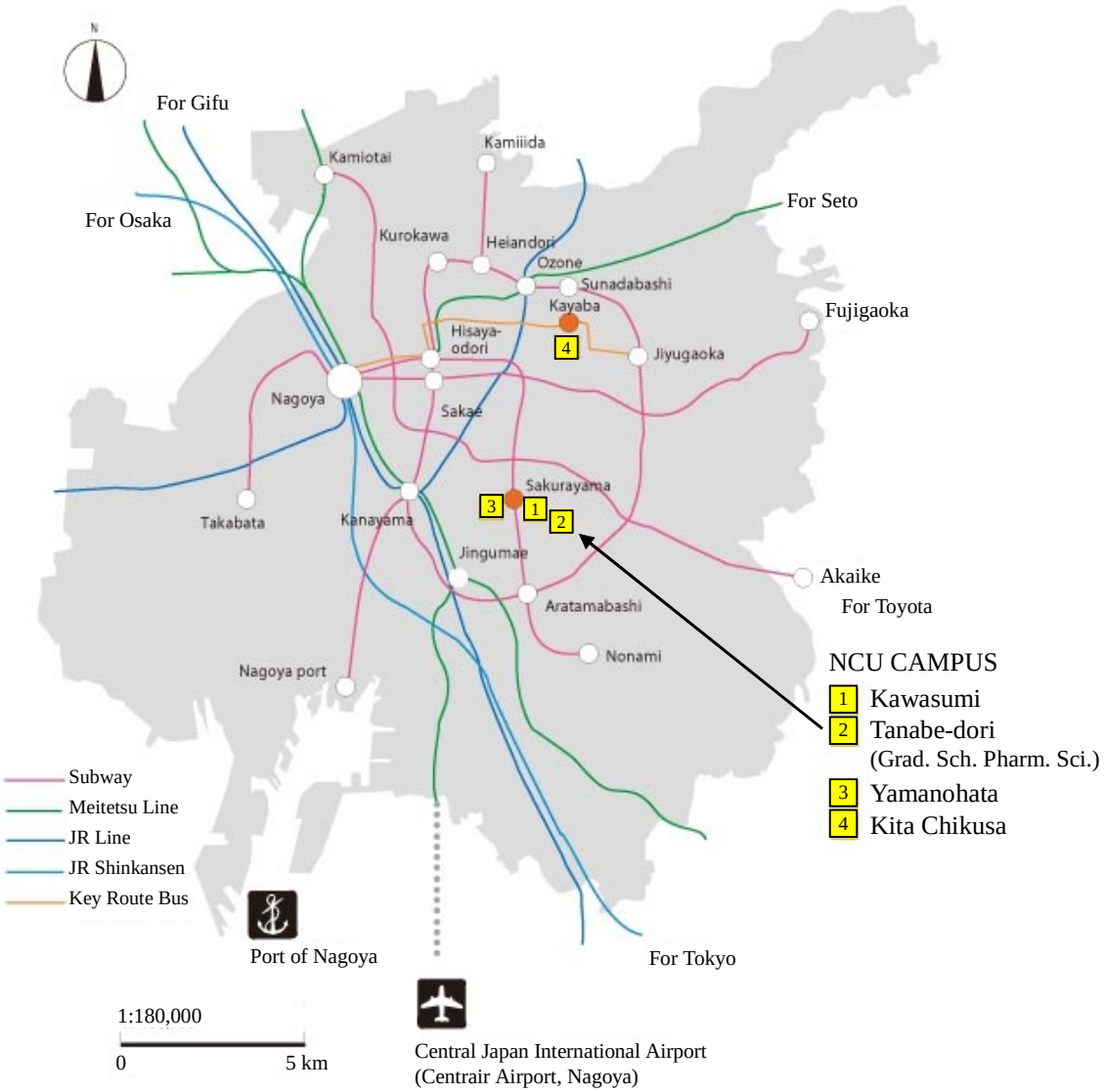
From Nagoya Station:

To Kawasumi Campus **[1]**: 20 minutes by Subway Sakuradori Line to Sakurayama Station, and right there.

✓ To Tanabe-dori Campus **[2]**/Grad. Sch. Pharm. Sci.: 22 minutes by Subway Sakuradori Line to Mizuhokuyakusyo Station, and 20-minute walk.

To Yamanohata Campus **[3]**: 20 minutes by Subway Sakuradori Line to Sakurayama Station, and 10-minute walk.

To Kita Chikusa Campus **[4]**: 40 minutes by Key Route Bus bound for Jiyugaoka to Kayaba, and right there.



Welcome

This conference represents the 4th conference jointly organized by the University of Southern California in US and its affiliated schools of pharmacy in Japan. The conference goal is to strengthen scientific research through international cooperation in pharmaceutical sciences and relevant disciplines. This 4th conference hosted by Nagoya City University focuses particularly on the combination of pharmaceutical sciences and life sciences and is expanded by inviting The University of Sydney as its partner in Australia as a country in the Pan-Pacific region.

The scientific program consists of symposium sessions, which feature lectures by invited speakers from affiliated universities, and a poster session of contributed papers. This conference is open to public and participation from any party will be welcome. The official language of the conference will be English.

Alumni Hall Building

accommodating Mizuno Hall for the Conference on the third floor

Overview of Nagoya City

Nagoya Castle

Atsuta Shrine

Twin Towers of Nagoya Station

Home Soccer Stadium for Nagoya Grampus Eight

Program

February 22 (Fri)

12:45 - 13:00 Opening Remarks

Hitoo Nishino, President (NCU)
Hajime Mizukami, Dean of Grad. Sch. Pharm. Sci. (NCU)

13:00 - 15:00 Session 1: Drug Discovery and Design

Moderators: Hiroo Toyoda (TUPLS), Naoki Umezawa (NCU)

S1 Interaction Between Inflammation and Fibrinolysis and its Pharmacological Control
Satoshi Fujii (NCU, Japan)

S2 Chemical and Apoptotic Properties of Hydroxy-Ceramides Containing Long-Chain Bases with Unusual Alkyl Chain Lengths
Mamoru Kyogashima (Aichi Cancer Center Research Institute; NCU, Japan)

S3 New Aspects of Microtubule Targeting Agents as Anti-Cancer Drugs
Yoshio Hayashi,[a,b] Yuri Yamazaki,[a,b] Mariusz Skwarczynski,[b] Yoshiaki Kiso,[b] G. Kenneth Lloyd[c] ([a]TUPLS; [b]Kyoto Pharmaceutical Univeristy, Japan; [c]Nereus Pharmaceuticals, USA)

S4 Structure-Based Biomolecular Engineering Targeting 'Sweets'
Koichi Kato (NCU; National Institutes of Natural Sciences; GLYENCE; Ochanomizu University; CREST, Japan)

15:15 - 17:15 Poster Session (Auditorium in Library Building)

17:30 - 19:30 Reception (Students' Hall)

February 23 (Sat)

9:00 - 10:30 Session 2: Drug Action and Therapy

Moderators: Paul Beringer (USC), Yuji Imaizumi (NCU)

S5 Potential Sites of Ethanol Action and Medications Development in Glycine Receptors Suggested by Hyperbaric Studies
Ronald L. Alkana,[a] Daya I. Perkins,[a] Rachel L. Kelly,[a] Jeffrey C. Tom,[a] James R. Trudell,[b] Edward J. Bertaccini,[b] Daryl L. Davies[a] ([a]USC; [b]Stanford Univeristy, USA)

S6 Individual Differences of Oxidative Metabolism of Medicines and Their Clinical Significance
Hiroshi Yamazaki,[a] Dimitrios Zonios,[b] Makiko Shimizu,[a] John Bennett,[b] Norie Murayama[a] ([a]SPU, Japan; [b]NIH/NIAID, USA)

S7 Cholesterol Metabolism in the Central Nervous System and Alzheimer's Disease
Makoto Michikawa (National Center for Geriatrics and Gerontology; NCU, Japan)

10:30 - 11:00 Break

11:00 - 12:30 Session 3: Drug Disposition and Delivery I

Moderators: Katsuhisa Inoue (NCU), Hideki Sakai (UT, Toyama)

S8 Role of ABC Transporters in Drug Absorption and Elimination
Hiroyuki Kusuhara (UT, Tokyo, Japan)

S9 Blood-Retinal Barrier Transporters: Role in Retinal Drug Delivery
Ken-ichi Hosoya, Masanori Tachikawa (UT, Toyama, Japan)

S10 Nonviral Gene Delivery Based on Physical Methods in Combination with Naked Plasmid DNA
Yoshinobu Takakura (KU, Japan)

12:30 - 14:00 Lunch Break

14:00 - 16:00 Session 4: Drug Disposition and Delivery II

Moderators: Tatsuo Akitaya (MU), Yoshiteru Watanabe (SPU)

S11 Inhalation Drug Delivery I: Effect of Particle Size of Dry Powder Mannitol on the Lung Deposition in Healthy Subjects
Hak-Kim Chan,[a] William Glover,[a] Stefan Eberl,[b] Evangelia Daviskas,[c] Jordan Verschuer[b] ([a]USYD; [b]Dept. PET and Nuclear Medicine; [c]Dept. Respiratory Medicine, Royal Prince Alfred Hospital, Austlaria)

S12 Inhalation Drug Delivery II: Understanding and Enforcing Drug Aerosolisation from Dry Powder Inhalation Medicines: Measurement and Modification of Particulate Systems to Improve Efficiency
Paul M Young (USYD, Austlaria)

S13 Inhalation Drug Delivery III: Predicting Adhesion and Cohesion in Non-Aqueous Propellant Systems
Daniela Traini (USYD, Austlaria)

S14 Sea-Urchin-Like Particles for Inhalation Drug Delivery to Lungs
Hirokazu Okamoto, Kazumi Danjo (MU, Japan)

16:00 - 16:15 Closing Remarks

Ronald Alkana (USC)
Hiroaki Yuasa (NCU)

Poster Session (15:15 - 17:15, February 22)

P1 RECENT APPLICATION OF TRIMETHYLSILYLDIAZOMETHANE TO THE SYNTHESIS OF HETEROCYCLES
Yoshiyuki Hari, Toyohiko Aoyama (NCU, Japan)

P2 RHODIUM-CATALYZED SYNTHESIS OF INDOLE COMPOUNDS FROM *N*-PROPARGYL ANILINES OR THE RELATED COMPOUNDS
Akio Saito, **Shoko Oda**, Miki Hayashi, Yuji Hanzawa (SPU, Japan)

P3 DISCOVERY OF HISTONE DEACETYLASE 6-SELECTIVE INHIBITORS AND THEIR EFFECT ON HUMAN CANCER CELLS
Yukihiro Itoh,[a] Takayoshi Suzuki,[a] Akiyasu Kouketsu,[a] Satoko Maeda,[b] Minoru Yoshida,[b] Hidehiko Nakagawa,[a] Naoki Miyata[a] ([a]NCU; [b]RIKEN, Japan)

P4 PYRAZOLONE DERIVATIVES WITH POTENT ANTI-PRION ACTIVITY: THEIR STRUCTURE-ACTIVITY RELATIONSHIP
Ayako Kimata,[a] Hidehiko Nakagawa,[a] Ryo Ohyama,[a] Tomoko Fukuuchi,[b] Shigeru Ohta,[c] Katsumi Doh-ura,[d] Takayoshi Suzuki,[a] Naoki Miyata[a] ([a]NCU; [b]Hiroshima International University; [c]Hiroshima Univeristy; [d]Tohoku Univeristy, Japan)

P5 NOVEL QUADRUPLE HYDROGEN-BONDING MODULE WITH FIVE-MEMBERED HETEROAROMATIC RING
Yosuke Hisamatsu, Naohiro Shirai, Shin-ichi Ikeda, Kazunori Odashima (NCU, Japan)

P6 SYNTHETIC STUDIES ON NEODYSIHERBAINE A
Toshihiro Hirai, Nobuyoshi Morita, Iwao Okamoto, Osamu Tamura (SPU, Japan)

P7 COMPARISON OF SKIN AND SPLEEN AS INJECTION SITE OF VACCINES FOR INDUCTION OF IMMUNE RESPONSE
Xin Guan, Makiya Nishikawa, Yoshinobu Takakura (KU, Japan)

P8 MECHANISM OF mRNA DEADENYLATION: EVIDENCE FOR A MOLECULAR INTERPLAY BETWEEN TRANSLATION TERMINATION FACTOR eRF3 AND mRNA DEADENYLASES
Yuji Funakoshi,[a,b] Yusuke Doi,[a] Nao Hosoda,[a] Naoyuki Uchida,[c] Masafumi Tsujimoto,[b] Shin-ichi Hoshino[a] ([a]NCU; [b]RIKEN; [c]UT, Tokyo, Japan)

P9 ANALYSIS OF PROTEINS INTERACTING WITH THE N-TERMINAL REGION OF NUCLEAR RECEPTOR FARNESOID X RECEPTOR (FXR)
Masae Ohno, Eiko Suzuki, Masaaki Kunimoto, Makoto Nishizuka, Shigehiro Osada, Masayoshi Imagawa (NCU, Japan)

P10 KIAA0319, A DYSLEXIA-ASSOCIATED GENE, INDUCES ABNORMAL BRANCHING OF NEURITES
Moe Ishii, Yukie Matsuda, Atsushi Baba, Mitsuharu Hattori (NCU, Japan)

P11 P90 RSK-1 ASSOCIATES WITH AND INHIBITS NEURONAL NITRIC-OXIDE SYNTHASE[1]
Tao Song,[a,b,c] Katsuyoshi Sugimoto,[b] Hideshi Ihara,[d] Akihiro Mizutani,[e] Naoya Hatano,[b] Kodai Kume,[b] **Toshie Kambe**,[a] Fuminori Yamaguchi,[b] Masaaki Tokuda,[b] Yoshiaki Miyamoto,[a] Yasuo Watanabe[a] ([a]SPU; [b]Kagawa Univeristy, Japan; [c]China Medical Univeristy, China; [d]Osaka Prefecture Univeristy; [e]UT, Tokyo, Japan)

P12 MOLECULAR MECHANISMS FOR OXIDATIVE DAMAGE AND STRUCTURE CHANGE OF DNA THROUGH REDOX COPPER-CATECHOL COMPLEX FORMATION
Motozumi Ando, Koji Ueda, Rena Makino, Nakao Kojima (MU, Japan)

P13 FUNCTIONAL ANALYSIS OF A PSEUDOKINASE, TRB3 DURING THE M PHASE
Satoshi Sakai,[a] Nobumichi Ohoka,[b] Makoto Nakanishi,[c] Kikuo Onozaki,[a] Hidetoshi Hayashi[a] ([a]Grad. Sch. Pharm. Sci., NCU; [b]UT, Tokyo; [c]Grad. Sch. Med. Sci., NCU, Japan)

P14 MEMBRANE FUSION LIPOSOMES CONTAINING SNARE PROTEINS INVOLVED IN EXOCYTOSIS OF MAST CELLS
Satoshi Tadokoro,[a] Hiroki Sakiyama,[a] Mamoru Nakanishi,[b] Naohide Hirashima[a] ([a]NCU; [b]Aichi Gakuin Univeristy, Japan)

P15 ENHANCEMENT OF THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR EXPRESSION BY COMPOUNDS TO ELEVATE INTRACELLULAR cAMP LEVELS
Yuka Kojima, Yutaka Masuda, Kimihiko Takada, Hidemi Ishii (SPU, Japan)

P16 DISCOVERY OF NEW THERAPEUTIC TARGETS USING LIVER *PTEN* DELETION ANIMAL MODEL
Bangyan Stiles, Vivian Galica, Ni Zeng, Jennifer-Ann Bayan, Lina He (USC, USA)

P17 TYPE 1 Na^+-DEPENDENT PHOSPHATE TRANSPORTER (NPT1) MAKES A LIMITED CONTRIBUTION TO THE URINARY EXCRETION OF ORGANIC ANIONS
Yushun Kuroiwa, Hiroyuki Kusuhara, Yuichi Sugiyama (UT, Tokyo, Japan)

P18 DEVELOPMENT OF EFFICIENT GENE TRANSFER SYSTEMS INTO CACO-2 CELL MONOLAYER BY ADENOVIRUS VECTOR
Naoya Koizumi,[a] Yoshiaki Yamagishi,[a] Hiroyuki Mizuguchi,[b,c] Makiko Fujii,[a] Yoshiteru Watanabe[a] ([a]SPU; [b]National Institute of Biomedical Innovation; [c]Osaka Univeristy, Japan)

P19 THERMOGENOUS EFFECTS OF PROCESSED ACONITE ROOT ON COLD-STRESSED MICE HYPOTHERMIA
Keita Kato, Toshiaki Makino, Hajime Mizukami (NCU, Japan)

P20 EFFECTS OF MORPHINE AND GABAPENTIN ON MECHANICAL ALLODYNIA INDUCED BY CHEMOTHERAPEUTIC DRUGS IN MICE
Punam Gauchan,[a] Kenichiro Ikeda,[a] Masahide Fujita,[a] Tsugunobu Andoh,[a] Atsushi Sasaki,[a] Atsushi Kato,[b] Yasushi Kuraishi[a] ([a]Grad. Sch. Med. Pharm. Sci; [b]Dept. Hospital Pharmacy, UT, Toyama, Japan)

P21 MODURATION OF GASTRIC PROTON PUMP ACTIVITY BY CLC-5
Yuji Takahashi,[a] Takuto Fujii,[a] Hiroshi Furuya,[a] Yoshiaki Tabuchi,[b] Akira Ikari,[c] Hisato Sakamoto,[d] Ichiro Naito,[e] Koji Manabe,[e] Shinichi Uchida,[f] Sei Sasaki,[f] Shinji Asano,[g] Magotoshi Morii,[a] Noriaki Takeguchi,[a] Hideki Sakai[a] ([a]Grad. Sch. Med. Pharm. Sci.; [b]Life Science Research Center, UT, Toyama; [c]University of Shizuoka; [d]Kitasato University; [e]Shigei Medical Research Institute; [f]Toyko Medical and Dental Univeristy; [g]Ritsumeikan Univeristy, Japan)

P22 UP-REGULATION OF AQUAPORIN-5 IN HUMAN GASTRIC ADENOCARCINOMA IS INVOLVED IN THE CANCER CELL DIFFERENTIATION
Takuto Fujii,[a] Tomoko Watanabe,[b] Takeshi Oya,[c] Tatsuma Fujita,[a] Naoki Horikawa,[b] Yuji Takahashi,[a] Magotoshi Morii,[a] Noriaki Takeguchi,[a] Kazuhiro Tsukada,[b] Hideki Sakai[a] ([a]Dept. Pharmaceutical Physiology; [b]Dept. Surgery II; [c]Dept. Pathology II, Grad. Sch. Med. Pharm. Sci., UT, Toyama, Japan)

P23 THE PEPTIDE TRANSPORTER IS NOT RESPONSIBLE FOR UPTAKE OF LEVOFLOXACIN IN HUMAN INTESTINAL CACO-2 CELLS
Shiro Fukumori, Toshiya Murata, Katsutoshi Tahara, Masato Taguchi, Yukiya Hashimoto (UT, Toyama, Japan)

P24 STEREOSELECTIVE GLUCURONIDATION AND OXIDATION OF CARVEDILOL IN HUMAN LIVER AND INTESTINAL MICROSOMES
Kazuya Ishida, Shigehiro Taira, Hiroki Morishita, Masato Taguchi, Yukiya Hashimoto (UT, Toyama, Japan)

P25 FUNCTIONAL CHARACTERISTICS OF HUMAN PROTON-COUPLED FOLATE TRANSPORTER/HEME CARRIER PROTEIN 1 (hPCFT/HCP1) HETEROLOGOUSLY EXPRESSED IN MAMMALIAN CELLS
Misato Sugiura,[a] Katsuhisa Inoue,[a] Yasuhiro Nakai,[a] Naoki Abe,[a] Kin-ya Ohta,[a] Mai Hatakeyama,[b] Yayoi Hayashi,[b] Hiroaki Yuasa[a] ([a]NCU; [b]Kinjo Gakuin Univeristy, Japan)

P26 INCREASING ANTICANCER ACTIVITY OF INTERFERON GENE TRANSFER BY PROLONGING THE DURATION OF TRANSGENE EXPRESSION
Lei Zang, Makiya Nishikawa, Masaru Mitsui, Yuki Takahashi, Yoshinobu Takakura (KU, Japan)

