ABSTRACTS

Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008

— Novel development and technological innovation in anti-influenza virus agents

Tokyo, Japan

September 29 – October 1, 2008
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Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008 (JCMWDDT 2008) will focus on important new scientific and technological developments in drug discovery process, particularly those relevant to anti-influenza virus agents. The workshop will create an environment for in-depth, informed discussions highlighting the importance of drug researches. It will also provide opportunities to re-emphasize the crucial position of medicinal chemistry in the drug discovery process and its pivotal role in linking and exploiting the associated biological sciences. JCMWDDT intends to create a forum for all scientists interested in medicinal chemistry and related fields.

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Design, synthesis and preliminary activity assay of influenza virus neuraminidase inhibitors

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Influenza is an acute viral infection of the upper respiratory tract that can affect millions of people every year. The catalytic activity of Neuraminidase (NA) is essential for influenza virus replication and infectivity. It has been considered a suitable target for designing agents against influenza viruses.

X-ray crystal structures of complexes of NA with inhibitors revealed that potent inhibition of NA is determined by the relative positions of the interacting inhibitor substituents rather than by the absolute position of the central ring. This led us to design and synthesis potential NA inhibitors in which the pyrrolidine ring and benzene ring served as a scaffold for substituents which would interact with the NA active site.

Recent years, two structural patterns of compounds have been designed and synthesized in our laboratory. The first pattern is a series of pyrrolidine derivatives (1) which were synthesized in good yields starting from 4-hydroxy-L-proline using a suitable synthetic strategy. These compounds showed potent inhibitory activity against NA (IC50 = 1.56–90.79 μM). Within this series, five compounds have the good potency (IC 50 = 1.56–2.71 μM) which are compared to the NA inhibitor Oseltamivir, and could be used as lead compound in the future. The second pattern is salicylic acid derivatives derived from p-amino salicylic acid. A new series of NA inhibitors (2) containing a hydrophobic side chain at C-2 and an amino or guanidine at C-5 were designed and synthesized. And the preliminary result showed that salicylic acid derivatives displayed inhibitory activities with IC50 value from 0.032 to 9.26 μM. The compound with two guanidine group at C-3 and C-5 and ethyl as hydrophobic side chain showed the best inhibitory activity (IC 50 = 0.032 μM). The other five compounds containing guanidine exhibited good activities (0.036~0.049 μM).

In conclusion, we have described the synthesis and properties of a series of pyrrolidine and p-aminosalicylic acid derivatives as influenza NA inhibitors. All of the compounds were shown to possess potent influenza NA inhibitory activity. We reported a more convenient and economical method of the synthesis of pyrrolidine and p-aminosalicylic acid NA inhibitors. Compared to the other research, 4-hydroxyproline and p-aminosalicylic acid we used appeared to be an ideal starting material because of its low cost and commercial abundance. We also established a consistent QSAR model which was critical to predictive structure-based drug design and discovering potent compounds that would potentially be useful for antiviral therapy. The compounds we have got all showed potent NA inhibitory activity, and this finding could be used to design further influenza NA inhibitors.

Keywords: Influenza, Influenza virus, Neuraminidase, Pyrrolidine, Salicylic acid, QSAR

References

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Infection disease models with silkworms to evaluate the therapeutic effects of drug candidates

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Preclinical tests using animal models are necessary for evaluating the therapeutic effects of drug candidates, since most drug candidates obtained by in vitro screening are inappropriate as medicines due to their problems of pharmacodynamics in the human body. Although mammalian models, such as mice, rats, dogs, cats, and rabbits, have been used to examine the pharmacodynamics of drug candidates, both the high cost and the ethical issues of sacrificing mammals for drug analysis can delay the development of potentially therapeutic drugs. The use of invertebrate animals for the evaluation of drug candidates can overcome these problems. We propose the use of silkworms, Bombyx mori, as model animals to evaluate the properties of drug candidates. The lower cost and smaller space required for the maintenance of silkworms compared to mice allows for a larger number of animals to be handled in limited facilities. Because of the long history of the silk industry, the methods for taking care of silkworms are well established in Asian countries. Silkworms are ideal for use in a large-scale drug screening system, as they are large enough to be used in injection experiments, for making hemolymph preparations, and for isolating organs such as the midgut, which are essential processes for studying the pharmacodynamics of drugs in individual animals. The silkworm has a number of cytochrome P450s and sulfur or glucose conjugation enzymes, which are involved in drug detoxification.