P27 A NEW CLASS OF LIGAND FOR VITAMIN D RECEPTOR: DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF LIGANDS WITH ADAMANTINE RING
Nobuko Yoshimoto,[a,b] Yuka Inaba,[a,b] Sachiko Yamada,[b] Masato Shimizu,[b] Keiko Yamamoto[a] ([a]SPU; [b]Tokyo Medical and Dental Univeristy, Japan)

P28 INHIBITION OF DRUG METABOLIZING ENZYMES BY XANTHENE DYES
Kenji Furumiya, Takaharu Mizutani (NCU, Japan)

P29 KINETIC STUDY ON METHYLESTERIFICATION OF DICARBOXYLIC ACYLCARNITINES IN ACIDIC METHANOL SOLUTION
Hiromi Nakayama,[a] Yasuhiro Maeda,[a] Tetsuya Ito,[b] Hajime Togari,[b] Naruji Sugiyama,[c] Yukihisa Kurono[a] ([a]Grad. Sch. Pharm. Sci.; [a]Grad. Sch. Med. Sci., NCU; [c]Aichi Gakuin Univeristy, Japan)

P30 TRANSCRIPTIONAL REGULATION OF L-TYPE AMINO ACID TRANSPORTER 1 (LAT-1) GENE IN THE RETINAL CAPILLARY ENDOTHELIAL CELLS UNDER THE GLUCOSE DEPLETED CONDITIONS
Ryo Matsuyama, Masatoshi Tomi, Shin-ichi Akanuma, Masanori Tachikawa, Ken-ichi Hosoya (UT, Toyuama, Japan)

P31 APPLICATION OF THE MULTI-DIMENSIONAL HPLC MAPPING METHOD FOR *N*-GLYCOSYLATION PROFILING OF CELLS, TISSUES AND ORGANS
Hirokazu Yagi,[a] Noriko Takahashi,[a,b,c] Koichi Kato[a,b,c,d] ([a]NCU; [b]GLYENCE; [c]Ochanomizu University; [d]National Institutes of Natural Sciences, Japan)

P32 P-GLYCOPROTEIN (PGP) AND RENAL CLEARANCE (CL_R) OF FEXOFENADINE IN CYSTIC FIBROSIS (CF)
Paul Beringer,[a,b] S. Liu,[a] L. Hidayat,[a] S. Louie,[a] G. Burckart,[a] A. Rao,[b] B. Shapiro[b] ([a]Sch. Pharm.; [b]Sch. Med., USC, USA)

P33 INHIBITORY EFFECTS OF FLUORINATED TOLUBUTAMIDE ANALOGS ON CYP2C9 ISOZYMES
Noriko Matsuyama, Takaharu Mizutani, Ken-ichi Saeki (NCU, Japan)

P34 DEFICIENCY OF HIPPOCAMPAL LONG-TERM POTENTIATION AFTER PERIPHERAL NERVE INJURY AND ITS RELATION WITH THE EXTRACELLULAR GLYCINE LEVEL REGULATED BY GLYCINE TRANSPORTER 1
Daisuke Kodama, Hideki Ono, Mitsuo Tanabe (NCU, Japan)

P35 MOLECULAR GENETIC MINING OF THE *ASPERGILLUS* SECONDARY METABOLOME
Yi-ming Chiang,[a] Edyta Szewczyk,[c] James F. Sanchez,[a] Nancy Keller,[d] Berl R. Oakley,[c] **Clay C. C. Wang**[a,b] ([a]Sch. Pharm.; [b]Coll. Letters, Arts and Sciences, USC; [c]Ohio State Univeristy; [d]University of Wisconsin, USA)

P36 CLINICAL PHARMACIST TRAINING IN COLLABORATION WITH MEDICAL EDUCATION
Hiroyuki Kamei, Manako Han-ya, Fumiko Ohtsu, Tadao Taguchi, Kotaro Iida, Masayuki Nadai, Tsutomu Yoshida, Tadashi Nagamatsu, Rikio Shinohara, Mikio Nishida, Masami Hirano, **Kazuhisa Matsuba** (MU, Japan)

P37 STUDY OF INHIBITION OF VPA-GLUCURONIDASE BY CARBAPENEM ANTIBIOTICS
Yutaka Nakamura, Keiko Nakahira, **Takaharu Mizutani** (NCU, Japan)

P38 CATALITIC ACTIVITIES OF THE FLAVIN-CONTAINING MONOOXYGENASE 3 (FMO3) VARIANTS FOUND IN JAPANESE
Satomi Nagashima, Makiko Shimizu, Hiroshi Yano, Norie Murayama, Hiroshi Yamazaki (SPU, Japan)

P39 MAGNITUDE OF EFFECT AND SITE OF ACTION OF DOPAMINE ON THE RENAL CIRCULATION IN PATIENTS WITH SEVERE HEART FAILURE
Uri Elkayam, **Tien Ng**, Anilkumar Mehra, Moshe Garty (USC, USA)

P40 WHAT IS A GOAL OF RADIO(-HEALTH) CHEMISTRY IN THE 6-YEAR EDUCATION PROGRAM FOR PHARMACY?
Ikuko Takahashi, Masayuki Hiramatsu, Tadashi Nagamatsu, **Mikio Nishida** (MU, Japan)

P41 INSULIN REGULATES PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 EXPRESSION IN HEPATOCYTES AND IN MICE LIVER THROUGH STATIN-SENSITIVE PATHWAY: IMPLICATIONS FOR CLINICAL EFFECTS
Ryu Miyagawa,[a] Yuki Sato,[b] Tomomi Nakamura,[a] Satoshi Fujii[a] ([a]NCU; [b]Hokkaido University, Japan)

P42 CHARGE-INDUCED PHASE SEPARATION IN BINARY CHARGED COLLOIDS
Koki Yoshizawa, Nao Wakabayashi, Junpei Yamanaka, Masakatsu Yonese (NCU, Japan)

P43 THE EFFECT OF ISCHEMIA ON ERECTILE FUNCTION IN A RABBIT MODEL
Yuko Abe, Yuji Hotta, Kana Okumura, Chigusa Kikuchi, Toru Maeda, Kazunori Kimura (NCU, Japan)

P44 VISUALIZATION OF LOCAL Ca^{2+} TRANSIENTS IN SMOOTH MUSCLE CELLS USING TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPE
Hisao Yamamura, Yuji Imaizumi (NCU, Japan)

Symposium Abstracts

S1

INTERACTION BETWEEN INFLAMMATION AND FIBRINOLYSIS AND ITS PHARMACOLOGICAL CONTROL

Satoshi Fujii
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The fibrinolytic system, which controls the formation and activity of plasmin, plays a key role in regulating hemostasis and thrombosis. Fibrinolytic system also regulates several other biological processes such as tissue remodeling (for instance intimal hyperplasia after vascular injury and atherosclerosis) by controlling local proteolysis and cell adhesion and migration via either inhibiting plasminogen activators (PAs) or interference with the binding between cellular integrins or urokinase-type PA (uPA) receptor and vitronectin. While much is studied about the function of the PA system, it remains a focus of intensive investigation because the list of biological pathways and human diseases that are modulated by normal and pathologic function of fibrinolytic components continues to increase.

We aimed to study the normal and pathologic functions of the PA system and to identify the means of pharmacological control. Particular attention is paid to the vascular functions of plasminogen activator inhibitor-1 (PAI-1), a key major physiologic regulator of the PA system and an acute-phase reactant abundantly produced in liver. Atherosclerosis is accelerated by inflammatory processes and increased expression of PAI-1. Thus, the effects of inflammatory factors on PAI-1 production in liver were elucidated. Prototypical pro-inflammatory cytokines, interleukin (IL)-1β and IL-6, increased the PAI-1 mRNA and protein in a well-differentiated human liver cell line (HepG2). Transient transfection and luciferase assay of the PAI-1 promoter demonstrated that the PAI-1 promoter activity was increased by IL-1β and IL-6. Systematic deletion assay of the PAI-1promoter demonstrated that the region from –239bp to –210bp containing a putative CCAAT-enhancer binding protein (C/EBP) binding site was critical. Point mutation in C/EBP site abolished both IL-1β and IL-6 responses. Antibody interference electrophoretic mobility-shift assays showed that among C/EBP isoforms C/EBPδ (but not C/EBPα or C/EBPβ) binding and C/EBPδ protein were increased by IL-1β and IL-6. Down-regulation of C/EBPδ induced with small interfering RNA decreased secretion of PAI-1. IL-1β and IL-6 exert directionally similar effects on PAI-1 expression and the induction involves distinct signaling pathways with a common mediator, C/EBPδ. Lipid-lowering agents, statins, lowered PAI-1 expression induced by cytokines. In hypertensive patients high levels of PAI-1 in plasma were closely correlated with the lack of favorable vascular remodeling in brachial arteries and with the reduction of endothelial function in coronary arteries.

Taken together, PAI-1 mediates the interaction between inflammation and fibrinolysis. Pharmacological intervention to inhibit PAI-1 expression may not only diminish atherothrombosis but also provide vascular protection by regulating PAI-1 expression.

References

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4. Dong J., Fujii S., Imagawa S., Matsumoto S., Matsushita M., Todo S., Tsutsui H., Sobel B.E., *Am J Physiol*, 292, C209-215 (2007)

S2

CHEMICAL AND APOPTOTIC PROPERTIES OF HYDROXY-CERAMIDES CONTAINING LONG-CHAIN BASES WITH UNUSUAL ALKYL CHAIN LENGTHS

Mamoru Kyogashima[a,b]

[a]*Division of Molecular Pathology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan,*
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Ceramide (N-acylated long-chain base) is a basic component of sphingolipids [1]. Abundant free ceramides are present in skin, and act as a water reservoir and a barrier due to their physicochemical properties, but in most cells, free ceramides are bioactive and play important roles in cell signaling for differentiation, growth, and apoptosis; and their amounts are strictly regulated throughout sphingolipid metabolism. Therefore “drug discovery” based on the elucidation of ceramides is important for cancer therapy [2].

Long chain bases of sphingolipids are usually composed of octadeca types of (4E)-sphingenine (d18:1) or 4D-hydroxysphinganine (t18:0), the latter of which is usually minor. We purified and analyzed free ceramides from equine kidneys using electrospray ionization mass spectrometry with low energy collision-induced dissociation. In addition to usual ceramide molecular species of non-hydroxy-ceramide (Cer 1), we identified two types of monohydroxy-ceramides (Cer 2 and Cer 3) and one type of dihydroxy-ceramide (Cer 4). Furthermore, these hydroxyl-ceramides contained non-octadeca long-chain bases (NOD-LCBs) such as d16:1/t16:0, d17:1/t17:0, d19:1/t19:0 and d20:1/t20:0, in addition to d18:1/t18:0. Because mammalian serine palmitoyltransferase has been reported to use palmitoyl-CoA as the best substrate, pentadecanoyl- /heptadecanoyl-CoA as some effective substrates and other acyl-CoAs as far less effective substrates, respectivel, details of the synthetic pathways for NOD-LCBs and the ceramides containing them are still uncertain.

The apoptosis inducing activities of these hydroxyl-ceramides towards tumor cell lines were compared. Both monohydroxy-ceramides (Cer 2 and Cer 3) exhibited stronger activity than usual ceramide (Cer 1). Interestingly, dihydroxy-ceramide of Cer 4 exhibited similar or rather weaker activity than Cer 1. These results suggest that single hydroxylation of ceramides, enhances apoptotic activity but double hydroxylations of ceramides attenuate the activity.

Lengths of the alkyl chain and hydroxy groups in ceramides significantly influence the physicochemical properties of sphingolipids, probably critically affecting the formation of microdomains and cell signaling. Various roles and the metabolic pathway of ceramides, especially hydroxy-ceramides with NOD-LCBs, remain to be investigated for “drug discovery” of cancer therapy.

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S3

NEW ASPECTS OF MICROTUBULE TARGETING AGENTS AS ANTI-CANCER DRUGS

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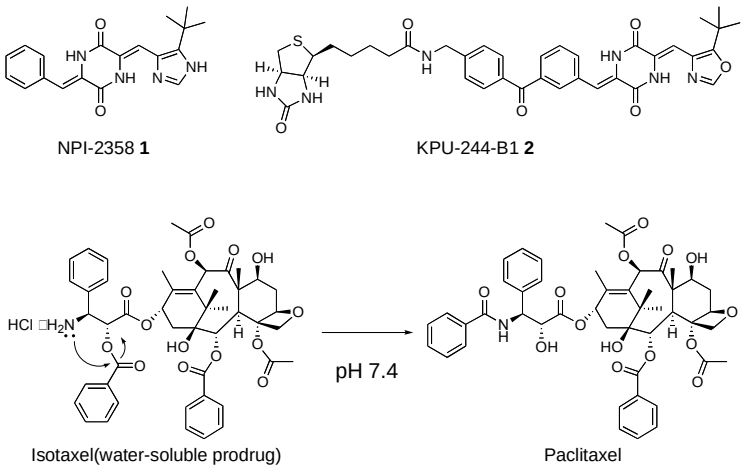
Microtubule targeting agents (MTAs) such as taxoids have revolutionized the treatment of cancer and improved patient survival. However, in clinical use, several drawbacks were observed: ineffectiveness against MDR tumors; side effects due to low selectivity; and low solubility for i.v. administration due to the drugs' hydrophobic nature. Consequently, there is a significant need to develop new and improved MTAs so that they are suitable and practical in clinical oncology. An interesting aspect is that some MTAs induce tumor-selective vascular collapse, thus deviating blood supply from the tumor tissues. These MTAs are referred to as a "vascular disrupting agents" (VDAs). We are currently performing two MTA projects: the development of novel VDAs and water-soluble taxoid prodrugs.

From a natural diketopiperazine, phenylahistin,[1] we developed a potent VDA, NPI-2358 **1**, which is now in Phase I clinical trial as an anticancer drug in the US.[2] Although, it is believed that **1** recognizes around colchicine binding site on tubulin, the three-dimensional structure of **1** could not be overlapped to that of colchicine. Hence, to understand the precise binding mode of **1**, we developed a biotin-tagged photoaffinity probe **2** and tubulin photoaffinity labeling was performed. A specific labeling towards both tubulin units, but competitive to colchicine, was detected, suggesting that the probe specifically recognizes around the colchicine binding site on tubulin.

We also developed a series of novel water-soluble taxoid prodrugs, "isotaxoids", that are the 2'-*O*-isoforms of taxoids.[3] Isotaxoids exhibited improved water-solubility and appropriate kinetics for parent drug formation via a pH- dependent O-N intramole- cular acyl migration reaction. The fact that an auxiliary is not used and byproducts are not produced from this prodrug is useful in improving the problematic MTAs.



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S4

STRUCTURE-BASED BIOMOLECULAR ENGINEERING TARGETING 'SWEETS'

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Recent advances in structural biology have made possible the high throughput structure determination of proteins, which offers the basis for rational drug design and biomolecular engineering. In these approaches, recombinant proteins subjected to NMR and X-ray crystallographic analyses are produced by using bacterial or cell-free expression systems and therefore do not possess sugar chains. However, many of the proteins in the living systems express sugar chains, which play crucial roles in regulation of glycoprotein functions and even in glycoprotein-fate determination in cells. To elucidate the underlying mechanisms of the sugar functions, we have been developing a systematic method for structural glycobiology by combined use of a 920 MHz ultra-high field NMR spectroscopy and multi-dimensional HPLC techniques for glycosylation profiling and sugar library construction.

In this presentation, I will illustrate several examples of our projects to address the structural basis of:

- Glycoform-dependent effector functions of immunoglobulin G glycoproteins as therapeutic antibodies;
- Quality control of glycoproteins in cells through interactions with a variety of intracellular lectins operating as molecular chaperones, cargoreceptors, and ubiquitin ligases.

On the basis of the structural data obtained, we have performed biomolecular engineering targeting intra- and inter-molecular sugar-protein interactions.

Insights into molecular mechanisms for sweet-tasting and taste-modifying activities of curculin, a sweet protein isolated from a plant grown in Malaysia, will also be discussed on the basis of structural and mutational data.

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S5

POTENTIAL SITES OF ETHANOL ACTION AND MEDICATIONS DEVELOPMENT IN GLYCINE RECEPTORS SUGGESTED BY HYPERBARIC STUDIES

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Alcohol is the number one drug of abuse in the world. Available pharmacological strategies for treating alcohol use disorders are limited due, in part, to our lack of knowledge regarding the molecular targets on which alcohol acts in the brain. Part of the difficulty lies in the physical-chemical nature of ethanol's mechanism, which limits the ability to use standard pharmacological approaches to determine how and where ethanol acts. To address these issues, our laboratory has developed and used increased atmospheric pressure (hyperbaric exposure) as a mechanistic ethanol antagonist. These behavioral and cellular studies indicate that increased atmospheric pressure is a novel ethanol antagonist that can be used to help identify molecular targets for ethanol in ligand-gated ion channels (LGICs) (1,2). Recent evidence suggests that position 52 in Loop 2 of the extracellular domain of α1 WT glycine receptors (GlyRs) is a site of action for ethanol and pressure antagonism of ethanol (3). The current study tests the hypothesis that the polarity at this position (A52) or its homologous position in α2WT GlyRs (T59) is a key determinant of ethanol action and pressure antagonism of ethanol.

These positions were mutated to investigate the roles of molecular volume, weight, polarity and hydrophobicity:(1) α1A52(small, non-polar), α1A52S(small, polar), α1A52T(intermediate, polar), α1A52C(intermediate, intermediate-polar), α1A52F(bulky, non-polar) and α1A52Y(bulky, polar); (2) α2T59(intermediate, polar) and α2T59A(small, non-polar). GlyRs expressed in *Xenopus* oocytes were tested using glycine EC_2 ± EtOH(25-200mM) under 1 and 12ATA conditions using two-electrode voltage clamp (-70mV).

EtOH sensitivity was significantly reduced in GlyRs with polar residues at position 52 or 59 compared to those with non-polar substitutions at these positions. Pressure antagonized the effects of EtOH in GlyRs with non-polar residues at position 52 or 59, but not in GlyRs with polar substitutions at these positions. Correlation analyses confirmed that the polarity and hydrophobicity of the residue at positions 52 and 59 in α1 and α2GlyRs, but not molecular volume or weight, determines sensitivity to EtOH and to pressure antagonism of EtOH (r^2s>0.85).

These findings support the hypothesis that physical-chemical properties of the residue in the extracellular domain at positions 52 and 59 in α1 and α2GlyRs, respectively, can determine sensitivity to ethanol and pressure antagonism of ethanol. Taken together, the findings indicate that pressure can be used in conjunction with molecular manipulations to identify structural-functional sites of ethanol action within GlyRs and possibly other LGICs that can be targeted for the development of pharmacotherapeutic agents (Support: NIAAA/NIH AA013890(DLD), 013922(DLD), 013378 (JRT), 03972(RLA), USC School of Pharmacy

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S6

INDIVIDUAL DIFFERENCES OF OXIDATIVE METABOLISM OF MEDICINES AND THEIR CLINICAL SIGNIFICANCE

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Voriconazole ((2*R*, 3*S*)-2-(2, 4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1, 2, 4-triazol-1-yl)-2-butanol) is a novel azole antifungal agent metabolized mainly by cytochrome P450 (P450 or CYP) 2C19 into voriconazole *N*-oxide (1). To confirm the precise roles of P450 isoforms in voriconazole clearance in individuals, we studied the oxidative metabolism of voriconazole catalyzed by human liver microsomes genotyped for the *CYP2C19* gene and therapeutic monitoring in patients after voriconazole treatments. Recombinant and liver microsomal CYP3A4 produced a new methyl hydroxylated metabolite from voriconazole, detected by LC/UV and LC/MS/MS and confirmed by ^{1}H- and ^{13}C-NMR analyses (2). The voriconazole 4-hydroxylation to *N*-oxidation metabolic ratios in liver microsomes from the wild-type *CYP2C19*1/*1* individuals (0.07) were lower than those observed in other genotypes (0.20-0.27) at a substrate concentration of 25 μM based on the reported clinical plasma level. The primary oxidative metabolism of voriconazole was efficiently catalyzed by polymorphic CYP2C19 and abundant CYP3A4 to form *N*-oxide of voriconazole in human liver microsomes.

In order to confirm these findings and to further understand voriconazole metabolism in human, we analyzed 171 serum samples in 62 predominantly Caucasian patients (range 1-17 samples/patient) treated with voriconazole between 2005 and 2007 in NIH. There was no difference between median voriconazole and median *N*-oxide voriconazole serum levels in patients heterozygous for *CYP2C19* mutations and the wild type ($p > 0.05$). There was a better correlation of serum *N*-oxide of voriconazole levels and voriconazole dose ($r = 0.26$, $p < 0.01$) than voriconazole levels and voriconazole dose ($r = 0.14$, $p < 0.05$). There was an inverse correlation of the voriconazole metabolic ratio (*N*-oxide of voriconazole/voriconazole) and voriconazole dose adjusted for weight, implying saturation of voriconazole metabolism in higher doses ($r = 0.48$, $p < 0.001$).

In conclusion, the present study suggested that voriconazole was efficiently catalyzed by polymorphic CYP2C19 and abundant CYP3A4 to form *N*-oxide of voriconazole and that CYP3A could also mediate the methyl hydroxylation of voriconazole to yield the polar metabolite (4-hydroxyvoriconazole) more than *N*-oxide of voriconazole. The effect of *CYP2C19* genotype on the levels of voriconazole and *N*-oxide of voriconazole was modest and probably not clinically relevant in our Caucasian population, while voriconazole levels did not correlate well with the given dose. This information is worth understanding voriconazole disposition extensively metabolized in humans.

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S7

CHOLESTEROL METABOILSM IN THE CENTRAL NERVOUS SYSTEM AND ALZHEIMER'S DISEASE

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Because the cholesterol metabolism in the central nervous system (CNS) is segregated from the systemic circulation by the blood-brain barrier, the profile of the lipid transport system in CNS is quite different from that in the systemic circulation. The lipoprotein found in CNS is the high-density lipoprotein (HDL)-like particle, and apolipoprotein E (ApoE) is one of the major apolipoproteins regulating cholesterol transport in CNS by generating HDL from astrocytes and supplying HDL-cholesterol to neurons. In addition, ApoE4, one of the isoform of ApoE, is shown to be a strong risk factor for the development of Alzheimer's disease; however how ApoE4 is involved in AD pathogenesis remains undetermined. In this symposium, the mechanisms underlying ApoE-isoform dependent regulation of cholesterol metabolism will be presented. We found that cholesterol release to generate HDL induced by lipid-free ApoE is modulated by two major factors: the presence or absence of inter-molecular dimer formation and the intra-molecular domain interaction.

The role of cholesterol in the amyloid cascade, which is widely accepted idea to explain pathogenesis of Alzheimer's disease, will be also presented. We found that cholesterol plays a key role promoting tauopathy including enhanced tau phosphorylation, impaired neurite outgrowth and synaptogenesis. We also found that oligomeric Aβ disrupts cholesterol metabolism in neurons, leading to tauopathy. ApoE released from astrocytes generates HDL and supply HDL-cholesterol to neurons to maintain cholesterol homeostasis. However, we found that the ability of ApoE to generate HDL is isoform-dependent, that is, ApoE3 has greater ability to generate HDL than ApoE4. Thus the weaker ability of ApoE4 to maintain cholesterol homeostasis than ApoE3 may result in altered cholesterol metabolism in neurons leading to earlier development of Alzheimer's disease (tauopathy).

S8

ROLE OF ABC TRANSPORTERS IN DRUG ABSORPTION AND ELIMINATION

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Drug disposition determines a pharmacological/toxicological drug response. Cumulative studies have shown that ABC transporters, such P-glycoprotein, Multi drug resistance associated proteins (MRPs) and BCRP, act as detoxification systems in the body. The MRP family consists of 9 isoforms. Among this family, MRP2 is a well-known transporter playing an important role in the biliary excretion of amphipathic organic anions including glutathione- and glucuronide conjugates. Recently, we and other groups have found that MRP3 and MRP4 are also involved in drug disposition. MRP3 is abundantly expressed in the basolateral membrane of the liver and intestine. It was found that the liver of *Mrp3*$^{-/-}$ mice accumulates glucuronide conjugates, resulting in a marked decrease in their plasma concentrations[1)]. We found that the plasma concentrations of methotrexate (MTX) achieved at steady-state were 2-fold lower in *Mrp3*$^{-/-}$ mice than that in wild-type mice although the liver concentration and biliary excretion rate were unchanged. The hepatic uptake and biliary excretion across the bile canalicular membrane were unchanged between wild-type and *Mrp3*$^{-/-}$ mice. Therefore, the increase in the elimination rate from the systemic circulation can be ascribed to a reduction in the sinusoidal efflux clearance. The reduced bioavailability of MTX is accounted for not only by reduced hepatic availability, but also by a reduction in the fraction absorbed and/or intestinal availability. Saturable mucosal-to-serosal transport of MTX was observed only in the everted intestinal sacs from the duodenum along the intestine. In the everted sacs from the duodenum of *Mrp3*$^{-/-}$ mice there was a significant reduction, indicating that Mrp3 is involved in the basolateral efflux of MTX in the duodenum. Mrp4 substrates were given by constant infusion. MRP4 is abundantly expressed in the brush border membrane of the kidney and, to lesser degree, in the sinusoidal membrane of the liver. In vitro transport studies using membrane vesicles overexpressing MRP4 identified diuretics (hydrochlorothiazide and furosemide)[2)], acyclic nucleotides (adefovir, tenofovir)[3)], and cephalosporins (ceftizoxime and cefazolin)[4)] as new MRP4 substrates. These drugs were given by constant intravenous infusion, and the plasma, kidney and urine concentrations were determined. It was found that functional impairment of Mrp4 causes drug accumulation, suggesting that Mrp4 is involved in the tubular secretion of drugs[2-4)]. In conclusion, Mrp3 mediates sinusoidal efflux in the liver, and basolateral efflux in the intestine in conjunction with uptake transporters, and Mrp4 mediates the urinary excretion of drugs.

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S9

BLOOD-RETINAL BARRIER TRANSPORTERS: ROLE IN RETINAL DRUG DELIVERY

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Retinal diseases (e.g., age-related macular degeneration, diabetic retinopathy, glaucoma, and retinitis pigmentosa) accompany severe vision loss and pose a serious socioeconomic burden. The treatment of these retinal diseases is challenging, since the ocular barriers that effectively protect the eye from foreign materials also hinders efficient absorption of pharmaceuticals. The inner blood-retinal barrier (inner BRB) forms complex tight junctions of retinal capillary endothelial cells to prevent the free diffusion of substances between the circulating blood and the neural retina.[1] Thus, understanding of the inner BRB transport mechanisms could provide a basis for strategies of drug delivery to the retina.

The information of inner BRB transport is very limited due in part to the difficulty in precise determination of inner BRB transport properties. We have employed the integration plot, Retinal Uptake Index (RUI), and microdialysis methods[2] to evaluate the *in vivo* contribution of the inner BRB influx and efflux transport. Conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB) established from transgenic rats harboring the temperature-sensitive simian virus 40 large T-antigen gene were also employed to clarify the molecular mechanism of the inner BRB transport *in vitro*.[3] In addition, for the purpose of measuring reliably the expression levels of various transporter genes in the inner BRB *in vivo*, we have used magnetic beads coated with anti-rat CD31 antibodies to isolate rat retinal vascular endothelial cells and quantify transporter gene levels in the inner BRB using real-time quantitative PCR analysis as an *ex vivo* experiment.[4]

Using a combination of newly developed and other conventional methods, we have elucidated various mechanisms as to how vitamins, amino acids, creatine, nuclosides, and related drugs are supplied to the retina and how organic anions are effluxed from the retina. We have identified the responsible transporters. GLUT1, SR-BI, CRT, xCT, LAT1, TauT, ENT2, Oatp1a4, and mdr1a are involved in the inner BRB influx and efflux transport of these compounds. Mrp4, 6 and ABCA9 are also predominantly expressed at the inner BRB.[5]

Identification and characterization of all the transporters at the inner BRB will provide a basis for more successful strategies for drug delivery to the retina.

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S10

NONVIRAL GENE DELIVERY BASED ON PHYSICAL METHODS IN COMBINATION WITH NAKED PLASMID DNA

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Plasmid DNA (pDNA) is an attractive nonviral vector in gene therapy in terms of safety, productivity and versatility. Development of highly efficient gene delivery technologies, including hydrodynamic delivery and electroporation-guided gene transfer methods has almost solved the problem of the low level of transgene expression of nonviral vectors. We have been working on in vivo gene delivery based on the physical methods in combination with naked pDNA for optimization of in vivo gene therapy and DNA vaccination for cancer.

Systemic Interferon Gene Delivery with Naked pDNA

We examined the therapeutic effect of interferon (IFN)-α and IFN-α gene transfer by iv injection of pDNA by the hydrodynamics-based procedure[1]. A significant amount of IFNs was observed in the liver and blood circulation following gene expression. In the liver metastasis experiment, IFN-expressing pDNA showed a profound reduction of liver metastasis and a prolonged survival[2]. In order to prolong the duration of gene expression, we designed CpG-reduced pDNA encoding IFNs, in which about 80 % of the CpG sequences were depleted. Hydrodynamic delivery of CpG-reduced vectors resulted in more sustained production of IFNs and a better therapeutic effect against the lung metastasis[3].

Local Delivery of siRNA-expressing Vectors with Naked pDNA

RNA interference (RNAi) is a post-transcriptional gene silencing event in which short double-stranded RNA (siRNA) degrades target mRNA in a sequence-specific manner. Silencing oncogenes or other genes contributing to tumor cell malignancy or progression by delivery of siRNA-expressing vector offers a therapeutic treatment for cancer. We optimized the condition of local delivery of siRNA-expressing naked pDNA combined with electroporation[4,5]. We selected β-catenin and hypoxia-inducible factor 1α (HIF1α as the target gene involved in tumor growth. Intratumoral delivery of the siRNA-expressing pDNA targeting to these target lead to a significant inhibition of the tumor growth. Systemic delivery of HIF1α-targeted vector by the hydrodynamic method also exhibited a significant effect[6].

Genetic Vaccination Using HSP70-based pDNA in Naked Form

We designed DNA vaccines using heat shock protein 70 (Hsp70), which can present a broad repertoire of tumor antigens to antigen presenting cells (APCs) and elicit innate immunity. The pDNA expressing the secretable Hsp70-model antigen peptide fusion proteins were used to induce the peptide-specific immune responses. Further incorporation of poly-histidine molecules for enhancing cytosolic delivery following cellular uptake or cell-penetrating peptide (CPP) for intercellular delivery of the expressed fusion protein was examined. Our results demonstrated that these DNA vaccine systems in naked form combined with electroporation could be useful for cancer immunotherapy[7].

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S11

EFFECT OF PARTICLE SIZE OF DRY POWDER MANNITOL ON THE LUNG DEPOSITION IN HEALTHY SUBJECTS

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Particle size is an important determinant of in vitro aerosol performance for dry powder inhalation aerosols. It has been shown that as the particle size of powders is decreased, the fine particle fraction (defined as % mass of particles < 5 µm in the aerosol) is increased (Chew and Chan, 1999; Chew et. el., 2000). However, there is a lack of *in vivo* studies focusing on the effect of particle size of dry powder aerosols on lung deposition and distribution.

We investigated the dose and distribution of radiolabelled powder aerosols of mannitol in the lungs using single photon emission tomography (SPECT). Three different sized radiolabelled powders were produced by co-spray drying mannitol with 99mTc-DTPA. The primary particle size distribution of the powders measured by laser diffraction showed a volume median diameter of 2, 3, and 4 µm with span 2.3, 2.0 and 2.1, respectively, which corresponded to an aerodynamic diameter of 2.7, 3.6, 5.4 µm and geometric standard deviation of 2.6, 2.4 and 2.7 when the powders were dispersed using an Aeroliser® dry powder inhaler. Three capsules each containing approximately 20 mg (i.e. a total of 60 mg containing 60–90 MBq) of each of the radiolabelled powders were inhaled by eight healthy volunteers using the Aeroliser® inhaler. Images of aerosol deposition in the lungs were acquired using fast, multi-bed position SPECT.

The results showed that the lung dose markedly decreased with increasing aerosol particle size (mean ± S.E.M.: 44.8 ± 2.4, 38.9 ± 0.9, 20.6 ± 1.6% for 2.7, 3.6, 5.4 µm, respectively, $p < 0.0001$). The sites of deposition of the 2.7 and 3.6 µm aerosols were similar (dose ratio of peripheral to central lung, or penetration index, PI = 0.63 ± 0.05, 0.60 ± 0.03, respectively, $p > 0.3$), but different to the 5.4 µm aerosols (PI = 0.52 ± 0.04, $p < 0.02$). The lung dose followed the *in vitro* powder dispersion performance, with the % lung dose being related to fine particle fraction by a slope of 0.8.

The SPECT results provide direct evidence that the lung deposition of dry powder aerosols strongly depends on the particle size. The lung dose of the 2.7 and 3.6 µm aerosols using the Aeroliser® was double compared to that of the 5.4 µm aerosols and the deposition of the smaller particles was more peripheral.

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S12

UNDERSTANDING AND ENFORCING DRUG AEROSOLISATION FROM DRY POWDER INHALATION MEDICINES: MEASUREMENT AND MODIFICATION OF PARTICULATE SYSTEMS TO IMPROVE EFFICIENCY

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Dry powder drug particulates are either agglomerated with similar sized powder or blended with larger inert carrier material to form ordered mixes. Although this approach overcomes issues with powder handling, aiding metering and flow, the drug particulates are still highly cohesive and adhesive. This results in the performance of many marketed products being less than 20% [1]. The key to successful delivery of such systems is therefore to understand the inter-particulate mechanisms and relative sheer forces that are present between the formulation during storage and the aerosolisation process.

For example it is commonly believed that during the dynamic process of mixing, drug particulates will become preferentially adhered to areas containing a higher activity, or regions coined “active sites” [2, 3]. Such active sites maybe due to a combination of physico-chemical factors including morphology (peaks and troughs) and surface chemistry (face specific crystallinity and amorphous content). Here, the author investigates the variation in surface physicochemical properties and probes there effect on ultimate aerosolisation performance.

Specifically, the presentation focuses on recent advances in technologies that may be improve the understanding and the impact of physico-chemical carrier properties on drug aerosolisation efficiency (namely surface energy, adhesion measurement and phase imaging). In addition, we investigate current strategies for manipulating performance in such systems. For example, model drug (salbutamol sulphate or bovine serum albumin) and carrier systems (lactose monohydrate) were used via conventional formulation approaches (e.g. micronisation or sieve fractionation) or after particle engineering (e.g. controlled corrugation through spray drying or crystal growth and etching). Variations between particle engineered systems and conventional formulations were compared to gain a greater incite into the formulation variables that affected performance.

This study highlights the importance for high-tech approaches in both the measurement and preparation of drugs for inhalation and accentuates the challengers that must be overcome to ensure better therapeutic outcomes in future medicines

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S13

INHALATION DRUG DELIVERY III: PREDICTING ADHESION AND COHESION IN NON-AQUEOUS PROPELLANT SYSTEMS

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Since it was originally developed in the 1950s, the pressurized metered dose inhaler (pMDI) has become popular with both patients and clinicians. With worldwide production exceeding 500 million devices annually[1], and different manufacturers launching ever more sophisticated devices, pMDIs are now one of the most prescribed dosage forms used in the treatment of asthma[2]. Furthermore, due to the poor solvent properties of hydrofluoroalkane (HFA) propellants, most currently marketed HFA-134a and HFA-227 products are suspension formulations[3]. After the Montreal Protocol in 1996, all aspects of pMDI reformulation, from the classical chlorofluorocarbons to the novel hydrofluoroalkane (HFAs) propellants have been investigated. Interestingly, although many areas have been studied, little research has focussed on particle-particle and/or particle-device interactions, an area that has critical interconnections within pMDI systems.

In the early 1990's, pMDI formulators attempting to replace chlorofluorocarbons (CFCs) propellants with alternatives were beginning to report problems with drug aggregation (cohesion) and drug loss onto canister walls (adhesion), as a function of time[4]. It is interesting to note however, that such observations were generally empirical in nature and, at best, were only studied in a semi-quantitative manner. Furthermore, with these early observations, and the logic that precludes stable suspension of micronised particulates, it became clear that there was considerable risk for irreversible cohesion of drug material and/or adhesion to the internal canister walls; consequently, an increase in the force of cohesion and adhesion may result in a decrease in emitted dose[5] and aerosolisation performance. The aim of this review is to challenge current theory and literature, setting the basis for formulation and canister selection based on an informed, rather than empirical approach, endeavouring to remove some of the apparent 'claims of magic' in the patent literature. It is likely that the future will see a development in the way suspension pMDIs are formulated: moving beyond the empirical stage, surface science/engineering will become an integral tool for formulating and answering these and many other fundamental cross-disciplinary questions**.**

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S14

SEA-URCHIN-LIKE PARTICLES FOR INHALATION DRUG DELIVERY TO LUNGS

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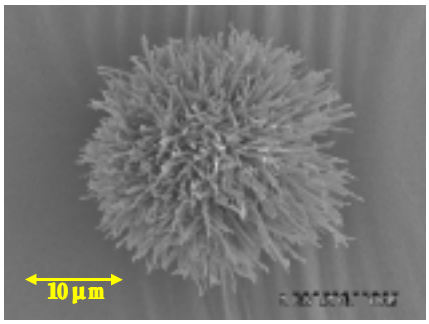
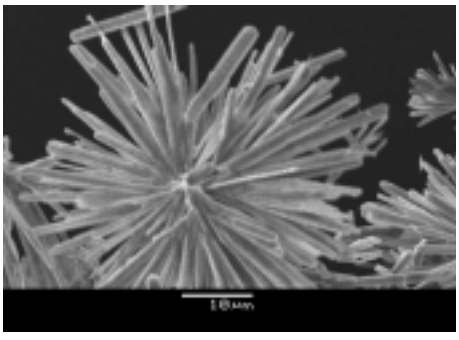
Recently, precipitation of powders with supercritical carbon dioxide (SCF) has been attracting much attention as a method to produce tiny particles with high functionality. It has been generally recognized that large porous particles are preferable to small non-porous particles with the same aerodynamic diameter for pulmonary drug delivery (1, 2). In the present study, we developed unique sea-urchin-like particles of lactose and sulbutamol sulfate by using a SCF system with a V-shaped nozzle. We optimized the operating conditions to obtain the sea-urchin-like particles and evaluated their performance.

Particle design of lactose, a model hydrophilic drug, was performed by supercritical carbon dioxide process with a V-shaped nozzle. An aqueous solution of lactose was dispersed from one end of the V-shaped nozzle in a carbon dioxide (CO_2)/ethanol (EtOH) admixture flowing from the other end of the nozzle. The influences of operating conditions to particle size and morphology of precipitants were examined. In the result, the effect of temperature and pressure was not obvious, while the flow rates of CO_2 and EtOH affected particle size and morphology of the precipitants drastically. When the ratio of the flow rates of CO_2 and EtOH (CO_2/EtOH) flow rate ratio was fixed, particle size and morphology of the precipitants were not influenced by the flow rate of CO_2 or EtOH, suggesting that the CO_2/EtOH flow rate ratio was the essential factor to determine the particle size and morphology. The unique shaped precipitant like “sea-urchin” was obtained when CO_2/EtOH flow rate ratio was 4 in this study. The precipitant was very feathery, so it will be widely applied to pharmaceutical use such as DPI.

The same operating conditions were successfully applied to sulbutamol sulfate (SS) to prepare sea-urchin-like particles. The fine particle fraction of SS powder was maximized when a 10% SS solution was introduced into the SCF system. Thus the sea-urchin-like particles are promising inhalation for better drug delivery to lungs.



Lactose



Sulbutamol Sulfate

Acknowledgement
The authors acknowledge Mr. Takeaki Furudate, Dr. Tetsuo Yamaguchi, and Mr. Shusei Ito for their contribution in this study.

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Poster Abstracts

P1

RECENT APPLICATION OF TRIMETHYLSILYLDIAZOMETHANE TO THE SYNTHESIS OF HETEROCYCLES

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Trimethylsilyldiazomethane ($TMSCHN_2$, Me_3SiCHN_2) is a stable, safe and easy-handled reagent and can be used not only as substitute for dangerous diazomethane but also for various types of organic reactions by the use as its lithium or magnesium bromide salts ($TMSC(Li)N_2$ and $TMSC(MgBr)N_2$). Minutely, $TMSCHN_2$ and its metallo derivatives can work as C1–unit and [C–N–N]azole–unit introducers, and an alkylidenecarbene generator. One of our research interests is to extend the usefulness of $TMSCHN_2$ in organic synthesis in light of the development of environmentally benign synthetic reactions. In the poster session, our recent results on the synthesis of heterocycles using $TMSCHN_2$ and its metallo derivatives will be presented as shown below.