I report that pathogenic microorganisms, such as Staphylococcus aureus and Candida albicans, were lethal to silkworms, and clinically-used antibiotics had therapeutic effects in silkworms. Moreover, the effective doses of antibiotics in this silkworm infection model were similar to those in mammalian models. Further, the availability of antibiotics by oral administration, and the drug distribution and metabolism were similar between silkworms and mammals.
Japan's governmental approaches to facilitate drug development process

Makoto Shimoaraiso

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There is no doubt that the supply of the safe and efficient drugs to the patients in a timely manner is the key of public health. In order to facilitate the drug development process, the Government of Japan, especially the Ministry of Health, Labour and Welfare (MHLW), has been playing an active role. In this presentation, the governmental approaches taken recently will be introduced.

There are three major administrative organizations regarding pharmaceutical affairs in Japan; the MHLW, the Pharmaceuticals and Medical Devices Agency (PMDA), and the National Institute of Biomedical Innovation (NIBIO). The MHLW is in charge of pharmaceutical regulatory affairs, whereas the PMDA is responsible primarily for review process and advices for clinical trials. The NIBIO is engaged in drug research and development including orphan drugs. These three organizations handle cooperative activities for approving new drugs from the very first stage.

From the development to the approval stage, the PMDA conducts advices, such as clinical trial consultations, in order to streamline the development process. In addition to that, the MHLW has notified guidelines/guidance for different stages of the development, such as “the guideline for stability testing of biotechnological/biological products”, and “the general consideration of clinical trials”. There are also guidelines for specific therapeutic categories, for example, “the guidelines on clinical evaluation methods of antibacterial drugs”. These guidelines are, or being tried to be, harmonized to other countries.

Products designated as orphan drugs (the criteria include less than 50,000 patients) are entitled to priority measures such as guidance and advice by the NIBIO. The orphan drugs are also entitled to priority review, and in 2006, four products of anti-influenza virus vaccines were designated as orphan drugs in Japan.
Effective detection of the epidermal growth factor receptor mutation by the peptide nucleic acid-locked nucleic acid PCR Clamp

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Gefitinib is an inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and has been treated to the advanced non-small cell lung cancer (NSCLC) patients. In 2004, spring, it had been shown that the responsiveness to gefitinib had been linked to the presence of EGFR mutations (Lynch TJ, et al. N Engl J Med 2004; 350:2129-2139). So it is very important to get result of the EGFR mutation in each patient more sensitive and more rapidly. Recently, a rapid and sensitive detection system for EGFR mutation named the peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp has been introduced (Nagai Y, et al. Cancer Res 2005; 65:7276-7282). It has been shown that the PNA-LNA PCR clamp method can detect EGFR mutations in the presence of 100-to 1,000-fold background of wild-type EGFR and that screening of 30 non-small cell lung cancer cell lines has resulted in detection of specific mutations in 12 cell lines. We have applied this PNA-LNA PCR clamp method to detect EGFR mutations in trans-bronchial lung biopsy (TBLB) specimens of NSCLC patients and have studied the correlation between EGFR mutations and effect of gefitinib. The PNA-LNA PCR clamp method has been confirmed to be more sensitive and rapid than the conventional sequence method, namely 9 of 10 patients who had responded to gefitinib have been detected EGFR mutations by the PNA-LNA PCR clamp method but only 7 of 10 patients have been detected EGFR mutations by the conventional sequence method.
Design and synthesis of p53-MDM2 binding inhibitors
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The tumor suppressor p53 is a powerful anti-tumor molecule, and the activation of p53 by antagonizing its negative regulator MDM2 (murine double minute 2) or HDM2 (human double minute 2) might offer a new therapeutic strategy. Nutlins were one of the most potent small molecular inhibitors of p53-MDM2 binding, which demonstrated promising bioactivity in vitro and in vivo.