1) 2-Azaazulene synthesis by using $TMSCHN_2$

Previously, the one-pot synthetic method of furans (or pyridones) from $TMSCHN_2$, acyl isocyanates and propiolates (or *N*-phenylmaleimide) was established.[1,2] On the basis of the results, the reaction of $TMSCHN_2$ with thiobenzoyl isocyanates was investigated. Interestingly, the reaction proceeded in the different mode from that with acyl isocyanates to afford unexpected diazoketones, which could be converted into 2-azaazulenes by the intramolecular Buchner reaction.[3]

2) Indole synthesis by using $TMSC(Li)N_2$

Reactions of $TMSC(Li)N_2$ with *N*-tosyl and *N*-pivaloyl *o*-acylanilines were examined. The former preferentially gave 3-substituted indoles, the intramolecular N-Li insertion products, *via* alkylidenecarbene intermediates generated from $TMSC(Li)N_2$ and *o*-acylanilines;[4] on the contrary, in the latter case, the rearrangement reaction of alkylidenecarbene intermediates predominantly proceeded to afford *o*-alkynylanilines, intermediates for the synthesis of 2-substituted indoles.[5] These results suggest that the reaction mode of alkylidenecarbene intermediates can be controlled by the sort of *N*-protecting group of *o*-acylanilines. Furthermore, the one-pot synthesis of 2,3-disubstituted indoles was achieved by application of the 2-substituted indole synthesis described above.[6]

3) Pyrazole synthesis by using $TMSC(MgBr)N_2$

It was found that $TMSC(MgBr)N_2$ smoothly reacted with carbonyl compounds to quantitatively give 2-diazo-2-(trimethylsilyl)ethanols. For the synthesis of multi-substituted pyrazoles, the [3+2]cycloaddition of 2-diazo-2-(trimethylsilyl)ethanols with propiolates was examined. As results, the two-step synthetic method of multi-substituted pyrazoles from carbonyl compounds by using $TMSC(MgBr)N_2$ was developed.[7]

$TMSCHN_2$ would be a very practical reagent for various organic syntheses because it has unique reactivity and is commercially available. Therefore, to provide new synthetic approach using $TMSCHN_2$ will greatly contribute to the finding of new bioactive compounds for drug discovery.

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P2

RHODIUM-CATALYZED SYNTHESIS OF INDOLE COMPOUNDS FROM *N*-PROPARGYL ANILINES OR THE RELATED COMPOUNDS

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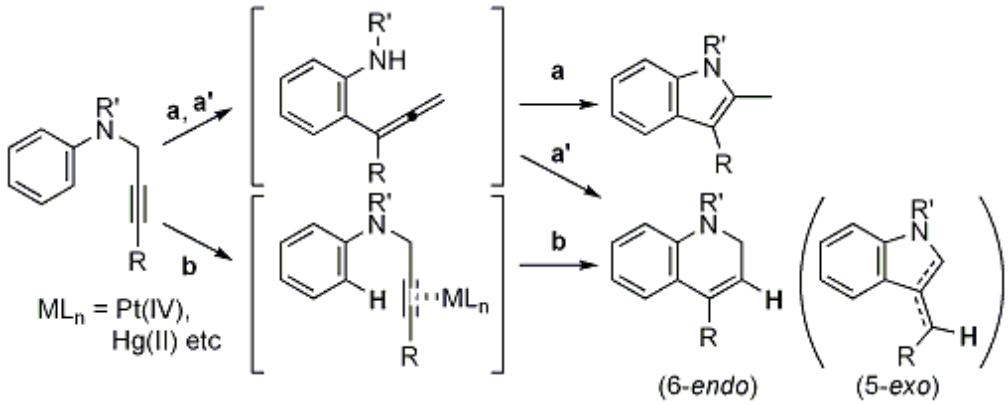
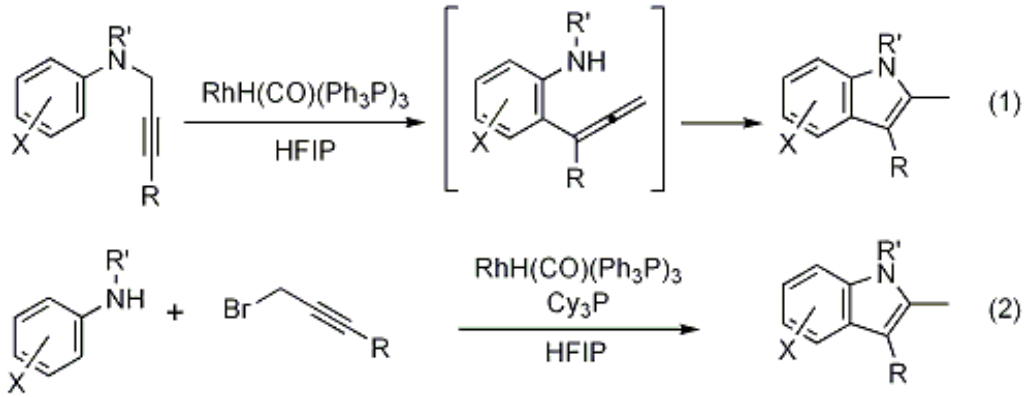
The indole nucleus is present among a wide range of biologically active substances and pharmaceutical agents, and the synthesis of the important structure has been a steady topic of interest for many years. While a number of methods have already been well documented for the construction of the nucleus, few catalytic procedures has been established for the construction of 2,3-disubstituted indoles. Regioselective annulation of *N*-propargyl aniline derivatives is a divergent method for preparing indole or quinoline nucleus. Transition metal-catalyzed reaction of *N*-propargyl anilines have been reported to bring about quinoline nucleus through intramolecular hydroarylation (path **b**)[1] or amino-Claisen rearrangement (path **a'**).[2] We present herein mild and facile synthesis of 2,3-disubstituted indole compounds from *N*-propargyl aniline derivatives catalyzed by cationic Rh(I) catalyst (path **a**).[3]



The Rhodium catalyst, $RhH(CO)(Ph_3P)_3$, in HFIP (hexafluoroisopropanol) efficiently catalyzes the formation of indole compounds via amino-Claisen rearrangement of *N*-propargyl anilines (eq 1). In addition, a mixture of anilines and 1-bromo-1-butyne was converted into indoles in one-pot by the Rh-catalyst (eq 2).



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P3

DISCOVERY OF HISTONE DEACETYLASE 6-SELECTIVE INHIBITORS AND THEIR EFFECT ON HUMAN CANCER CELLS

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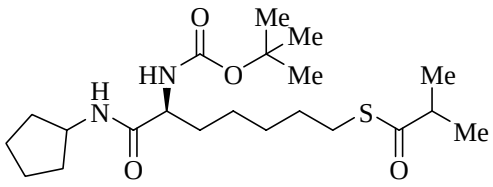
Histone deacetylases (HDACs) catalyze the deacetylation of the acetylated lysine residues of histone and are involved in various fundamental life process such as gene expression and cell cycle progression. Thus far, eighteen HDAC family members have been identified and some of the HDAC isoforms have been reported to play important roles in cell functions and be associated with the proliferation of cancer cells. HDAC6, one of HDAC family members, is unique in that it deacetylates non-histone proteins such as α-tubulin, HSP90 and cortactin, and is involved in microtubule stabilization, molecular chaperon activity and cell motility. Furthermore, recent studies have revealed that HDAC6 is associated with several disease states. Therefore, HDAC6-selective inhibitors are of great interest not only as tools for probing the biological functions of the isoform, but also as therapeutic agents having few side effects. In order to uncover novel histone deacetylase 6 (HDAC6)-selective inhibitors and to elucidate the structural requirements for their inhibitory activity, we designed and prepared a series of thiolate analogues based on the structure of an HDAC6-selective substrate and evaluated their properties by Western blotting and enzyme assays. Several thiolate analogues were found to be potent and selective HDAC6 inhibitors. Study of the structure-selectivity relationship revealed that the presence of a bulky alkyl group and *tert*-butylcarbamate group in these compounds is important for HDAC6-selective inhibition.[1,2] Compounds **1** and **2**, the most selective and active compounds in this series, exerted a synergistic inhibition of cancer cell growth in combination with paclitaxel.[2] They also blocked the growth of estrogen receptor α-positive breast cancer MCF-7 cells that had been treated with estrogen.[2] These results and detailed studies will be presented.



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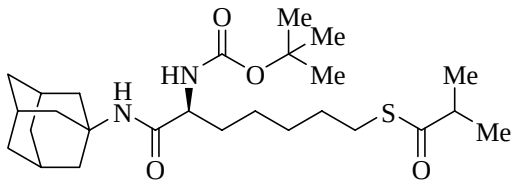

2

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P4

PYRAZOLONE DERIVATIVES WITH POTENT ANTI-PRION ACTIVITY: THEIR STRUCTURE-ACTIVITY RELATIONSHIP

Ayako Kimata,[a] Hidehiko Nakagawa,[a] Ryo Ohyama,[a] Tomoko Fukuuchi, [b] Shigeru Ohta, [c] Katsumi Doh-ura,[d] Takayoshi Suzuki,[a] Naoki Miyata[a]

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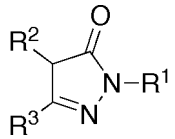
The prion disease or transmissible spongiform encephalopathy (TSE), such as Creutzfeldt-Jakob disease (CDJ), Gerstmann-Sträussler-Scheinker disease (GSS) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle, are known to be invariably fatal neurodegenerative disease. This disease is characterized by the deposition of protease-resistant isoform of prion protein (PrP^{Sc}). It is considered to be the main component responsible for the pathogenesis. PrP^{Sc} is known to be in the abnormally folded ß-rich conformation, which is converted from constitutive cellular prion protein (PrP^{C}). Therefore, compounds that inhibit conversion to or deposition of PrP^{Sc} would serve as anti-prion agents. We explored for the anti-prion activity of various pyrazolone compounds (Fig. 1), and found highly potent anti-prion compounds having IC_{50} values in nanomolar range for the formation of PrP^{Sc} in ScN2a and F3 cell lines,[1] whereas quinacrine, one of the known anti-prion agents, had IC_{50} value of 400 nM in the same assay system. Their anti-prion activity was neither directly correlated with antioxidant activity,[2] which is known as a typical biological activity of pyrazolone derivatives, nor with copper-chelating activity, which may affect the copper-containing prion proteins. Although the mechanisms of action of pyazolone derivatives are underway, we identified pyrazolone derivatives as a new series of potent anti-prion compounds.



1: R^1 = phenyl, R^2 = H, R^3 = 4-nitrophenyl (IC_{50} = 3 nM)

Fig. 1

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P5

NOVEL QUADRUPLE HYDROGEN-BONDING MODULE WITH FIVE-MEMBERED HETEROAROMATIC RING

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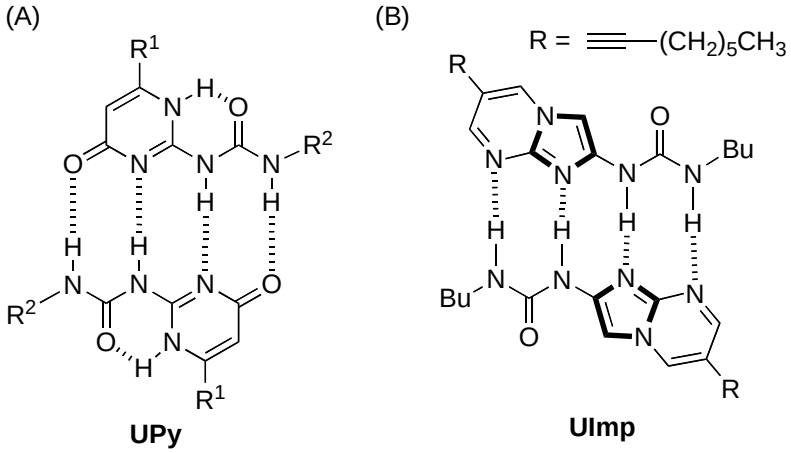
The development of quadruple hydrogen-bonding module with DDAA hydrogen-bonding array (D = hydrogen-bond donor; A = hydrogen-bond acceptor) has attracted considerable attention as the building blocks for the novel supramolecular structures and materials. Despite the considerable progress achieved in resent years,[2] DDAA module has been mainly restricted to the ureidopyrimidinone (UPy) reported by Meijer et al.[1] In the view of the range of properties required from the various supramolecular structures and materials, there is clearly a need for alternative strong quadruple hydrogen-bonding modules. In addition, the UPy modules can exist in three different tautomeric forms depending on the environment, which can increase the complexity of the assembling systems. Therefore, it has been important to develop the new DDAA modules, which is free from the tautomeric problem.

In poster presentation, we report the novel DDAA hydrogen-bonding module (UImp) with imidazo[1,2-a]pyrimidine ring as the five-membered heterocyclic ring.[3] The UImp module was synthesized in three steps, and capable of forming strong quadruple hydrogen-bonded dimer (K_{dimer} >1.1 x 10^5 M^{-1}) in $CDCl_3$.



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P6

SYNTHETIC STUDIES ON NEODYSIHERBAINE A

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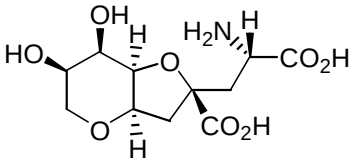
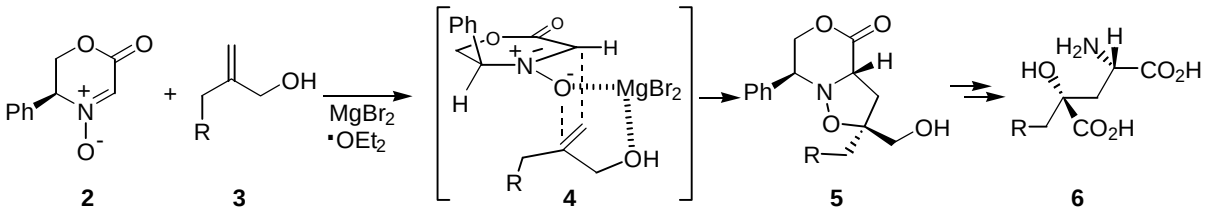
Neodysiherbaine A (**1**),[1] isolated from Micronesian Sponge *Dysidea herbasea* in 2001, is a selective agonist for non-NMDA type glutamate receptors, and hence **1** is anticipated to become a useful tool for investigation of the central nervous system. These biological properties and the low availability from natural sources make **1** an attractive synthetic target. Structurally, neodysiherbaine A (**1**) is characterized by its possession of 4-oxy-4-substituted glutamic acid moiety and poly-hydroxylated tetrahydropyran framework. We previously reported the synthetic method of 4-hydroxy 4-substituted glutamic acids based on 1,3-dipolar cycloaddition of nitrone **2** with allyl alcohol **3** in the presence of magnesium bromide.[2] This cycloaddition proceeds via chelated transition state **4** to give cycloadduct **5** having correct stereochemistry for the synthesis of **6**. Thus, synthesis of **1** may be possible by the use of cycloaddition of nitrone **2** with an allyl alcohol **3** carrying poly-hydroxylated tetrahydropyran moiety as R group derived from an appropriate sugar. We planed a synthetic route from D-mannose as the starting material.

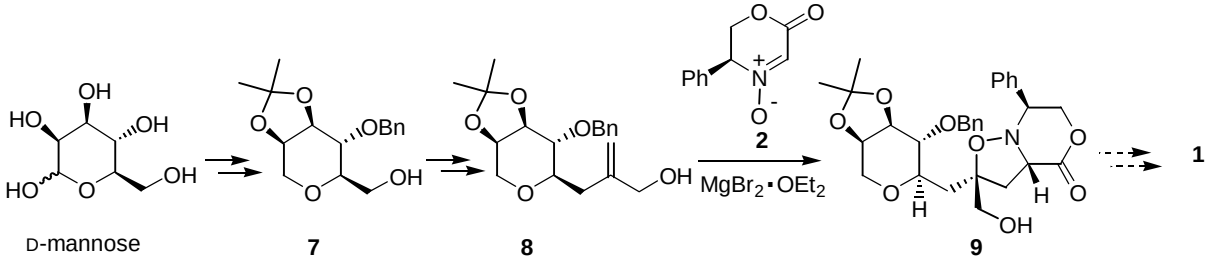


neodysiherbaine A (**1**)



2 **3** **4** **5** **6**

Alcohol **7** was prepared from D-mannose in six steps. Oxidation of **7** followed by Wittig reaction, hydrogenation, Mannich reaction, and reduction gave allyl alcohol **8**. With allyl alcohol **8** in hand, we next examined the crucial cycloaddition of nitrone **2** with ally alcohol **8**. Nitrone **2**, on treatment with allyl alcohol **8** in the presence of magnesium bromide at room temperature for five days, underwent stereoselective cycloaddition to afford cycloadduct **9** exclusively in 80% yield. Transformation of **9** toward neodysiherbaine A (**1**) including reductive cleavage of the *N-O* bond and construction of tetrahydrofuran ring by intramolecular S_N2 reaction is under intense investigation.



D-mannose **7** **8** **9**

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P7

COMPARISON OF SKIN AND SPLEEN AS INJECTION SITE OF VACCINES FOR INDUCTION OF IMMUNE RESPONSE

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Antigen immunogenicity is dependent not only on the physicochemical and immunological properties of antigens but on their encounter with antigen-presenting cells (APCs). Vaccination with antigens is often performed by intradermal injections because of easy accessibility for administration and the presence of Langerhans cells, the APCs in the epidermis. Upon recognition of antigens, Langerhans cells and dermal dendritic cells migrate to local lymph nodes where they present the antigens to T cells, which initiates a variety of immunological responses.[1] Because spleen is the largest organ in the lymphatic system and contains various types of immunocytes, including lymphocytes, macrophages, dendritic cells, it can be a promising target organ for vaccines as far as the presence of APCs is concerned. However, its applicability as a target site of vaccines has hardly been explored. Therefore, in this study we have selected the spleen and skin as injection site of DNA and protein vaccines and evaluated how the injection site influences the induction of antigen-specific immune responses.

Plasmid vectors expressing ovalbumin (OVA; pOVA) and SIINFEKL, a typical MHC class I-restricted epitope of OVA (pPep-ER), were used as DNA vaccines. OVA and its cationized derivative, HMD-OVA, were used as protein vaccines. C57BL/6 mice were injected with one of these vaccines into the dorsal skin or spleen twice with a week interval. Each pDNA injection was followed by electroporation. Seven days after the last immunization, the blood of mice was collected and the level of anti-OVA antibody was measured as an indicator of humoral immune response. Then, splenocytes were isolated and were restimulated in vitro for 5 days with mitomycin C-treated EG7 cells, a mouse lymphoma EL4 clone stably expressing OVA. Then ^{51}Cr release assay was performed to evaluate the antigen-specific cytotoxic T lymphocyte (CTL) activity. Separately, immunized mice were challenged with EG7 cells by an intradermal inoculation, and the tumor size and survival of EG7-bearing mice were recorded.

Although the spleen produced a larger amount of transgenes than skin after injection of pDNA followed by electroporation,[2] intradermal injection of pDNA induced more efficient immune response compared with injection into the spleen. To exclude the possible damages of electroporation, protein vaccines were used and immunological responses were evaluated. However, similar results to those with pDNA were obtained; intradermal injection of OVA or HMD-OVA produced more efficient immune response than its injection into the spleen, with an exception of the generation of anti-OVA antibody. These results indicate that spleen is unlikely to be a good site for immunization despite the presence of a large number of APCs.

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P8

MECHANISM OF mRNA DEADENYLATION: EVIDENCE FOR A MOLECULAR INTERPLAY BETWEEN TRANSLATION TERMINATION FACTOR eRF3 AND mRNA DEADENYLASES

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Control of mRNA decay is a fundamentally important step in determining the amount of protein produced from the mRNA by translation, and mRNA decay is intimately linked to and regulated by translation. In eukaryotes, decay of most mRNAs is initiated by shortening of the poly(A)-tail at the 3' end, referred to as deadenylation. Deadenylation is the first and rate-limiting step and also the most efficient step in controlling mRNA decay. Two major deadenylase complexes, Caf1-Ccr4 and Pan2-Pan3, play central roles in this process. However, the molecular mechanism triggering deadenylation remains elusive.

Previously, we showed that translation termination factor eRF3 interacts with poly(A) binding protein (PABPC1) through its N-domain.[1] And in yeast, eRF3 regulates the initiation of mRNA decay at the poly(A) tail shortening step through the interaction with PABPC1 in a manner coupled to translation termination.[2,3] Thus, the translation termination triggers mRNA decay.

In this study, we examined the mechanism of mRNA deadenylation and obtained evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases.[4,5] We demonstrate that eRF3-mediated deadenylation is catalyzed by both of the two major mRNA deadenylase complexes, Caf1-Ccr4 and Pan2-Pan3, in yeast and humans. Interestingly, translation termination complex eRF1-eRF3, Pan2-Pan3 and Caf1-Ccr4 competitively bind to the PABC domain of PABPC1. In each complex, eRF3, Pan3 and Tob, respectively, mediate PABPC1-binding. The PAM2 motifs found in both eRF3 and the two deadenylase complexes are responsible for their binding to PABPC1 and mRNA deadenylation. The termination complex and the deadenylase complex are exchanged on PABPC1 in a translation-dependent manner. Recruitment of the two deadenylase complexes to PABPC1 leads to the activation of both enzymes. From these results, we suggest a mechanism of mRNA deadenylation by Pan2-Pan3 and Caf1-Ccr4 in cooperation with eRF3 and PABPC1.

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P9

ANALYSIS OF PROTEINS INTERACTING WITH THE N-TERMINAL REGION OF NUCLEAR RECEPTOR FARNESOID X RECEPTOR (FXR)

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Nuclear receptors comprise a family of transcription factors and regulate gene expression involved in growth, development and homeostasis. Agonists or antagonists of nuclear receptors are currently used as drugs for cancer, type II diabetes mellitus and inflammatory diseases. Study of nuclear receptors promises to provide discoveries of therapeutic agents for various diseases. Based on structural and functional similarities, nuclear receptors are divided into several domains including the N-terminal domain (A/B), the DNA binding domain (C), the hinge region (D) and the C-terminal domain (E/F). Nuclear receptors have two regions for transactivation, a constitutive activation function (AF-1) in the A/B domain and a ligand-dependent activation function (AF-2) in the E/F domain. Further, the A/B domain as well as the E/F domain is required for the complete ligand-dependent transactivation. Generally, DNA-binding transcription activators require for coactivators, which function as a bridge between transcription factors and the basal transcriptional factors including RNA polymerase II. Although coactivators required for the AF-2 activity have been well studied, coactivators for the AF-1 activity are poorly understood.

To gain insights into AF-1 and to understand the molecular mechanism of the ligand-dependent transactivation, we isolated proteins associated with AF-1 by GST pull-down assay using the A-C domain of farnesoid X receptor (FXR) and nuclear extract from HeLa cells. By mass spectrometry analyses, these proteins identified as DNA-PK catalytic subunit (DNA-PKcs), Ku70 and Ku80. These factors compose heterotrimeric complex, called DNA-PK. DNA-PK is involved in multiple nuclear processes, such as DNA repair, telomere maintenance, DNA recombination and transcriptional regulation. We also showed that these proteins associated with the A-C domains of several other nuclear receptors. Next, we examined which domains of FXR are required for the interaction with these factors. As a result, the A/B domain of FXR interacted with DNA-PKcs, but not Ku70/Ku80. Moreover, the D-F domain of FXR as well as the A-C domain of FXR was interacted with these factors. We also found that Ku80 and Ku70 directly interacted with FXR *in vitro* by pull-down assay using bacterially expressed Ku80, Ku70 and FXR. Furthermore, immunoprecipitation assay revealed that DNA-PKcs, Ku80 and Ku70 interacted with FXR. Finally, we examined the effect of ectopic expression of Ku80 and Ku70 on the FXR-mediated promoter activity. Luciferase assay using reporter plasmid containing the FXR responsive promoter revealed that Ku80 and Ku70 up-regulate the FXR-mediated promoter activity. Taken together, these findings suggest that Ku80 and Ku70 function as coactivators for FXR.

P10

KIAA0319, A DYSLEXIA-ASSOCIATED GENE, INDUCES ABNORMAL BRANCHING OF NEURITES

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Dyslexia (Reading Disability; RD) is one of the congenital disorders characterized by a specific difficulty with learning to read, in spite of the normal intelligence.[1] Recent studies indicated that KIAA0319 gene is associated with the onset of RD by analysis in SNPs of RD families.[2] KIAA0319 encodes a putative membrane-spanning protein, and it is reported that KIAA0319 is involved in the regulation of neuronal migration in the developing brain.[3] Because KIAA0319 mRNA is highly expressed also in the adult brain, it may have other functions than the regulation of neuronal migration. We therefore examined the effect of KIAA0319 on neuronal cell shape.

Primary cultured hippocampal neurons were prepared from embryonic day 17 mouse. The neurons were transfected with expression plasmid for green fluorescent protein-fused KIAA0319 protein (full-length or a variety of deletion mutants). We found that neurons expressing full-length KIAA0319 protein formed complex neurites and fine branches by a few days after transfection. Similar results were obtained in neurons expressing a mutant of KIAA0319 protein which lacks the intracellular region. On the other hand, expression of a mutant of KIAA0319 protein which lacks the portion of the extracellular region had no effect on neuronal shape. Thus, it was suggested that KIAA0319 was involved in the regulation of neuronal shape by binding to certain extracellular molecule.

We next tried to identify the proteins that bind to the extracellular region of KIAA0319 protein. Postnatal day 8 mouse brain lysate was mixed with Protein G-Sepharose bound either to Fc region of Immunoglobulin G (Control Fc) or to Fc-fused the extracellular region of KIAA0319 protein. The proteins bound to the beads were eluted by the addition of SDS-PAGE loading buffer containing 2% SDS and 5% β-mercaptoethanol. As a result, we obtained the candidate ligand (approximately 105 kDa) which specifically binds to the extracellular region of KIAA0319 protein. We are currently trying to identify this protein by mass spectrometry.

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P11

P90 RSK-1 ASSOCIATES WITH AND INHIBITS NEURONAL NITRIC-OXIDE SYNTHASE[1]

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The p90 ribosomal S6 kinases (RSKs) are a family of serine-threonine kinases that lies at the terminus of the Ras/extracellular signal-regulated kinase 1/2 (ERK1/2)-mitogen-activated protein kinase cascade[2]. Nitric oxide (NO), generated by NO synthases (NOSs), is important for both physiological processes and pathological alteration in mammals[3]. For example, synthesis by neuronal NOS (nNOS) has been implicated in the pathogenesis of brain injury from hypoxia-ischemia[4].

Evidence is presented that RSK1 directly phosphorylates nNOS on Ser^{847} in response to mitogens. The phosphorylation thus increases greatly following epidermal growth factors (EGF) treatment of rat pituitary tumor GH3 cells and is reduced by exposure to the MEK inhibitor PD98059. Furthermore, it is significantly enhanced by expression of wild-type RSK1 and antagonized by kinase-inactive RSK1 or specific reduction of endogenous RSK1. EGF treatment of HEK293 cells, expressing RSK1 and nNOS, led to inhibition of NOS enzyme activity, associated with an increase in phosphorylation of nNOS at Ser^{847}, as also the case with in vitro assay. In addition, these phenomena were significantly blocked by treatment with the RSK inhibitor Ro31-8220. Cells expressing mutant nNOS (S847A) proved resistant to phosphorylation and decrease of NOS activity. Within minutes of adding EGF to transfected cells, RSK1 associated with nNOS and subsequently dissociated following more prolonged agonist stimulation. EGF-induced formation of nNOS/RSK1 complex was significantly reduced by PD98059 treatment. Treatment with EGF further revealed phosphorylation of nNOS on Ser^{847} in rat hippocampal neurons and cerebellar granule cells. This EGF-induced phosphorylation was partially blocked by PD98059 and Ro31-8220.

Together, these data provide substantial support that RSK1 associates with and phosphorylates nNOS on Ser^{847} following mitogens-stimulation and suggests a novel role for RSK1 in the regulation of NO function in brain.

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P12

MOLECULAR MECHANISMS FOR OXIDATIVE DAMAGE AND STRUCTURE CHANGE OF DNA THROUGH REDOX COPPER-CATECHOL COMPLEX FORMATION

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Some metals generate reactive oxygen species (ROS) through the reaction with several redox compounds. ROS oxidize biomolecules including DNA in the living system and interfere with their original functions. DNA damage induced by oxidative stress plays an important role in carcinogenesis as well as DNA adduct formation. Catechol, which is detected usually in our daily lives, for example in cigarette smoke and occasionally in foods, has been categorized as a group 2B carcinogen ('possibly carcinogenic to human') by the International Agency for Research on Cancer. This carcinogenicity is considered to be attributed to ROS generation by reacting with redox metals such as copper.

In order to understand underlying mechanisms of oxidative DNA damage, we investigated the interaction between Cu(II), catechol and DNA in the copper/catechol-mediated DNA damage by biochemical and spectrophotometric approaches. UV/Vis spectrophotometry showed that Cu(II) and catechol formed redox complexes producing H_2O_2 and Cu(I). Cu(II) inhibited the DNA cleavage by a certain restriction enzyme, BamHI (G|GATCC), not EcoRI (G|AATTC), indicating a sequence-specific binding of copper to GG. Therefore, the DNA damage by the copper-catechol complexes may undergo in the close proximity between the complexes and DNA. We found by CD analysis that DNA changed the conformation under the damaging conditions. Cu(I) was crucial in the conformation change as well as DNA damaging. Elucidating the characteristics of the DNA damaging complexes will provide a new clue to understanding molecular mechanisms of the oxidative DNA damage and to establishing a novel chemotherapy.

P13

FUNCTIONAL ANALYSIS OF A PSEUDOKINASE, TRB3 DURING THE M PHASE

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Cell cycle progression is strictly and correctly regulated by multiple molecules. Many of them regulated active or inactive states by the between phosphorylated and dephosphorylated status of the molecules related to cell cycle.

The CDC25 dual-specificity phosphatases (CDC25A, B and C) are key regulators of cell cycle progression through activation of cyclin-dependent kinases (CDKs) at G1/S and G2/M transition. Despite sharing a common biochemical mechanism of action, each CDC25 has a unique characteristic and a specific role in cell cycle regulation. In *Drosophila,* reinitiation of mitosis is regulated by expression of CDC25. There are two CDC25 homologs in *Drosophila*, *String* and *Twine*. It was reported in *Drosophila* that the degradation of String/CDC25 was induced by Tribbles via proteasome dependent pathway and delayed mitotic entry. Previously we identified TRB3 (tribbles-related protein 3) as one of the human orthologs of Tribbles, which induced by endoplasmic reticulum stress and contributed to cell growth inhibition. In this study we examined whether the cell cycle of mammalian cells is also regulated via CDC25 degradation or directly affected by TRB3.

We obtained three members of TRB family (TRB1, 2 and 3) interacted with CDC25A and regulated its expression. HeLa cells were synchronized at G1/S phase by double thymidine block and the expressions of endogenous CDC25A and TRBs were detected. TRB3 protein was expressed in a cell cycle dependent manner and its expression was inversely correlated to that of CDC25A. Knockdown of TRB3 expression caused the up-regulation of CDC25A expression and the delay of G2/M transition.

These results suggest that TRB3 contributes to down regulation of CDC25A expression and directly affect at mitosis progression. TRB3 would be a potential target of cancer and other diseases related to a cell cycle disorder.

P14

MEMBRANE FUSION LIPOSOMES CONTAINING SNARE PROTEINS INVOLVED IN EXOCYTOSIS OF MAST CELLS

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In mast cells, cross-linking of high-affinity receptor for IgE (FceRI) by multivalent antigen cause the activation of an intracellular signaling cascade and leads to the exocytotic release of granular contents (degranulation), resulting in allergic responses.[1] The molecular mechanism of exocytotic release has been studied intensively in neuronal cells, and some key proteins, such as SNARE proteins and some regulatory proteins which associate with SNARE proteins and regulate their conformation and activity, have been identified.[2] In mast cells, several groups including ours showed the involvement of SNARE proteins in exocytotic release, although different isoforms of SNARE proteins function in mast cells.[3,4] Recent studies have suggested that syntaxin-3, -4 and SNAP-23 are possible SNARE proteins on the plasma membrane responsible for exocytosis, and VAMP-7, -8 are possible candidates for SNARE on the granule membrane.

In the present study, we developed a SNARE-containing liposome fusion assay to determine which of the SNARE isoforms are involved in exocytotic membrane fusion in mast cells. We found that the combination of SNAP23/syntaxin-3/VAMP-8 showed the most efficient liposome-liposome fusion activity. SNAP23/syntaxin-4/VAMP-8 also had substantial fusogenic activity. These results also suggest that both syntaxin-3 and syntaxin-4 might contribute to degranulation of mast cell. In contrast, VAMP-7 containing liposomes had poor activity despite presence of isoforms of syntaxin. These results implicate that VAMP-7 might not contribute to degranulation of mast cell. In some kinds of secretory cells, membrane fusion is also observed between secretory granules. It is thought that granule-granule fusion allows granules far from the plasma membrane to secrete intragranular contents. We previously reported that syntaxin-3 resides not only on the plasma membrane but also on the secretory granules in mast cells. Thus, syntaxin-3 on the secretory granule might be involved not only in fusion with the plasma membrane but also in granule-granule fusion to facilitate exocytotic release.

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P15

ENHANCEMENT OF THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR EXPRESSION BY COMPOUNDS TO ELEVATE INTRACELLULAR cAMP LEVELS

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Thrombin-activable fibrinolysis inhibitor (TAFI) is a plasma zymogen that is activated by thrombin. [1,2] Activated TAFI prevents fibrin clot lysis by removing carboxyl-terminal lysine residues from partially degraded fibrin that mediate positive feedback in the fibrinolytic cascade. So it has been suggested that TAFI plays a crucial role in mediating the balance between coagulation and fibrinolysis. However, the molecular mechanism responsible for the regulation of TAFI gene expression remains to be fully elucidated. In the present study, we investigated the ability of forskolin and dibutylyl cyclic AMP (DBcAMP) that elevate intracellular cAMP level to modulate human TAFI gene expression in a cultured human hepatoma cell model.

Analysis of TAFI mRNA expression was performed by real-time polymerase chain reaction (RT-PCR). TAFI promoter region was isolated by PCR and reporter plasmid for TAFI promoter assay was constructed by using the luciferase reporter vector pGL3 Basic. TAFI antigen levels in culture medium were assayed sandwich-ELISA. Standard TAFI was isolated from normal human plasma.

Human hepatoma HepG2 cells stimulated with forskolin showed an increased release of TAFI antigen from the cells into conditioned medium. A similar enhancement in the level of the TAFI release was also observed upon exposure of the cells to DBcAMP. The induction of TAFI antigen levels by forskolin or DBcAMP was inhibited by KT5720, a cAMP-depndent protein kinase (protein kinase A) inhibitor. These TAFI antigen levels were correlated with intracellular TAFI mRNA abundance, suggesting that TAFI expression might be regulated through transcriptional and/or post-transcriptional levels. The possibility of transcriptional regulation was supported by the results that pretreatment of the cells with actinomycin D inhibited the expression of TAFI antigen levels. TAFI promoter activity measured by luciferase reporter plasmid was stimulated more than 2-fold by treatment with forskolin or DBcAMP, as compared to untreated control cells. Moreover, a half-life time of TAFI mRNA in the DBcAMP-treated cells prolonged to twice of that in the control cells, indicating that the post-transcriptional regulation also participated elevation of TAFI expression in the DBcAMP-treated cells.

Thus, it was suggested that the cAMP/protein kinase A signaling pathway plays an important roles in regulation of TAFI mRNA expression in human hepatoma cells, and enhancement of TAFI mRNA expression by activation of protein kinase A was regulated via both the activation of TAFI promoter and increased stability of the mRNA.

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P16

DISCOVERY OF NEW THERAPEUTIC TARGETS USING LIVER *PTEN* DELETION ANIMAL MODEL

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Hepatocelluar carcinoma (HCC) is the 5th most common cancer worldwide, with most of the cases occurring in Asia and Africa. The prognosis of HCC is extremely poor due to late diagnosis and lack of appropriate treatment. A lack of experimental models has significantly limited the research in the field of liver cancer.

We have developed an animal model that lacks the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) in the liver using the Cre-Lox system. PTEN negatively regulates growth factor signaling through PI3K/AKT pathway. Mutation or deletion of *PTEN* is associated with 30% of liver cancer and 80% of the metastatic liver cancer presents abnormal *PTEN* expression. The two phased development of liver cancer in our liver specific *Pten* deletion mice mimics human liver cancer phenotype and provides a unique model to study this often fatal disease. This animal model allows us to understand the mechanism of fatty liver transformation to liver cancer. We have shown that metabolic alterations by PTEN regulation in the liver led to fatty liver development. We showed that this metabolic development may be associated with a role of PTEN on mitochondrial function regulation, a process critical to energy balance in the cells. Cellular energy status is regulated by a fuel gauge sensor protein AMPK. When cellular energy levels are low, AMPK signals compete with cell growth signals (like those provided by *Pten* deletion) so that carbon sources can be used for ATP production rather than for cell growth. Our data indicated that PTEN may inhibit growth potential by regulating metabolism and mitochondrial biogenesis through AMPK, a global metabolic regulator and NRF-1 (nuclear respiratory factor-1), a mitochondrial biogenesis regulator. Understanding factors that promote the progression of fatty liver to liver cancer is an important step towards management of this malignancy.

P17

TYPE 1 Na^+-DEPENDENT PHOSPHATE TRANSPORTER (NPT1) MAKES A LIMITED CONTRIBUTION TO THE URINARY EXCRETION OF ORGANIC ANIONS.

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Many endogenous and xenobiotic anions are removed from the body through the active secretion *via* the renal organic anion transport systems. On the basolateral membrane of renal proximal tubular cells, OAT1 and OAT3 mediate the uptake of anionic compounds or drugs. On the apical membrane, unidentified carrier-mediated transport system is involved in the efflux of organic anions. From the recent studies[1, 2], Type 1 Na^+-dependent Phosphate transporter (NPT1) was expected to play an important role in this process. We investigated *in vivo* function and contribution of murine Npt1 to the renal secretion of anionic compounds with Npt1-knockout mice.

Npt1-knockout C57BL/6J mice were generated and used in this study. Disruption of Npt1 gene and protein were confirmed by genotyping PCR and western blotting analysis. To clarify *in vivo* function of Npt1, drug infusion study was performed. Prototypical organic anion, *p*-aminohippurate (PAH), or other anionic compounds were infused *via* jugular vein, and plasma concentration, urinary excretion rate and concentration in the kidney were determined. In addition to this infusion study, membrane vesicle transport study was examined. Mouse renal brush-border membrane vesicles (BBMVs) were purified from Npt1-knockout and wild-type mice[3] and we investigated the potential-sensitive transport of PAH in these vesicles.[4]

Protein expression level of Npt1 in the mouse brush-border membrane was more abundant in male than female. This means that Npt1 appear to be more contributive in male mice. In the infusion study of organic anions, however, there were no significant differences in the pharmacokinetics between Npt1-knockout and wild-type mice. Although *in vivo* contribution of Npt1 seems to be negligible, the potential-sensitive transport of PAH was significantly diminished in the BBMVs from Npt1-knockout mice, which was observed in the BBMVs from wild-type mice.

From our studies, Npt1 mediates the potential-sensitive transport of PAH, but accounts for limited *in vivo* contribution to the renal efflux of organic anions and it might be suggested that other transporter(s) play a major role in this elimination process.

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P18

DEVELOPMENT OF EFFICIENT GENE TRANSFER SYSTEMS INTO CACO-2 CELL MONOLAYER BY ADENOVIRUS VECTOR

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The Caco-2 cell monolayer model is widely used as a tool for evaluating human intestinal permeability. However, a number of drug-metabolizing enzymes are low expressed in the Caco-2 cell monolayer, compared with human intestinal epithelium. Development of efficient gene transfer systems into the Caco-2 cell monolayer appears to be crucial to the basic research of drug intestinal permeability. In contrast, recombinant adenovirus (Ad) type 5 vectors are potentially useful for gene transfer to a wide variety of cells and tissues both in vitro and in vivo. They can be grown easily to high titer, and can transfer the genes to both diving and non-dividing cells. Previously, Kesisoglou *et al.* reported that Caco-2 cell monolayer (differentiated Caco-2 cells) are resistant to transduction by Ad vector. [1] In the present study, we examined the infectious mechanism of Ad vector into Caco-2 cell monolayer, furthermore, we developed the simple method for efficient gene transfer into Caco-2 cell monolayer by Ad vector.