In principle of optimize the bioavailability and/or pharmacokinetic property of Nutlins, modifications were carried out on N-1 subsistent position, while 2, 4, 5-triphenyl of Nutlins was maintained to keep its MDM2 inhibitory. All the designed compounds were synthesized and tested for their cytotoxic activities in vitro against several human cancer cell lines including PC3, KB, K562, A549 and HCT116, most of these compounds exhibited potent cytotoxic activities. ADMET study forecasted that some of the compounds had better pharmacokinetic properties. Among them, compound L2, exhibited good pharmacokinetic property and the most potent cytotoxic activity, with IC50 value ranging from 0.96 μM to 6.86 μM against a variety of cancer cell lines.

![Chemical structures](image)

Figure 1

Immuno-precipitation study was carried out to testify if L2 has MDM2 inhibitory. Result showed that L2 has equal MDM2 inhibitory as Nutlin-1 (Figure 1). Cell-cycle distribution analysis on human non-small lung cancer cells A549 also showed that L2 acted on the G0/G1 phase and G2/M phase of the cell cycle arrest. Cell apoptosis study turned out that Nutlin-1 induced A549 cell apoptosis by 33.31% at 12 μM while L2 induced apoptosis by 68.78% at the same concentration.

Above results demonstrated that L2 could induce both cell apoptosis and MDM2 inhibitory. Interestingly, L2 induced more cell apoptosis than Nutlin-1 while they have the same ability in MDM2 inhibitory. This indicated that, besides the MDM2 inhibitory, L2 might take part into other oncogene pathways. Further study is under investigation.

Keywords: p53-MDM2, Binding inhibitors, Small molecule, MDM2 inhibitory

References

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With the advent of a post genome era, clinical studies associated with drug development are likely to change drastically. In particular, phase-1 clinical studies, where healthy adults are enrolled as trial subjects, noticeable improvements are expected. With the introduction of pharmacogenomics, evidence-based medications based on genome information will become available and result in improved safety and efficacy.

Clinical investigations into single nucleotide polymorphisms (SNPs) in drug metabolism have already been set out for clinical trials in classified subject groups of extensive metabolizers or poor metabolizers. In particular, the frequency of CYP2C19 in poor metabolizers within the Japanese population is relatively high (approximately 20%), and genetic variations result in differences in kinetics and pharmacological action, e.g. clinical response to proton pump inhibitors which are mainly metabolized by 2C19 in the liver.

We introduced a novel fully-automated genotyping system and applied it for the genotyping of CYP2C19. The completed system is based on the analysis of a melting curve of probe DNA bound to the target SNP site using a fluorescence quenching probe. The system enables automated and multiple SNP-genotyping from sample preparation. This full analyzing automation system can be translated to clinical studies, e.g. classification by genetic variations of metabolizing enzymes or transporters.
Synthesis and biological evaluation of pentacyclic triterpenes as anti-tumor agents

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Pentacyclic triterpenes are widely distributed throughout the plant kingdom. A variety of biological properties have been ascribed to pentacyclic triterpenes including anti-inflammatory, hepatoprotective, gastroprotective, anti-ulcer, anti-HIV, anti-cancer, anti-diabetic, cardiovascular, hypolipidemic, antiatherosclerotic and immunoregulatory effects. Recently, there is a growing interest in the elucidation of chemotherapeutic potential of naturally occurring pentacyclic triterpenes for the prevention and treatment of cancer. Interest in developing even more potent anti-cancer agents based on pentacyclic triterpenes has led to the discovery of a series of highly active synthetic triterpene derivatives such as CDDO and related compounds which are being evaluated in clinical and/or pre-clinical trials. While the mechanism of action has not been fully determined, it has been shown that some pentacyclic triterpene analogs act at various stages of tumor development, including inhibition of tumorigenesis, inhibition of tumor promotion, and induction of tumor cell differentiation. Herein, we report semi-synthesis of a series of natural and synthetic pentacyclic triterpenes as potential chemopreventive and chemotherapeutic agents against tumors.
Drug discovery and therapeutics using silkworm as experimental animal

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Genome Pharmaceuticals Institute is the bio-venture company of cooperation between industry and academia that is established to rely on Prof. Sekimizu's research to put into practical use. We aim to develop novel drugs using silkworm as experimental animal. We previously constructed various disease silkworm models including bacterial (¹) or virus (²) infection, diabetes (³) and so on.