The primary receptor, the coxsackievirus and adenovirus receptor (CAR), and the secondary receptor, αv integrins and heparan sulfate glycosaminoglycans, are the tropism determinants of Ad type 5. First, we examined the expression levels of CAR on the Caco-2 cell monolayer by Western blotting analysis. The expression levels of CAR in Caco-2 cells were increased by Caco-2 cell differentiation levels-dependent manner. In contrast, the expression levels of transgene into Caco-2 cells by Ad vector were decreased by Caco-2 cell differentiation levels-dependent manner. Cohen *et al.* reported that CAR is a one of the component protein in the tight junction, and Ad vector is hardly accessible to CAR sequestered within tight junction in the epithelial cells. [2] Our data resulted in that expression of transgene into Caco-2 cell monolayer (differentiated Caco-2 cells), which is formed tight junction strand, is lower than undifferentiated Caco-2 cells. Then, we tried the modulation of tight junction with sodium caprate (C10), which can be open the intestinal epithelial tight junction, for efficient transduction of Ad vector to the Caco-2 cell monolayer. The co-transfection with Ad vector and C10 mediated higher transgene expression into the Caco-2 cells monolayer than Ad vector alone. Furthermore, the tight junction barrier in the Caco-2 cell monolayer was restored by washout of C10. This system could be a powerful tool for gene transfer into Caco-2 cell monolayer in studies of gene function as well as drug intestinal permeability.

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P19

THERMOGENOUS EFFECTS OF PROCESSED ACONITE ROOT ON COLD-STRESSED MICE HYPOTHERMIA

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Root of aconite (*Aconitum carmichaeli*, Ranunculaceae) is one of the important medicinal herbs in traditional Chinese and Japanese medicine (kampo medicine) to treat *Yang* deficiency, severe *coldness*, and general debilitation. Japanese Pharmacopoeia 15th Edition registers autoclaved aconite as "processed aconite root", which has much lower toxicity than unprocessed one, and it is prescribed in several herbal kampo formulae for the diseases induced by *coldness*. The pharmacologically active ingredients of aconite root are considered as diterpene-type alkaloids, which have strong analgesic as well as cardiotoxic effects, though aconitine, a main alkaloid of aconite root, is degraded by heat-processing. In the present study, we evaluated the thermogenous effects of processed aconite root on mice hypothermia induced by chronic cold-stress, and investigated its pharmacological mechanisms and active ingredients.

Male ddY mice (7 week-old) were reared in 4°C environment with free access to drinking water and food for 10 days. Powdered processed aconite root had been administered *ad libitum* from 3 days before (day -3) to the end of rearing (day 10) in cold environment as a food additive. The rectal body temperature was measured every two days from day -3 to day 9. On day 10, mice was sacrificed, and blood, retroperitoneal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) were collected. The concentration of triiodothyronin (T3) in the serum, and uncoupling protein (UCP)-1 level in BAT was measured by ELISA and western blot analysis, respectively. To find the active ingredients, powdered processed aconite root was extracted with NH_3-alkalized diethylether, and the residue was extracted again by boiling water. The aqueous extract was further separated into 80% EtOH soluble and insoluble factions.

Rectal body temperature of mice significantly decreased about 1 degree centigrade 5 days after rearing in cold environment, and the decrease had remained until day 10. In the mice fed processed aconite root, the decrease of body temperature significantly recovered in dose dependent manners, and the mice fed processed aconite root (1 g/kg/day) had maintained the body temperature at normal levels. When processed aconite root had been administered to the mice rearing in the environment of general temperature (25°C), the body temperature had not been affected. The similar thermogenous effects on hypothermia mice was appeared in 80% EtOH soluble fraction of the aqueous extract of processed aconite root, and the fraction containing diterpene-type alkaloids was inactive. In control hypothermia mice, serum T3 concentration, weight of BAT and UCP-1 level in BAT were increased, while the weight of WAT was decreased. The serum T3 concentration was decreased by the treatment with processed aconite root. On the other hand, the augmentation of UCP-1 level in BAT and the reduction of WAT weight in hypothermia were further proceeded in the mice treated with processed aconite root.

Processed aconite root has thermogeneous effect on hypothermia mice induced by cold-stress, and this pharmacological effect was earned by further up-regulation of UCP-1 level in BAT which was already enhanced by hypothermia. Although the pharmacologically active ingredients of aconite root have been considered as dierpene-type alkaloids such as aconitine and its degraded compounds, it is suggested that processed aconite root would contain other active compounds than aconitine, which has lower molecular weight and is soluble in boiling water as far as the traditional usage to treat *coldness* is concerned.

P20

EFFECTS OF MORPHINE AND GABAPENTIN ON MECHANICAL ALLODYNIA INDUCED BY CHEMOTHERAPEUTIC DRUGS IN MICE

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Many cancer patients suffer from pain, which interferes with sensory functions and decreases the quality of life. Cancer-related pain arises from the malignancy directly or indirectly. The latter includes pain caused by the treatment to alleviate the cancer such as surgery, radiation and chemotherapy.

Chemotherapy-induced peripheral neuropathy is a dose-limiting adverse effect of anti-cancer drugs, including the vinca alkaloid vincristine, the taxane paclitaxel and the platinum-based drug oxaliplatin. They are clinically used in many chemotherapeutic regimens. Many patients receiving the anti-cancer drugs experience on going pain and hypersensitivity toexternal stimulus such as light touch[1], but there is no accepted care for these pains except for dose decrease or drug withdrawal. The management of pain is important to improve not only quality of life but also cancer therapy and it is important to investigate the effective drugs decreasing these symptoms. In the present study, therefore, we examined the effects of three types of analgesic drugs on allodynia in mouse models of chemotherapy-induced neuropathy.

Male C57BL/6 mice (6 weeks old at the start of experiment) were used. Paclitaxel (5mg/kg), vincristine (0.1mg/kg) and oxaliplatin (3mg/kg) were injected intraperitoneally. These doses were selected based on clinical doses prescribed to patients with cancer. Mechanical allodynia was tested by using von Frey filament with strength of 2.83 mN^2.

Paclitaxel, vincristine and oxaliplatin produced mechanical allodynia from day 3 post-injection. The allodynia reached the peak 10–14 days after injections and almost subsided by 40, 35 and 21 days after injections of paclitaxel, vincristine and oxaliplatin, respectively. The aspirin-like drug diclofenac, the opiate morphine and the anticonvulsant gabapentin were tested at the peak time points of mechanical allodynia. Morphine (3 and 5 mg/kg s.c) dose-dependently inhibited mechanical allodynia induced by all drugs, but diclofenac (30 mg/kg, i.p) was without effects. Gabapentin (30 and 100 mg/kg, p.o) dose-dependently inhibited mechanical allodynia induced by paclitaxel and oxaliplatin, but not vincristine.

Morphine and gabapentin may help to attenuate peripheral neuropathy caused by chemotherapeutic drugs. The use of morphine is limited because of its side effects and tolerance. Although the efficacy of gabapentin depends on the types of anticancer drug, it may be a potential therapeutic agent for the treatment of neuropathy in patients receiving paclitaxel and oxaliplatin.

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P21

MODURATION OF GASTRIC PROTON PUMP ACTIVITY BY CLC-5

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In gastric parietal cells, protons are actively secreted by H^+,K^+-ATPase, but it has not been established what molecule contributes to apical Cl^- transport for HCl secretion. Recently, we found that CLC-5 was expressed in the gastric parietal cells. CLC-5 belongs to CLC Cl^- channel family that includes nine members in mammals. CLC-5 is a Cl^-/H^+ antiporter, and essential for renal proximal tubular endocytosis. Here, we examined the interaction between CLC-5 and gastric H^+,K^+-ATPase.

We constructed a tetracycline-regulated expression system of CLC-5 in the HEK293 cells stably expressing H^+,K^+-ATPase. The ATP-hydrolyzing activity, $^{86}Rb^+$ transporting activity and phosphorylation level of the H^+,K^+-ATPase were examined by using SCH 28080, a specific inhibitor of the pump. Ouabain-sensitive K^+-dependent ATP-hydrolyzing activity (Na^+,K^+-ATPase activity) was measured as a control. In hog gastric tubulovesicles (TV), distributions of H^+,K^+-ATPase, CLC-5 and caveolin-1, a marker of caveolae, were examined.

We found that CLC-5 protein is highly expressed in TV compared with the gastric mucosa. CLC-5 was co-localized with H^+,K^+-ATPase in hog parietal cells, while distribution of CLC-5 was apparently different from that of basolateral Na^+,K^+-ATPase. CLC-5 and H^+,K^+-ATPase in the TV sample were co-immunoprecipitated and they were predominantly found in the caveolae fractions**.** Exogenous expression of CLC-5 in the HEK293 cells (tet-on cells) significantly increased H^+,K^+-ATPase activity, while the CLC-5 expression had no effect on endogenous Na^+,K^+-ATPase activity. In the plasma membrane of the cells, expression level of H^+,K^+-ATPase was not changed by the CLC-5 expression. $^{86}Rb^+$ transporting activity in the tet-on cells was significantly higher than that of control cells. Furthermore, we found that expression of CLC-5 significantly elevated the phosphorylation level of H^+,K^+-ATPase in the cells. In contrast, exogenous expression of CLC-2 in the HEK293 cells stably expressing gastric H^+,K^+-ATPase had no effect on H^+,K^+-ATPase activity. These results suggest that CLC-5 up-regulates gastric H^+,K^+-ATPase activity.

P22

UP-REGULATION OF AQUAPORIN-5 IN HUMAN GASTRIC ADENOCARCINOMA IS INVOLVED IN THE CANCER CELL DIFFERENTIATION

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Aquaporins (AQPs) are water channel proteins expressed in various organs and tissues. Three AQP isoforms (AQP3, 4 and 5) have been reported to be expressed in glandular cells in the rat stomach. So far abnormal expression of AQP has been reported in several cancer tissues, but the function of AQP in human cancer has been little known. Here, we compared the protein expression levels of AQP3, 4 and 5 between human gastric cancer tissue and its accompanying normal mucosa in the upper or middle part of the stomach. It was found that AQP5 was significantly up-regulated in the intestinal type of gastric adenocarcinomas but not in diffuse type of adenocarcinomas. AQP5 was localized in the apical membrane of the cancer cells. Expression of AQP4 was down-regulated both in intestinal and diffuse types of adenocarcinomas. No significant expression of AQP3 was observed in the cancer and normal tissues.

To clarify the function of AQP5 in gastric adenocarcinomas, we transiently expressed AQP5 in a poorly differentiated human gastric adenocarcinoma cell line (MKN45). Interestingly, AQP5-expressing cell increased a number of differentiated cells. In addition, the AQP5 expression increased an activity of alkaline phosphatase, a marker for the intestinal epithelial cell type of cancer cells and expression level of laminin, an epithelial cell marker. On the other hand, the expression of AQP5 significantly decreased total cell number. Treatment with $HgCl_2$, an inhibitor of water permeability of aquaporins, significantly suppressed a number of differentiated cells and an activity of alkaline phosphatase but increased total cell number. Furthermore, the differentiated state induced by the AQP5 expression reversibly returned to the undifferentiated state after treatment with $HgCl_2$. The AQP5-mediated cell differentiation was stimulated under the hypertonic conditions and inhibited under the hypotonic conditions.

These results suggest that the increased water permeability via AQP5 is involved in induction of differentiation and also in maintaining the differentiated state of human gastric cancer cells.

P23

THE PEPTIDE TRANSPORTER IS NOT RESPONSIBLE FOR UPTAKE OF LEVOFLOXACIN IN HUMAN INTESTINAL CACO-2 CELLS

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We previously performed pharmacokinetic analysis of the transcellular transport of levofloxacin across Caco-2 cell monolayers in order to characterize the membrane transport responsible for intestinal absorption of levofloxacin.[1] The apical influx clearance of levofloxacin in the cells was greater than any other membrane transport clearance, suggesting that the membrane transporter is involved in the apical uptake of levofloxacin in Caco-2 cells; however, it is still unclear which transporter is involved in the apical uptake of levofloxacin in Caco-2 cells.[1] On the other hand, Yamaguchi *et al.* reported that a common dipeptide carrier-mediated system can transport sparfloxacin in the rat intestinal mucosa.[2] The primary aim of the present study was to investigate the involvement of peptide transporters for absorption of levofloxacin across human intestinal epithelial cells. In order to evaluate the characteristics of apical and basolateral peptide transporters in Caco-2 cells, we first performed pharmacokinetic analysis of transcellular transport of glycylsarcosine (Gly-Sar) in the cells grown on porous membrane filters. Transcellular transport of Gly-Sar at the medium pH 6 in Caco-2 cells was greater in the apical-to-basolateral direction than in the opposite direction. The influx clearance at the apical membrane was much greater than the basolateral efflux clearance, indicating that the apical peptide transporter plays an important role in the directional transcellular transport of Gly-Sar across Caco-2 cell monolayers. We then evaluated the effect of various compounds on the uptake of Gly-Sar and levofloxacin at the apical membrane of the cells. Although the apical uptake of [^{3}H]Gly-Sar was significantly inhibited by 5 mM Gly-Sar, Ala-Ala, and also levofloxacin, the apical uptake of [^{14}C]levofloxacin was not changed by 5 mM Gly-Sar and Ala-Ala. In contrast, the uptake of [^{14}C]levofloxacin was significantly inhibited by nicotine, enarapril, and fexofenadine, and was significantly increased by novobiocin. These findings suggested that the peptide transporter is not responsible for the apical uptake of levofloxacin into the Caco-2 cells, and that some transporters are involved in the influx and efflux of levofloxacin at the apical membrane of the cells.

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P24

STEREOSELECTIVE GLUCURONIDATION AND OXIDATION OF CARVEDILOL IN HUMAN LIVER AND INTESTINAL MICROSOMES

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Orally administered carvedilol undergoes stereoselective first-pass metabolism, and the blood concentration of *R*-carvedilol is approximately 2-fold higher than that of *S*-carvedilol.[1] Carvedilol is metabolized extensively *via* aliphatic side-chain oxidation, aromatic ring oxidation, and conjugation pathways.[2,3] However, it is still unclear which enzyme is responsible for the stereoselective presystemic clearance of carvedilol. Furthermore, in spite of the expression of UDP-glucuronosyltransferases (UGTs) and cytochrome P450 (CYP) 3A4 in intestinal epithelial cells, it is also unclear whether the intestine is responsible for the stereoselective presystemic clearance of carvedilol.[4] The aim of the present study is to investigate the mechanism for the stereoselective presystemic clearance of carvedilol. We examined the glucuronidation and oxidation of carvedilol in human liver microsomes (HLM) and human intestinal microsomes (HIM). The glucuronidation of carvedilol in HLM and HIM was evaluated in the presence of UDP-glucuronic acid, whereas the oxidation of the drug was evaluated in the presence of NADPH. The glucuronidation and also oxidation activities for *S*-carvedilol in HLM and HIM were higher than those of *R*-carvedilol, indicating that glucuronidation and oxidation of carvedilol are stereoselective for *S*-enantiomer both in the liver and intestine. In addition, the oxidation of *R*-carvedilol in HLM was inhibited by quinidine, whereas that of *S*-enantiomer was inhibited by quinidine and furafylline. In contrast, the oxidation of *R*- and *S*-carvedilol in HIM was inhibited by ketoconazole. The findings suggest that oxidation of *R*-carvedilol in the liver is catalyzed by CYP2D6, whereas that of *S*-carvedilol is catalyzed by not only CYP2D6 but also CYP1A2, and that oxidation of *R*- and *S*-carvedilol in the intestine is catalyzed by CYP3A4.

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P25

FUNCTIONAL CHARACTERISTICS OF HUMAN PROTON-COUPLED FOLATE TRANSPORTER/HEME CARRIER PROTEIN 1 (hPCFT/HCP1) HETEROLOGOUSLY EXPRESSED IN MAMMALIAN CELLS

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The absorption of folate (vitamin B_9) occurs primarily in the upper small intestine and involves a carrier-mediated process with a low pH optimum, which operates efficiently within the acidic microclimate of the intestinal surface. Although the molecular entity of such a transporter had long been unclear, proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1, SLC46A1), which was originally cloned as a heme transporter, has recently been redefined as a folate transporter by studies by Qiu et al.[1] and by us[2] independently. Although this transporter is most likely the molecular entity responsible for carrier-mediated intestinal folate absorption, it is yet to be fully proven. We here report updated functional characteristics of human PCFT/HCP1 (hPCFT/HCP1) to further prove that.

The uptake of folate by hPCFT/HCP1 transiently expressed in HEK293 cells was optimum within a range of pH 4.0 to 5.5 and saturable with a Michaelis constant (K_m) of 1.7 μM at pH 5.5. Furthermore, it was significantly reduced when the inward H^+ gradient was collapsed by nigericin, a K^+/H^+-exchanging ionophore, under K^+-rich condition, indicating that proton-coupled cotransport is the most likely mode of operation for this transporter. However, hyperpolarization of the plasma membrane by the use of valinomycin, a K^+ ionophore, failed to alter folate uptake, indicating insensitivity to membrane potential. Affinities of folate derivatives for hPCFT/HCP1 were in the order of folate $\geqq$ methotrexate (MTX; an antifolate analog) > tetrahydrofolate. 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and sulfobromophthalein (BSP), which are inhibitors of intestinal folate transport, inhibited folate uptake by hPCFT/HCP1, but thiamine pyrophosphate (TPP), a substrate/inhibitor of reduced folate carrier 1 (RFC1), did not.

In MDCKII cells stably expressing GFP-hPCFT/HCP1, this transporter was localized at the apical membrane and, in a consistently polarized manner, specific MTX uptake was observed only through the apical membrane. Apical membrane vesicles were prepared to examine transport across the membrane more specifically. The uptake of MTX, as well as folate, by the membrane vesicles was accelerated by an inward H^+ gradient (pH_{out}/pH_{in} = 5.5/7.5), compared with an ungradiented near neutral pH condition (pH_{out}/pH_{in} = 7.5/7.5), and showed overshoot, initial and transient accumulation compared to equilibrium uptake. This observation supports the proton-coupled cotransport mechanism. Further, the uptake of neither MTX nor folate was altered by inside-negative K^+ diffusion potential induced by valinomycin in the presence of an outward K^+ gradient (K^+_{out}/K^+_{in} = 0/100 mM), supporting membrane potential-insensitivity of hPCFT/HCP1-mediated transport.

Thus, hPCFT/HCP1 has been clearly demonstrated to function as a proton-coupled folate transporter, which can also transport MTX and reduced folates. The characteristics of hPCFT/HCP1 are in agreement with those expected for the long sought apical pH-dependent folate transporter in the small intestine. Information about its transport functions should be useful for optimizing oral drug therapy with folate derivatives and also for utilizing it for oral drug delivery.

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P26

INCREASING ANTICANCER ACTIVITY OF INTERFERON GENE TRANSFER BY PROLONGING THE DURATION OF TRANSGENE EXPRESSION

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Plasmid DNA (pDNA) is an attractive nonviral vector in gene therapy in terms of safety, productivity and versatility. Development of highly efficient gene delivery technologies, including hydrodynamic delivery, electroporation- or sonoporation-guided gene transfer methods, has almost solved the problem of the low level of transgene expression of nonviral vectors, but it has had little effect on the short duration of expression, another important drawback. Interferon (IFN) gene transfer is considered to be useful for immunotherapy because IFNs have antiproliferative and immunomodulatory activities which are capable of contributing to the host's defense against tumors.[1] In a previous study, we demonstrated that repeated hydrodynamic injections of IFN-expressing pDNA were effective in inhibiting metastatic tumor growth in mouse liver.[2] To avoid multiple injections and possible side effects induced by high-level IFNs, it is important to achieve a sustained expression of IFNs after in vivo gene transfer. It has been suggested that the loss of transgene expression is attributed, at least partially, to the presence of unmethylated CpG dinucleotides or CpG motifs in conventional pDNA.[3] Therefore, in the present study, several plasmid vectors with different numbers of CpG motifs were constructed, and the profile of transgene expression, such as the level and duration, was compared. Several types of pDNA encoding firefly luciferase with different numbers of CpG motifs were prepared and hydrodynamically injected into mice. A conventional, CpG replete pCMV-Luc showed a transient transgene expression in the liver, whereas pGZB-Luc, all CpGs were replaced with TpGs except for those in Ori and luciferase cDNA, showed a partly sustained transgene expression. Furthermore, pDNA with no CpG motifs, pCpG-ΔLuc, showed a remarkable sustained transgene expression. Based on these findings, IFN-γ-expressing pDNA with fewer CpG motifs, pGZB-Muγ[4] and pCpG-Muγ, were constructed. Hydrodynamic delivery of any of these pDNA resulted in a sustained IFN-γ concentration at therapeutic levels, and significantly inhibited metastatic tumor growth in the lung. These results indicate that the duration of transgene expression of IFN as well as the anticancer activity of IFN gene transfer can be significantly increased by reducing the number of CpG motifs in IFN-expressing pDNA.

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P27

A NEW CLASS OF LIGAND FOR VITAMIN D RECEPTOR: DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF LIGANDS WITH ADAMANTINE RING

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1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$, **1**] plays an important role in calcium resorption and bone formation, cellular differentiation and proliferation, and immune responses.[1] The majority of these actions are mediated by the vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily and functions as a ligand-dependent transcriptional factor.[1,2] Upon ligand binding, the VDR undergoes conformational change to form the AF2 surface to allow binding of a coactivator.[3] Thousands of vitamin D analogues have been synthesized to date. Most of them act as a VDR agonist, whereas only two types of analogs[4,5] act as a VDR antagonist.

To develop novel VDR antagonists for treatment of metabolic bone disease such as Paget disease and to investigate the molecular basis of the VDR antagonism, we designed eight 1,25-$(OH)_2D_3$ analogs having an adamantane ring at the side chain. These compounds were expected to work as an antagonist interfering the appropriate folding of helix 12 (H12) of the VDR to form the active conformation. Four analogs (25AD25OHD) which have both an adamantine ring and a hydroxyl group at C25 are diastereomers at C20 and C25. The other four (24AD24OHD) which have the same ring and group at C24 are diastereomers at C20 and C24. All analogs were successfully synthesized by a convergent method using A-ring phosphine oxide, CD-ring fragment and the side chain moiety. Each stereochemistry at C25 or C24 was determined by Kusumi-Mosher method. Binding affinity for the VDR was evaluated by the competitive binding assay using [^{3}H]-1,25-$(OH)_2D_3$. All synthetic compounds specifically bound to the VDR, indicating they are all ligand of the VDR. Transcriptional activity was evaluated by the transient transfection assay in Cos7 cells. 25AD25OHD compounds showed little activity, whereas 24AD24OHD compounds showed significant activity. 25AD25OHD compounds inhibited the transcriptional activity of the natural hormone, 1,25-$(OH)_2D_3$ **1**, concentration dependently, indicating they are antagonists for the VDR. 24AD24OHD compounds inhibited the activity of the hormone **1** to their maximum efficacy, concentration dependently, indicating they are partial agonists for the VDR. Interaction between the ligand and the VDR was studied by the docking of each ligand into the VDR-LBD. Our docking analysis provided useful information to understand the molecular basis of the VDR antagonism as well as the partial agonism. In conclusion, we have discovered a new class of VDR ligands which have an adamantane ring at the side chain. They are promising candidates for the therapeutic agent to treat the metabolic bone disease.

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P28

INHIBITION OF DRUG METABOLIZING ENZYMES BY XANTHENE DYES

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We have reported that Erythrosine, one of Xanthene dyes, among synthetic food dyes inhibited UDP-glucuronosyltransferase 1A6 (UGT1A6) [1]. We clarified Xanthene dyes had an inhibitory effect on human UGT1A6 activity by combination of Xanthene structure and halogens on its [2]. The purpose of this study is to investigate whether Xanthene dyes inhibit cytochrme (CYP)3A4 and P-glycoprotein (P-gp) or not.CYP3A4 is a major CYP isoform responsible for the metabolism of many drugs including erythromycin, cyclosporine, codeine, and concerned with drug metabolism of more than 50 % in body. P-gp, multiple drug resisitant protein 1 (MDR1), plays a role in the blood-brain barrier, preventing drug distribution into the brain and in cancer chemotherapy.

Erythrosine, Phloxine, and Rose Bengal of Xanthene dyes storongly inhibited human CYP3A4, with IC_{50} values = 5.5, 7.9, and 21.2 μM, respectively. Meanwhile, Azo dyes, Indigoid dyes, and Triphenylmethane dyes did not inhibit human CYP3A4 activity. Erythrosine, Phloxine, and Rose Bengal well inhibited human P-gp, with IC50 values = 15.6, 23.5, and 11.7 μM, respectively. Azo dyes , Indigoid dyes, and Triphenylmethane dyes did not inhibit human P-gp activity. These results showd that Xanthene dyes with halogens on its have an inhibitory effect on human CYP3A4 activity and P-gp activity, as well as UGT1A6.

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P29

KINETIC STUDY ON METHYLESTERIFICATION OF DICARBOXYLIC ACYLCARNITINES IN ACIDIC METHANOL SOLUTION

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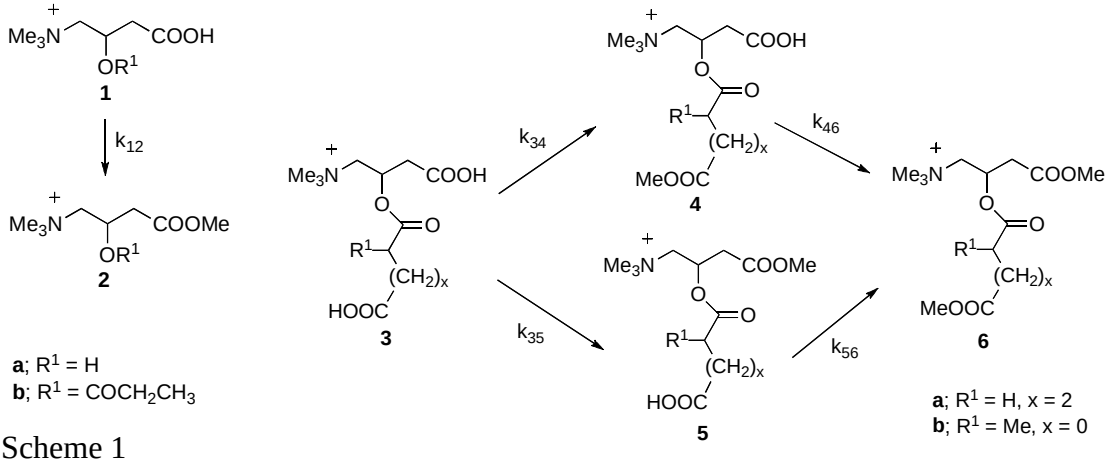
Acylcarnitine (**1**, **3**) analysis of dried blood spots by tandem mass (MS/MS) has been used to screen newborns for organic acidemias and fatty acid oxdation defects.[1] Glutarylcarnitine (**3a**, Scheme 1) having two carboxyl groups in the molecule is an indicator of glutaric aciduria. We have developed new method by HPLC-MS/MS for diagnosis of these diseases, and analyzed acylcarnitines in serum of patients.[2] Cation-exchange type solid phase extraction cartridge was used for the purification of acylcarnitines, and methanol was used for washing and elution. When **3a** was extracted by this method, methylesterified product (**4a**) was obtained. This reaction occurred having priority to carboxylic group of glutaric acid side. When carnitine (**1a**) and propionylcarnitine (**1b**) were applied to the caryridge, however, corresponding methylesters (**2**) were not obtained. We have examined the kinetics of **1** and **3** in acidic methanol solution.

After **1** or **3** was dissolved in 1% HCl methanol solution, concentrations of **1**-**6** were measured with time by HPLC-MS/MS. The kinetic constants k_{12} for **1a** and **1b** were 0.26 and 0.48 (hr^{-1}), respectively. Although production of **4a** was fast in the reaction of **3a** with methanol, a little amount of **5a** was formed. Formed **4a** changed to **6a** gradually. The kinetic constants in this reaction were calculated by nonlinear least-squares method (Multi[3]) and the values of k_{34}, k_{35} and k_{46} were 6.3, 1.6 and 0.7 (hr^{-1}), respectively. When **5a** was reacted with methanol, the constant k_{56} was 7.3 hr^{-1}. In the case of methylmalonylcarnitine (**3b**), **5b** was produced more than **4b**.

The methylesterification of **3a** was carried out preferentially at the carboxyl group of glutaric acid side. The reaction was much faster than those of **1** to **2**. Since acylcarnitines are extracted by methanol from dried blood spots at newborn screening, it is important not to extract them under acidic conditions.



Scheme 1

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P30

TRANSCRIPTIONAL REGULATION OF L-TYPE AMINO ACID TRANSPORTER 1 (LAT-1) GENE IN THE RETINAL CAPILLARY ENDOTHELIAL CELLS UNDER THE GLUCOSE DEPLETED CONDITIONS

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The inner blood-retinal barrier (inner BRB), which is formed by the retinal capillary endothelial cells with tight-junctions, expresses L-type amino acid transporter 1 (LAT-1) to supply the essential amino acids to the retina.[1] It is known that retinal neovascularization is occurred under the ischemia of diabetic retinopathy. Although LAT-1 is thought to be a key role in supplying the essential amino acids for proliferation of endothelial cells, regulation of LAT1 at the inner BRB under the ischemic conditions has not been clarified yet. The purpose of this study was to clarify regulation of LAT-1 in a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells)[2] as an in vitro model of the inner BRB under the glucose depleted conditions.

[^{3}H]L-Leucine uptake activity and the expression level of LAT-1 mRNA were examined in TR-iBRB2 cells following culture under the glucose depleted conditions. To determine the transcriptional activity of LAT-1 gene under glucose depleted conditions, promoter activity of the LAT-1 gene (-1958 to +70) was assayed using dual luciferase reporter assay system.

[^{3}H]L-Leucine uptake and the expression of LAT-1 mRNA in TR-iBRB2 cells cultured without glucose for 24 h were 2.5- and 4-fold greater than that cultured with glucose, respectively, suggesting that LAT-1 transport activity is induced under the glucose depleted conditions. Moreover, both increased activities were decreased in the presence of actinomycin D, an RNA synthesis inhibitor, supporting that transcriptional activation of LAT-1 expression takes place under glucose depleted conditions. Transcriptional activity of the 2 kbp LAT-1 promoter under the glucose depleted conditions was 1.7-fold greater than that of control condition. Moreover, mutagenesis of the E-box at -162 to -155 decreased the activation of LAT-1 promoter under the glucose depleted conditions.

These results suggest that glucose depleted sensitivity of LAT-1 expression is transcriptionally regulated in the retinal capillary endothelial cells, and E-box at -162 to -155 mediates this regulation.

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P31

APPLICATION OF THE MULTI-DIMENSIONAL HPLC MAPPING METHOD FOR *N*-GLYCOSYLATION PROFILING OF CELLS, TISSUES AND ORGANS

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N-glycans play important roles in mediation of cell-cell communication and virus infection, determination of the fates of their carrier proteins in and out of cells, and modulation of protein functions as hormones and antibodies. Cellular expression patterns of *N*-glycans depend on developmental stages and environmental factors. The recently emerging glycomics projects aim at comprehensive identification and characterization of *N*-glycans expressed by whole cells, tissues, organs, and bodies. However, unlike DNA and proteins, carbohydrates are highly branched and exhibit microheterogeneities, which hamper detailed structural analyses.

We have been developing HPLC mapping, which is a powerful method to identify the structures of *N*-glycans[1]. In this method, the structural determination of *N*-glycans was based on their elution positions on the three kinds of HPLC columns. The accumulated HPLC data of approximately 600 different *N*-glycans are available in the web application GALAXY (http://www.glycoanalysis.info/)[2]. This method is applicable for *N*-glycosylation profiling of proteins dealing with a variety of glycans such as sialyl, sulfated and glucuronyl oligosaccharides, which play important roles in cell-cell communications[3]. Here, we demonstrate the utility of the multi-dimensional HPLC mapping method in structural glycomics illustrating examples of *N*-glycosylation profiling of (i) influenza viruses grown by different host cells, and (ii) quail and chicken intestinal epithelial cells, as potential intermediate hosts for avian influenza virus transmission to humans[4], (iii) various tissues of the ascidian *Ciona intestinalis*[5], and (iv) *Opisthorchis viverrini*, a kind of parasite inducing a number of hepatobiliary diseases[6].

This work was a collaboration with Prof. Yasuo Suzuki (Chubu University). The funding of this study was provided in part by CREST/JST.

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P32

P-GLYCOPROTEIN (PGP) AND RENAL CLEARANCE (CL_R) OF FEXOFENADINE IN CYSTIC FIBROSIS (CF)

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Pgp is structurally related to CFTR and serves a complementary role in modulating chloride channel function. Compensatory upregulation in Pgp has been found in a CFTR knockout model suggesting that Pgp may be upregulated in patients with CF. Fexofenadine (FX) is not significantly metabolized and is a substrate for Pgp and OATP.[1-4] Therefore, its administration serves as useful probe for determining clinical implications of Pgp function on drug disposition. Probenecid (Pb) is an inhibitor of OATP and will be used pharmacologically to block the activity of this transporter in-vivo. Coadministration of FX and Pb will enable determination of the relative contribution of Pgp to the pharmacokinetic disposition of fexofenadine. We aim to evaluate FX pharmacokinetics as a probe for renal Pgp activity in CF patients with age-matched healthy volunteers (HV) control subjects.

Sixteen (n=8 CF, 8 HV) subjects underwent this prospective controlled study in which FX was received alone or in combination with probenecid (Pb). Iothalamate was given each day to measure glomerular filtration rate (GFR). Blood and urine samples were obtained at specified times over 12 hours each study day for determination of fexofenadine and iothalamate concentrations. Plasma concentrations were assayed using liquid chromatography-mass spectrometry and urine samples using HPLC. Pharmacokinetic analysis was performed using noncompartmental methods. Differences between groups were determined using a paired t-test or Man Whitney-U.

CF patients were younger (median (IQ range), 26 (25-28) vs 31 (29-38) p=0.05, and had lower body mass index 20.2 (18.8-21.5) vs 25.5 (24.5-26.2) p=0.002 respectively) when compared to HV subjects, but do not differ in GFR (113.8 (101.5-145.6) vs 105.8 (95.2-118.2) p=0.65 respectively). No significant differences were found between CF and HV in FX rCL when given alone (4.65 (3.4-5.8) vs 5 (4.4-5.9) p=0.65 or in combination with PB 1.8 (1.5-2.6) vs 1.3 (1.1-2.5) p=0.57 respectively). However, there was a difference in FX rCL when given alone compared to FX in combination with Pb. FX only vs. with inhibtion of OATP (Pb) resulted in 66% decrease in rCL (median (IQ range), 5 (4-5.8) vs. 1.7 (1.1-2.6) p=0.0002).

In conclusion, OATP appears to be the major transport mechanism for the tubular secretion of fexofenadine. No difference in renal clearance of fexofenadine in patients with CF noted indicating renal P-glycoprotein activity is not upregulated in patients CF.

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P33

INHIBITORY EFFECTS OF FLUORINATED TOLUBUTAMIDE ANALOGS ON CYP2C9 ISOZYMES

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Cytochrome P450 2C9 (CYP2C9) catalyzes the metabolism of a number of clinically used drugs, including *S*-warfarin, phenytoin, flurbiprofen, and tolbutamide (TB). Human CYP2C9 has three major polymorphic isozymes, CYP2C9.1 (wild-type), CYP2C9.2 (Arg144Cys), and CYP2C9.3 (Ile359Leu). We have previously reported that, when fluorinated benzo[h]quinolines were subjected to analysis of their inhibitory effects on drug metabolism by recombinant human CYP2C9 isozymes, the position-specific substitution by a fluorine atom(s) altered the CYP2C9 inhibition by benzo[h]quinolines in different manners depending on the polymorphic isozyme.[1]

In the present study, fluorinated derivatives of TB analogs, 1-benzensulfonyl-3-phenylurea (BSPU), 1-benzensulfonyl-3-cyclohexylurea (BSCU), and 1-benzensulfonyl-3-butylurea (BSBU), were used as inhibitors of the metabolism of 5-deoxyluciferine by recombinant human CYP2C9.1, CYP2C9.2, and CYP2C9.3 to investigate the structure-inhibition relationship. Fluorinated BSPUs, 2-F BSPU, 3-F BSPU, and 4-F BSPU, were synthesized from each fluorobenzensulfonamide and phenyl isocyanate. Fluorinated BSCUs, 2-F BSCU, 3-F BSCU, and 4-F BSCU, were synthesized from each fluorobenzensulfonamide and cyclohexyl isocyanate. And fluorinated BSBUs, 2-F BSBU, 3-F BSBU, and 4-F BSBU, were synthesized from each fluorobenzensulfonamide and butyl isocyanate. CYP2C9 activity was determined by measuring CYP2C9-catalyzed luciferin production from 5-deoxyluciferine at 37^{o}C for 30 min according to the manual of P450-Glo ™ Assays (Promega). The inhibitory activities of fluorinated BSPUs, BSCUs, and BSBUs were expressed as IC_{50}.

The most inhibitory activity was observed in BSPUs, but BSBUs showed very weak inhibition on CYP2C9 activity. In the case of 2-F BSPU, the inhibitory effect on CYP2C9.1 activity (IC_{50} = 15.2 μM) was almost the same as that on CYP2C9.2 activity (IC_{50} = 16.2 μM). However, the inhibitory effect on CYP2C9.3 activity was much weaker (IC_{50} = 102 μM). Moreover, the inhibitory effect of 3-F BSPU on CYP2C9.1 activity (IC_{50} = 8.8 μM) was also similar to that on CYP2C9.2 activity (IC_{50} = 9.9 μM), and the inhibitory effect of 3-F BSPU on CYP2C9.3 activity was again considerably weaker (IC_{50} = 32.1 μM). On the other hand, the inhibitory effect of 4-F BSPU on CYP2C9.1 activity (IC_{50} = 17.1 μM) was slightly weaker than that on CYP2C9.2 activity (IC_{50} = 12.4 μM), and the inhibitory effect of 4-F BSPU on CYP2C9.3 activity was only 2.3-fold weaker (IC_{50} = 40.0 μM) than that on CYP2C9.1.

In conclusion, the position-specific substitution by fluorine atom altered the CYP2C9 inhibition by tolbutamide analogs, BSPUs, BSCUs, and BSBUs, in different manners depending on the polymorphic isozymes. These results again suggest that inhibitory profiles obtained with fluorine-substituted analogs of the key inhibitor molecule may be useful as a new tool for phenotyping the polymorphic CYP isoforms.

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P34

DEFICIENCY OF HIPPOCAMPAL LONG-TERM POTENTIATION AFTER PERIPHERAL NERVE INJURY AND ITS RELATION WITH THE EXTRACELLULAR GLYCINE LEVEL REGULATED BY GLYCINE TRANSPORTER 1

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Pain is defined as an unpleasant sensory and emotional experience. Patients with chronic pain can present with not only persistent pain but also a complex set of additional symptoms, including chronic fatigue, anxiety, depression, insomnia and cognitive disturbance. Because of cognitive disturbance, which is often described as poor memory, the patients fail to remember their treatment instructions, thereby delaying their recovery. To investigate the mechanisms underlying the cognitive disturbance in chronic pain patients, we employed a murine neuropathic pain model prepared by partial ligation of the sciatic nerve, and examined whether synaptic transmission and its plasticity are affected in the hippocampus which has been considered to be involved in memory formation.

We first recorded field excitatory postsynaptic potentials and induced long-term potentiation (LTP) by tetanic stimulation in the CA1 area of hippocampal slices. In slices obtained from neuropathic mice developing mechanical hypersensitivity, LTP was maintained at a significantly lower level 60 min after tetanic stimulation than that from mice given a sham operation.[1] This may reflect some aspects of cognitive disturbance that frequently accompanies chronic pain patients. Impairment of the LTP in slices prepared from neuropathic mice was never observed when it was induced in the presence of the glycine transporter 1 (GlyT1) inhibitor NFPS (25 nM) which at this concentration alone did not significantly influenced LTP in slices prepared from sham-operated mice.[2]

Since extracellular glycine levels modulate NMDA receptor activities which play an essential role in the induction of LTP in the hippocampal CA1 area, we then recorded NMDA receptor-mediated synaptic currents using whole-cell patch clamp techniques, and their sensitivity to the extracellular glycine levels was examined. Bath application of glycine in the concentration range between 10 and 100 μM, potentiated NMDA receptor-mediated synaptic currents in slices obtained from both sham-operated and neuropathic mice. However, the currents tended to be more potentiated in slices obtained from neuropathic mice. It may reflect higher glycine uptake via GlyT1. Therefore, the extracellular concentration of glycine seems to be maintained at a lower level in the hippocampus after peripheral nerve injury. Increase in the extracellular level of glycine by its bath application or blockade of GlyT1 may normalize NMDA receptor activities and synaptic plasticity.