In spite of its appearance, silkworm takes after human in lots of ways such as analogous tissues or organs, similar sensitivities to pathogens and comparable effects of drugs, and it is low in cost, in little conflict with ethical problem and in no danger of biohazard. In addition, silkworm can be injected into not only hemolymph but also midgut. Therefore, silkworm is excellent tool for drug discovery and therapeutics.

We recently search for antibiotics using bacterial infection model (⁴) and develop “evidence-based” functional foods or supplements using silkworm, especially that activate natural immunity using muscle contraction assay (⁵-⁷). Moreover, we propose safety test of agricultural products, foods and environments using silkworm as “coal mine canaries” (⁸).

References

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4. This work is supported by funds from National Institute of Biomedical Innovation of Japan (NIBIO).
7. This work is supported by funds from Imagine Global Care Corporation.
8. This work is supported by funds from Japan Science and Technology Agency (JST) and from MASIS, Inc.
Novel selective estrogen receptor modulators (SERMs) with unusual structure and biological activities

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The preparation of small molecules with cell regulatory activities—agonists, antagonists, drugs, etc.—is a major component of “chemical genomics” having as its ultimate goal the development of ligands capable of selective regulation of the activity of all important protein targets.

The estrogen receptors (ERs), members of the family of nuclear receptors, have emerged as attractive pharmaceutical targets for therapeutic intervention in a wide variety of diseases, including osteoporosis and breast cancer. Two receptor subtypes are now known, ERα and ERβ (1), and they have different tissue distribution patterns. Because of the known importance of ERα as a pharmaceutical target, and the potential importance of ERβ as well (2), molecules that act as agonists or antagonists selectively on one or the other of the ER subtypes are currently being investigated for their therapeutic potential; those whose activity also shows tissue selectivity, termed selective estrogen receptor modulators (SERMs), are of particular interest (3-7).

This presentation will cover the efforts to prepare small molecule regulators of increasingly broad biological activity including the structural and chemical, as well as elemental diversity oriented syntheses based on estrogen receptors in breast tumors, with an eye to opportunities that still lie ahead in the future. Three series of ligands for the estrogen receptor, based on a three-dimensional structural motif, heterocycles of fused bicyclic core, as well as the isoelectronic and isostructural replacement of a C=C bond with a B-N bond have been designed and synthesized. Some of the synthesized compounds display good binding affinity for the ERs, and in transcription assays, modulated transcriptional activities (agonist or antagonist) can be obtained by slight modification of the ligands.

References
Synthesis and properties of isonucleosides incorporated oligonucleotides

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Antisense technology exploits oligonucleotide analogs bind to target RNAs via Watson-Crick hybridization, and either disables or induces the degradation of the target RNA. This work focuses on the synthesis and properties of modified, 20-bp antisense oligonucleotides incorporated with isonucleoside 1, 2, 3 or 4. UV melting experiment showed that 3’-overhang isonucleoside modification slightly increased stability of duplexes, especially DNA/RNA duplexes. Oligonucleotides with 3’-overhang isonucleoside showed better stability than native one. Furthermore, 4 incorporated oligonucleotide at the center position showed prominent activity to activate RNase H.

Figure 1. Modified positions of AON, which are incorporated with 2a or 7a.

Figure 2. RNase H cleavage of duplex.

Figure 3. CD spectra of duplexes.

References
Isolation of antiviral compounds from plant resources using silkworm bioassay

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Silkworms are a useful animal model of infection from bacteria pathogenic to humans. Actually, antibiotics clinically used to treat infected humans are effective in silkworms.

Using the silkworm infection model, we screened the antiviral activity of Kampo medicines and found that cinnamon bark, a component of Mao-to, had a therapeutic effect. Based on the therapeutic activity, we purified the antiviral substance. Nuclear magnetic resonance analysis of the purified fraction revealed that the antiviral activity was due to cinnzeylanine, which was previously isolated from *Cinnamomum zeylanicum*. Cinnzeylanine inhibits the proliferation of herpes simplex virus type 1 in Vero cells. These results suggest that the silkworm baculovirus infection model is useful for screening antiviral agents that are effective for treating humans infected with DNA viruses.