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P35

MOLECULAR GENETIC MINING OF THE *ASPERGILLUS* SECONDARY METABOLOME

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The recently sequenced genomes of several *Aspergillus* species including *A. nidulans* have revealed that these organisms have the potential to produce a surprising large range of natural product many of which are currently unknown. We have employed recently developed gene targeting procedures, in combination with natural products chemistry to identify novel secondary metabolites in *A. nidulans* and the gene clusters responsible for their biosynthesis.

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P36

CLINICAL PHARMACIST TRAINING IN COLLABORATION WITH MEDICAL EDUCATION

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In 2003, Meijo University constructed master's course, having the purpose of "developing clinical pharmacists who can understand pharmacotherapy in clinical sites and contribute to health care society". Meijo University does not have its own medical facility, so we established a joint master course program with a medical school for the clinical training of pharmacy students.

The educational features of master's course are as follows: 1) PBL education to train skills for integrating patient information, finding problems and solving those problems, 2) clinical communication skill like patient's interview for gathering patient's illness-related information, and 3) students in master's course train clinical pharmacy skills at bedsides with medical residents at affiliated university hospital for 15 months, that is similar to pharmacy residents system in U.S. Students train first the central pharmacy in the hospital and then experience nursing skills.

After that, master students are rotated to several clinic divisions for learning on-the-job training for pharmacotherapy. They train with medical students and medical residents in the ward and the ambulatory care under the guidance of physicians, pharmacists and faculty members of the Meijo University. Clinical clerkship is assessed formatively by preceptors of physicians and pharmacy faculty members. To enhance the effectiveness of clinical training, students receive feedback from them in the clinical case presentation for the following four items: 1) presentation style, 2) presentation contents, 3) documentation, and 4) responses to questions. A master course thesis is required at the completion of the program that is assessed as well.

Our faculty of pharmacy is connected with the medical school with the telecommunication education system, and students on both of campus can attend lectures on TV screen of this system. The clinical medicine conference given by clinicians and case presentations done by the graduate students training at the university hospital are held using this system. Pharmacy students, who have few opportunities to experience clinical medicine, can motivate themselves toward clinical pharmacy through this system.

The target of our education is to expand the pharmacist's responsibility from dispensing-centered pharmacy to more clinical field in the health care society. A new six-year pharmacy education system in Japan has started to train the health care pharmacists well equipped with pharmaceutical care in 2006. We propose a typical model for clinical pharmacist education, committing pharmaceutical care in the new era in Japan.

P37

STUDY OF INHIBITION OF VPA-GLUCURONIDASE BY CARBAPENEM ANTIBIOTICS

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The serum concentration of valproic acid (VPA) in epilepsy patients decreased by the administration of carbapenem antibiotics (CP), such as meropenem (MEPM), biapenem (BIPM) or imipenem (IPM), to a sub-therapeutic level. Studies to explain the decrease were carried out using almost rats by the following steps: absorption of VPA in the intestine, glucuronidation in the liver, disposition in blood and renal excretion. It is difficult to consider the inhibition of intestinal absorption, because CP are intravenously administered and do not reach the intestine at an effective concentration. The liver is the key organ for the decrease of VPA concentration by CP, because it has been reported that no decrease of the VPA level by CP was found in hepatectomized rats. The most likely mechanism in liver is the activation of UDP-glucuronosyltransferase by CP. We found a 35% increase of VPA-glucuronidation activity by the pre-incubation of human liver microsomes (HLM) with MEPM (1). We estimated that this increase fully compensates for the decrease of serum VPA level by CP (2).

In this work, we studied inhibition of VPA-glucuronidase by MEPM. The level of VPA-glucoronidase in HLM was one fifth of the level of human liver cytosol. MEPM inhibited VPA-glucuronidase in HLM (IC_{50} = 35 microM), and BIPM and IPM did not inhibit. Meanwhile, MEPM, BIPM and IPM did not inhibit VPA-glucuronidase activity in cytosol. These results suggest that MEPM partly decrease the VPA level by inhibition of VPA-glucuronidase in HLM.

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P38

CATALITIC ACTIVITIES OF THE FLAVIN-CONTAINING MONOOXYGENASE 3 (FMO3) VARIANTS FOUND IN JAPANESE

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The flavin-containing monooxygenase (FMO) is an NADPH-dependent enzyme that catalyzes the oxygenation of many nitrogen-, sulfur-, and phosphorous-containing chemicals and drugs.[1,2] FMO3 is considered a prominent form expressed in adult human liver.[3] The decreased capacity of the FMO3 to oxygenate xenobiotics including trimethylamine is believed to contribute to metabolic disorder.[2] Because of its strong linkage with the genetic disorder trimethylaminuria, considerable work has been done to relate coding region polymorphisms of the *FMO3* gene to interindividual differences in FMO3 phenotype.[1] The aim of this study is to clarify the function of the FMO3 variants recently found in a Japanese population.

N- and *S*-oxygenation activities were determined with FMO3 variants expressed in *Escherichia coli* membranes. The Glu158Lys and [Glu158Lys-Glu308Gly] FMO3 showed slightly decreased *N*-oxygenation of benzydamine and trimethylamine. Selective functional *S*-oxygenation of these variants of methyl *p*-tolyl sulfide or sulindac sulfide was comparable to that of wild-type FMO3. The [Glu158Lys-Thr201Lys-Glu308Gly] and [Val257Met-Met260Val] variants showed significantly decreased oxygenation of typical FMO3 substrates. Val257Met FMO3 had a lower catalytic efficiency for methyl *p*-tolyl sulfide and sulindac sulfide *S*-oxygenation. However, compared with wild-type FMO3, Val257Met FMO3 showed a similar catalytic efficiency for *N*-oxygenation of benzydamine and trimethylamine. The catalytic efficiency for benzydamine and trimethylamine *N*-oxygenation by Arg205Cys FMO3 was only moderately decreased, but it possessed decreased sulindac sulfide *S*-oxygenation activity. Kinetic analysis showed that Arg205Cys FMO3 was inhibited by sulindac in a substrate-dependent manner, presumably because of selective interaction between the variant enzyme and the substrate.

The results suggest that the effects of genetic variation of human FMO3 could operate at the functional level for *N*- and *S*-oxygenation for typical FMO3 substrates. Genetic polymorphism in the human *FMO3* gene might lead to unexpected changes of catalytic efficiency for *N*- and *S*-oxygenation of xenobiotics and endogenous materials.

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P39

MAGNITUDE OF EFFECT AND SITE OF ACTION OF DOPAMINE ON THE RENAL CIRCULATION IN PATIENTS WITH SEVERE HEART FAILURE

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Background: A "renal dose" of dopamine is often used to increase renal blood flow; however, data on the magnitude of effect and site of action in patients with heart failure are scarce.

Methods: We evaluated the renal effect of intravenous dopamine in doses of 1, 2, 3, 5, and 10 mcg/kg/min in 13 patients with chronic heart failure (CHF) undergoing cardiac catheterization. Renal artery cross sectional area (CSA) was measured with intravascular ultrasound and renal blood flow velocity-time integral (VTI) with the aid of an intravascular Doppler technique. RBF was calculated by the following formula: RBF=Heart rate x VTI x CSA. Renal vascular resistance (RVR) was calculated as follows: RVR=80 (mean renal artery blood pressure/RBF).

Results: Cross-sectional area increased and was significantly higher than baseline at 5 and 10 mcg/kg/min. The velocity-time integral was significantly higher than baseline at doses of 3 and 5 mcg/kg/min. Renal blood flow increased, whereas renal vascular resistance decreased, reaching statistical significance at 2 through 10 mcg/kg/min. Cardiac output gradually increased, reaching statistical significance at doses of 5 and 10 mcg/kg/min (5.5±0.5 and 6.1±0.7 versus 4.5±5.2 L/min at baseline), but the increase in renal blood flow appeared proportionately larger than corresponding increases in cardiac output.

	VTI (cm)	CSA (cm^2)	RBF (ml/min)	RVR (dynes*s*cm^{-5})
Baseline	22.5±2.62	0.301±0.040	557.6±60.6	13615±1317
1 mcg/kg/min	25.5±3.39	0.319±0.040	677.5±84.0	11681±1384
2 mcg/kg/min	28.1±4.70	0.336±0.040	761.8±94.3*	10200±1310*
3 mcg/kg/min	30.5±4.20*	0.350±0.048	831.0±103.0*	10018±1324*
5 mcg/kg/min	30.6±4.03*	0.362±0.048*	878.6±91.6*	9540±1269*
10 mcg/kg/min	27.9±3.74	0.378±0.064*	897.7±131.4*	9408±1620*

*$p<0.05$ vs. Baseline

Conclusions: Dopamine is associated with an increase in renal blood flow in patients with heart failure. This effect is due to dilation of both the large conductance and small resistance renal blood vessels. Further evaluation of the efficacy and safety of dopamine for improvement of renal function in hospitalized patients with heart failure is warranted.

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WHAT IS A GOAL OF RADIO(-HEALTH) CHEMISTRY IN THE 6-YEAR EDUCATION PROGRAM FOR PHARMACY ?

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A pharmacist is a profession to be authorized to deal with radio-pharmaceuticals by the Law. In the new Core-Curriculum, radio(-health) chemistry is included to teach the principle, knowledge and technical skills of radioisotopes. However, in many pharmacy schools, radio(-health) chemistry is elective and only a few schools conduct the laboratory practice. The goal of the class is not perfectly clear. While education on radio(-health) chemistry remains minor, utilization of radioactive materials in medical diagnosis and/or therapy has been increased. Moreover, serious dispute on a nuclear power plant for energy supply has not settled down without an opinion leader.

The purpose of the present study is to encourage pharmacy students to learn more radio(-health) chemistry, monitor possible risks to public health caused by radiation, counsel their concern about safety of the community and raise more support to the utilization of radioisotopes.

A questionnaire examination was carried out to 140 freshman and sophomore pharmacy students and examined what they already knew about negative and positive aspects on radiation associated with human health problems, nuclear fusion, nuclear power plants etc., and whether or not students already recognized that they should be professional about radio(-health) chemistry.

The results indicated that most students were already taught once before with the related subjects, but 80 % of the students were not aware of a pharmacist as an authority of radio-pharmaceuticals. To the question who can be a trustful leader to decide whether or not people should evacuate immediately in emergency with radioactivity leakage from the source, policeman, physician and others were named, but pharmacist was not nominated.

Efforts should be focused to educate pharmacy students that a pharmacist is a most acceptable specialist among other medical-chemical professions. The proposed goal of the learning radio(-health) chemistry is to develop roles in pharmacy to guarantee the safety of the public against hazardous radiation exposure and to enhance the application of radioactivity in medical field such as positron emission computed tomography and single photon emission computed tomography.

P41

INSULIN REGULATES PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 EXPRESSION IN HEPATOCYTES AND IN MICE LIVER THROUGH STATIN-SENSITIVE PATHWAY: IMPLICATIONS FOR CLINICAL EFFECTS

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Liver produces plasminogen activator inhibitor type-1 (PAI-1), the major physiologic inhibitor of fibrinolysis and a cardiovascular risk factor. We have previously shown that insulin stimulates PAI-1 expression in HepG2 human liver cell line. This study evaluated the underlying molecular mechanism of insulin as a regulator of PAI-1 synthesis. Insulin increased PAI-1 accumulation in the conditioned media after 24 hours. Insulin (10 μM) induced PAI-1 mRNA expression (real-time PCR). Transient transfection assay of the human PAI-1 promoter-luciferase construct demonstrated that insulin increased PAI-1 promoter (-829 to +36bp region) activity. Increase of PAI-1 mRNA was attenuated by U0126 and PD98059, specific inhibitors of mitogen-activated protein kinase kinase, and genistein, an inhibitor of tyrosine kinase. In contrast, GF109203X, an inhibitor of the protein kinase C pathway, and LY294002, an inhibitor of phosphatidylinositol 3-kinase, exerted no effects. An HMGCoA reductase inhibitor, simvastatin (10 μM), which translocates membrane-bound sterol regulatory element-binding protein (SREBP) to nuclei, also attenuated PAI-1 mRNA expression after stimulation with insulin. Simvastatin did not affect baseline PAI-1 mRNA expression. Intra-peritoneal injection of insulin (0.1 IU/kg) increased plasma PAI-1 antigen levels in mice at 3 hour. Plasma PAI-1 levels (ELISA) and hepatic PAI-1 mRNA expression (real-time PCR) were closely correlated. Because SREBP is involved in lipid metabolism, insulin-mediated PAI-1 production may provide a novel link between atherothrombosis and hyperlipidemia. Patients with insulin resistance may benefit from treatment with simvastatin with respect to diminution of PAI-1 expression in liver and consequent potential reduction of cardiovascular risk. We also report a potential insulin responsive element in the 3' region of the PAI-1 gene, mediating the adverse effects of hyperinsulinemia on cardiovascular system.

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P42

CHARGE-INDUCED PHASE SEPARATION IN BINARY CHARGED COLLOIDS

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Charged colloids are stabilized against aggregation due to the electrostatic interparticle repulsions. At the weak repulsions, the colloids take disordered “liquid” states, where the particle arrangements are almost random. When the interparticle repulsion becomes strong enough, the colloids take ordered “crystal” structures, where the particles are regularly arranged in BCC or FCC lattices. Here we examine the phase behavior of the binary mixtures of charged colloids having different charge numbers. We find that the present binary systems exhibit charge-induced two-phase separation into the liquid and crystal phases.

Three kinds of aqueous dispersions of polystyrene particles **(**SS-09, SS-11, and SS-22; the surface charge density = 0.53, 0.59 and 0.42 micro C/cm^2, respectively) and colloidal silica dispersion (KE-W10, 0.07 microC/cm^2) were purified by dialysis and ion-exchange method. The diameters were about 100 nm for all the particles. The particle concentrations in the binary systems were 1.17 vol% for all the cases. At this concentration, all the polystyrene dispersions took the crystal states, while the silica colloids were in the liquid state.

The binary colloids showed the phase separation structures where liquid and crystal states coexisted (Fig.1). Fig.2 shows the phase separation structures observed by using confocal laser scanning microscope at two evolution times. We report mechanism of the phase separation on the basis of the effective hard sphere model.

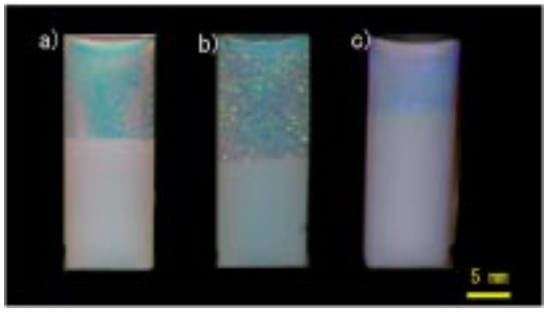

Fig.1
Charge induced phase separation in the binary colloids ($\Phi_{KE\text{-}W10}$ = 0.5)
a)SS-09+KE-W10, b)SS-11+KE-W10,
c)SS-22+KE-W10. Concentration of each particles =1.17vol% for all the cases. taken 31 days after preparation.

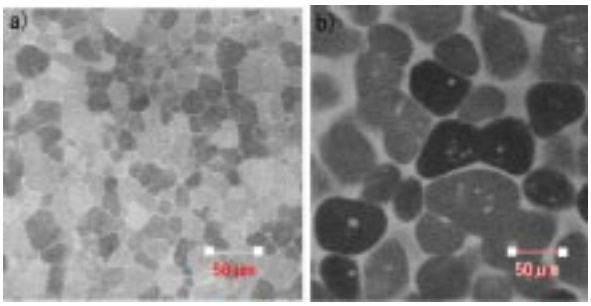

Fig.2
Confocal laser scanning micrographs
SS-11;1.638% KE-W10;0.702%
($\Phi_{KE\text{-}W10}$ = 0.3) about 100μm apart from the coverslip at a) 17.2min b)11.1hrs after preparation.

P43

THE EFFECT OF ISCHEMIA ON ERECTILE FUNCTION IN A RABBIT MODEL

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Young patients of arteriogenic erectile dysfunction(ED), who have suffered pelvic or perineal trauma such as in cases of bike accidents, tend to increase.[1] The elucidation of this pathology is very important task. Therefore, to research the damage in penile corpus cavernosum (CC) by traumatic arteriogenic ED, we produced penile ischemia model, and examined CC function.

Penile ischemia was created by the bilateral internal iliac artery ligation (BIIAL).[1,2] Male New Zealand White Rabbits were anesthetized intravenously with 30mg/kg pentobarbital. After lower abdominal incision, the bilateral internal iliac artery was exposed carefully, and each artery was ligated with 3/0 silk surgical suture. Rabbits were placed into four groups: sham surgery, BIIAL for 3days, BIIAL for 1week, BIIAL for 4weeks. We investigated the relaxant effect on isolated strips of rabbit penile CC using isometric tension study. The preparations were evaluated by acetylcholine (ACh), sodium nitroprusside (SNP), electric field stimulation (EFS). The relaxant response was investigated in preparation contracted by norepinephrine (NE).

In BIIAL groups, mean relaxant rate in ACh 10^{-4}M was 64% after 3days, and after 1week, it was decreased to 33 % ($P<0.01$). However, after 4weeks, the relaxant rate was 62%, which restored as almost equally as the rate after 3days. On the other hand, there is no marked change in the relaxant response by SNP or EFS.

In this study, we revealed that endothelial cell dysfunction occurred at early stage of ischemia when penile CC fell ischemia by sudden decrease of blood flow into penis. However, because the relaxant response by ACh recovered in BIIAL for 4weeks, we suggest that the other collateral circulation grew as compensatory change, and the endothelial function improved.

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P44

VISUALIZATION OF LOCAL Ca^{2+} TRANSIENTS IN SMOOTH MUSCLE CELLS USING TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPE

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In smooth muscles, cytosolic Ca^{2+} mobilization dramatically changes with complex spatiotemporal patterns at resting and exciting conditions. During action potentials, cytosolic Ca^{2+} signal is triggered by Ca^{2+} influx through voltage-dependent Ca^{2+} channel (VDCC) in plasma membrane [1-3]. The physiological significance of Ca^{2+} amplification by subsequent Ca^{2+} release through ryanodine receptor (RyR) from sarcoplasmic reticulum (SR) in excitation-contraction (EC) coupling is still a matte of topics in smooth muscles. On the other hand, at a rest, local Ca^{2+} dynamics play an essential role in the regulation of myogenic tone in smooth muscles [2, 4].

In the present study, depolarization-evoked local Ca^{2+} transients (Ca^{2+} hotspots) via Ca^{2+}-induced Ca^{2+} release (CICR) through RyR on subplasmalemmal SR were imaged using a total internal reflection fluorescence (TIRF) microscope in smooth muscle cells of murine urinary bladder. In addition, spontaneous Ca^{2+} releases (Ca^{2+} sparks) from RyR on subplasmalemmal SR were detected around a resting membrane potential by TIRF imaging in rabbit portal vein smooth muscle cells.

Upon depolarization from a holding potential of -60 to 0 mV for 50 ms under whole-cell voltage-clamp, the rapid elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$, as fluo-4 signals) occurred in a limited TIRF zone less than 200 nm from the chamber bottom, and the rate of $[Ca^{2+}]_i$ change reached the peak within 16 ms. The depolarization-evoked $[Ca^{2+}]_i$ increase in TIRF zone was abolished by the pretreatment with 100 μM Cd^{2+} or 10 μM ryanodine. The depolarization-induced outward currents, which were simultaneously recorded with Ca^{2+} images, were mainly due to the activation of large-conductance Ca^{2+}-activated K^+ (BK) channels, and were also reduced by these blockers. Similar $[Ca^{2+}]_i$ rise was induced by the application of 10 mM caffeine. When myocytes were stained with DM-BODIPY (-)-dihydropyridine (0.1 μM) or specific α1C antibody (1:100), the fluorescent signals of individual channel unit or their clusters were not uniformly distributed in the TIRF images and the sum of fluorescent areas occupied approximately 1 % of the plasma membrane in the image. In addition, at a holding potential of -40 mV, spontaneous Ca^{2+} releases (Ca^{2+} sparks) were observed in a TIRF zone and synchronized with spontaneous transient outward currents (STOCs) or membrane hyperpolarizations.

These TIRF analyses are useful to visualize and quantify the local Ca^{2+} transients (Ca^{2+} hotspot and Ca^{2+} spark) and elucidate its physiological significance in regulation of membrane excitability and myogenic tone in smooth muscles.

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