Now we are screening other Kampo formulae and spices for therapeutic effects of antiviral activities.
Synthesis and structural modification of tasiamide and the effect of these modification on *in vitro* anticancer activity

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Marine cyanobacteria are well-known to be a rich source of bioactive peptides and depsipeptides with pharmaceutical potential. Tasiamide (1), an acyclic peptide isolated from the marine cyanobacterium *Symploca* sp. by Moore's group in 2002, has been found to be cytotoxic against KB and LoVo cells. Herein, we detail the first total synthesis, stereochemical reassignment and structural modification of tasiamide and the effect of these modification on *in vitro* anticancer activity.
Spirohexalines A and B, novel undecaprenyl pyrophosphate inhibitors produced by *Penicillium* sp. FKI-3368

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is known as a major nosocomial pathogen which has also developed resistance to many other antibiotics. Moreover, MRSA getting resistant to the last-resort antibiotic, vancomycin, has been reported. These facts suggest that *S. aureus* would fully acquire resistance to vancomycin in near future. Therefore, a new class of drugs with novel modes of action against MRSA is in great demand.

Undecaprenyl pyrophosphate synthase (UPPS) is involved in the synthesis of polyisoprenoid in bacteria. UPPS catalyze the sequential cis-condensation of 8 molecules of isopentenyl pyrophosphate (IPP) with farnesyl pyrophosphate (FPP) to generate C55-undecaprenyl pyrophosphate, which is required as a lipid carrier of glycosyltransfer in the biosynthesis of a variety of cell wall polysaccharide components in bacteria. Therefore, UPPS is considered as a new target for development of antibacterial agents.

During the course of screening for UPPS inhibitors in the enzyme based assay system, the culture broth of *Penicillium* sp. FKI-3368, isolated from soil collected in Oahu Island, Hawaii, USA was found to show the potent inhibitory activity of the enzyme, and a novel compound designated spirohexaline B (2) together with viridicatumtoxin (spirohexaline A (1), Figure 1) were discovered from the fermentation culture of the fungus.

Both 1 and 2 dose-dependently inhibited UPPS activity with IC50 of 4.0 μM and 9.0 μM, respectively. Antimicrobial activity against 16 species of microorganisms was measured by the agar dilution method. Compound 1 exhibited potent antimicrobial activity against gram positive bacteria including clinically isolated MRSA with an MIC (μg/mL) range of 0.39 to 1.56, but showed no effect on gram negative bacteria. Compound 1 and 2 showed similar antimicrobial activity. But only 2 inhibited the growth of *Mycobacterium smegmatis*.

![Figure 1. Structures of spirohexalines A (viridicatumtoxin) and B.](image_url)

<table>
<thead>
<tr>
<th>Spirohexaline</th>
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<tbody>
<tr>
<td>A (viridicatumtoxin)</td>
<td>NH₂</td>
</tr>
<tr>
<td>B</td>
<td>CH₃</td>
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</tbody>
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Nosokomycins, novel anti-MRSA antibiotics, produced by *Streptomyces* sp. K04-0144

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**Background** Methicillin-resistant *Staphylococcus aureus* (MRSA) is now widespread, particularly in the hospital and hospital infection is a serious social problem. Therefore, it is important and necessary to find new antimicrobial agents and to devise new measures that are effective against MRSA infection. Our research group conducted the screening system for anti-MRSA compounds from microbial metabolites using silkworm larvae as an animal model.

**Materials & Methods** When MRSA (2.5×10⁷ CFU/larva) was injected into the blood of silkworm larvae (5th instar), almost all larvae (> 90%) of the larvae died within 3 days. An injection of vancomycin (1 mg/larva) proved effective in this MRSA-infection model (silkworm model), allowing the larvae to survive. Under the conditions, culture broths of actinomycete and fungal strains (over 7,000) were screened for anti-MRSA compounds.

**Results** In the course of this screening program, *Streptomyces* sp. K04-0144, isolated from a soil sample collected at Ishigakijima Island, Japan, was selected. From solid phase extracts of the 6 day-old culture broth of the strain, four active compounds designated nosokomycins A to D were purified by ODS column chromatography and HPLC using an ODS column. The structures of nosokomycins were elucidated by various NMR experiments and MS spectra. They are phosphoglycolipids structurally related to moenomycin A. An injection of nosokomycin A or B (50 mg/larvae) was effective in the silkworm model. Furthermore, nosokomycin A also proved active in MRSA-infected mice (mouse model). Other biological properties of nosokomycins are also presented.

**Discussion** Nosokomycins, showing anti-MRSA activity, were found to be effective not only in the silkworm model but also in the mouse model. Infection models using silkworm larvae are useful for screening works and drug evaluation as alternative *in vivo* models.

![Structures of nosokomycins and moenomycin A](image_url)
In vivo screening for antimicrobial activity of Thai Herbal Medicines using silkworm model

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Injection of *Staphylococcus aureus* or *Candida albicans* that are pathogenic to humans into silkworm hemolymph resulted in death of the larva within 2 days. The extracts of Thai herbal medicines from *Bauhinia malabarica* Roxb., *Sophora exigua* Craib, *Centella asiatica* Urban, *Azadirachta indica* (L.) Juss., *Nelumbo nucifera* Gaertn. had therapeutic effects on silkworms infected with *S. aureus*. The 50% effective doses of the extracts obtained by injection into the silkworm hemolymph were 0.59, 2.25, 2.10, 0.34 and 1.94 mg/g of larva, respectively. In addition, *Bauhinia malabarica* Roxb. and *Sophora exigua* Craib also had therapeutic effects on silkworms infected with *C. albicans*. The 50% effective doses of the extracts were 0.71 and 0.20 mg/g of larva.

**Keywords:** Antimicrobial, Herbal extracts, Silkworm
Nitric oxide (NO) is known as a biomarker for the study of anti-aging as the NO will be induced and released from the cell with the treatment of Traditional Chinese Medicines (TCM). Therefore, the development of a high sensitive NO is desirable for the detection and monitoring of NO concentration in the screening of anti-aging TCM. In this work a novel electrochemical sensor of nitric oxide has been successfully developed and potentially used for screening the anti-aging active components in TCM. The sensor was prepared by electrochemical deposition of carbon nano tube (CNT)-nickel composite sensing-film on the glass carbon electrode and coating with a thin Nafion layer on the top. The nano-nickel/CNT sensing-film showed a high sensitivity to the electrochemical oxidation of nitric oxide, as shown in Figure 1. The Nafion film eliminated the interferences and improved the selectivity. It has been demonstrated that the NO sensor can be used for the determination of NO released from drug with the linear concentration range from $8.0 \times 10^{-8}$ to $1.2 \times 10^{-4}$ M and the limit of detection (LOD) of $2.0 \times 10^{-8}$ M. The sensor has great potential used for the screening of active components in anti-aging TCMs.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Differential pulse voltammetry of bare GC electrode (a) and CNT/Nickel composite sensing film electrode (b) in the presence of $6 \times 10^{-6}$ M NO in PBS.}
\end{figure}

**Acknowledgement**

This work was supported by the National Scientific Foundation of China (NSFC Grant No: 20775055) and the start-up funding for ZC’ Luojia Professorship of Wuhan University (Grant No: 306276216).
Polysacchride from green tea purified by silkworm muscle contraction assay induces innate immunity by increasing the expression of various inflammatory cytokine mRNA in human leukocytes

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⁴ Genome Pharmaceuticals Institute Co., Ltd.

We recently developed a method to quantify innate immunity by measuring muscle contraction of silkworms (Ishii et al. J Biol Chem 2008; 283:2185-2191.). In this study, we demonstrated that hot water extract of green tea showed activity in muscle contraction assay. Green tea polyphenols [catechin and polyphenon (60)], which have been reported to modulate immunity, did not induce muscle contraction. Neither plant cellulose nor chitin did induce the contraction. Thus active substance(s) is different from these known ones. After a series of purification processes by ethanol precipitation, gel filtration, ion exchange chromatography, and superdex 200 HR chromatography, we found that the active substance was high molecular weight (> 669 kDa). The increase in specific activity at each step was evaluated by measuring activity by the muscle contraction assay and sugar content by the phenol sulphuric acid method. The analysis of the final fraction revealed that the activity measured by the muscle contraction and sugar contents comigrated on DEAE-cellulose chromatography and gel filtration with superdex 200HR. The activity of the purified fraction was lost by acid hydrolysis. This is the first report for an immune stimulant purified from green tea by using the muscle contraction assay. The purified product induced the production of IL-6 from mouse macrophage cells. Furthermore, it induced the expression of INF gamma, CCL20, IL6, IL10, IL8, TNFSF15, p21 and TNF alpha mRNA in human leukocytes. These results suggest that the immune stimulant purified from green tea is active against a wide spectrum of species including human mouse and silkworm.
Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities

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Flavonoids are polyphenolic compounds that are widely exist in plant kingdom, and the structure-activity relationship (SAR) of 24 flavonoids was studied on neuraminidase (NA) activity of influenza virus. Three typical influenza virus strains A/PR/8/34 (H1N1), A/Jinan/15/90 (H3N2) and B/Jiangshu/10/2003 were used as the source of NAs, the average of IC_{50}s of these compounds on these NAs was used in the SAR analysis. The order of potency for NA inhibition was as follows: aurones > flavon(ol)es > isoflavones > flavanon(ol)es and flavan(ol)es. The SAR analysis of flavonoids on influenza virus NAs revealed that for good inhibitory effect, the 4'-OH, 7-OH, C4=O and C2=C3 functionalities were essential, the presence of a glycosylation group greatly reduced NA inhibition. The in vitro anti-viral activities of 8 flavonoids were evaluated using a cytopathic effect (CPE) reduction method, the assay results confirmed the SAR as influenza virus neuraminidase inhibitors. The findings of this study provide important information for the exploitation and utilization of flavonoids as NA inhibitors for influenza treatment.

Keywords: Influenza virus, Neuraminidase, Flavonoids
Mechanisms and consequences of phagocytosis of influenza virus-infected cells

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The death of cells infected with viruses has been documented for many years and, until recently, was considered to be caused by viruses when their progeny burst out from host cells. However, the current understanding is that virus-infected cells undergo apoptosis, a physiological mode of cell death. Viruses manipulate the host cell's machinery for protein synthesis to propagate and produce progeny. The death of host cells upon infection should hamper the replication of virus and thus serves as a defense mechanism. However, accomplishment of the apoptotic process takes time, and some types of viruses may complete replication before the protein synthesis machinery breaks down. To overcome this problem, apoptosis in host cells serves another purpose in acting against viral invasion. In general, apoptotic cells become susceptible to phagocytosis, a biological event where a type of cell known as phagocyte captures, engulfs, and in most cases digests other cells. Cells undergoing apoptosis are recognized and engulfed by phagocytes at an early stage of the apoptotic pathway. If infected cells are phagocytosed and digested before the completion of viral replication, the dissemination of viruses and the development of viral diseases may be prevented.

In 1993, we reported for the first time that influenza virus-infected cells are induced to undergo apoptosis and subsequently found that such cells become susceptible to phagocytosis. Data from our in vitro and in vivo experiments have suggested that 1) alveolar macrophages and neutrophils phagocytose influenza virus-infected cells in an apoptosis-dependent manner; 2) the membrane phospholipid phosphatidylserine and viral neuraminidase-processed carbohydrates at the surface of target cells and phagocytes, respectively, are involved in the association of the two types of cells; and 3) phagocytic elimination of virus-infected cells leads to a reduction in the pathogenesis of influenza.

A large number of deaths due to influenza have been reported for many years worldwide, and new pandemics of influenza are predicted. Due to high genetic variability, the development of vaccines and antiviral agents against influenza virus faces difficulties. New strategies are thus needed for developing novel antiviral agents, and our findings suggest that one aiming at a raise in the level of phagocytosis of influenza virus-infected cells is a good candidate. Substances could be developed that augment either the susceptibility of virus-infected cells to phagocytosis or the phagocytic activity of macrophages and neutrophils. It is apparent that the mechanism underlying the phagocytosis of influenza virus-infected cells is not unique, but at least a part of it is common to the phagocytosis of all apoptotic cells. Therefore, the development of such antiviral agents depends on how soon we gain a deeper understanding of the mechanism of the phagocytic clearance of apoptotic cells.
Nuclear export inhibitors; a possible target for novel anti-influenza viral drugs

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² Central Research Center, AVSS Corporation 1-22, Wakaba-machi, Nagasaki 852-8137, Japan.

A virus is a unique pathogen which is incapable of replicating without a host cell. It utilizes the host cell environment and cellular factors for its propagation. The development of antiviral drugs is an important strategy; however, some viruses, especially RNA viruses, easily mutate to escape drugs designed to recognize viral gene products. Influenza virus resistant to amantadine (an inhibitor of viral M2 protein), is already spreading all over the world. In addition, the abuse of tamiflu, a newly developed neuraminidase inhibitor, has already resulted in the development of resistant influenza viruses. The development of novel drugs which target cellular proteins is therefore essential to make antiviral therapy more effective. Anti-viral drugs which target cellular factors may evoke less resistant viruses.

We focused on the nuclear import and export processes of virus infected cells. Nuclear import and export of proteins are among the most important processes for cellular homeostasis. The nuclear transport of proteins seems to be an attractive target for antiviral therapy because viruses such as human immunodeficiency virus (HIV) and influenza viruses that utilize this environment for their propagation. The transport of the RNP complex of influenza virus through the nuclear pore is an active process because the large complex (> 40 kDa) pass through the nuclear pore requires nuclear transport factors. Specific signals such as the nuclear localization signal (NLS) and nuclear export signal (NES) directly bind to the nuclear transport factors, importin and CRM1, respectively. All of the vRNP components (PB1, PB2, PA and NP) and a vRNP-associating factor, matrix protein (M1), have NLS, thus the nuclear import of vRNP is mediated by their viral NLSs. In contrast to the nuclear import process, less is known about the nuclear export of vRNP. M1 and vRNP components do not have NES. Only viral NS2 protein has NES, however, evidence suggests the existence of other host factors essential for the nuclear export of vRNP (Watanabe et al., Virus Res 2001; 77:31.). We explored the M1 binding host protein and determined to be heat shock cognate protein 70 (hsc70; Watanabe et al., FEBS Lett 2006; 580:5785.). The knockdown of endogenous Hsc70 resulted in the reduction of the viral production. Hsc70 also has NES. These results suggest that NES-containing protein such as Hsc70 could be a candidate for developing novel anti-influenza virus drugs. LMB is a CRM1-dependent nuclear export inhibitor and a recent study showed that LMB inhibits nuclear export of influenza viral RNP and viral production (Watanabe et al., Drug Discov Ther 2008; 2:77.).

To screen drugs which influence nuclear export of RNP complex, a cell system was established which expresses NES-GFP (Watanabe et al., Drug Discov Ther 2008; 2:7.). This system can therefore be used to easily detect the inhibitors of nuclear export. For example, the effect of 30 pg/mL LMB was detected within 10 min of treatment.
Catalytic asymmetric synthesis of oseltamivir phosphate directing toward its stable worldwide supply

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Oseltamivir phosphate (Tamiflu™) is an extremely important anti-influenza drug, which could protect humans against lethal flu pandemic. We developed a 12-step synthesis of oseltamivir phosphate using the asymmetric Diels-Alder type reaction between a siloxy diene and a fumarate catalyzed by a Ba-FujiCAPO complex. The asymmetric catalyst facilitated the reaction through the activation of the diene. Our synthesis may contribute to the stable worldwide supply of oseltamivir phosphate.
Clinical effects of probiotic bifidobacterium in the prevention of influenza virus infections and allergic diseases

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Probiotics are currently defined as “live microorganisms which when administrated in adequate amounts confer a health benefit on the host”. Bifidobacteria and lactobacilli are among the most well known members of probiotics. *Bifidobacterium longum* BB536 is a probiotic strain originated from a healthy infant and has being broadly applied in food industry and well demonstrated for its efficacy in the prevention and treatment of a broad spectrum of animal and/or human disorders. The present presentation shows the clinical results on the effects of BB536 in the prevention of influenza virus infections of elderly, and in the prevention and treatment of Japanese cedar pollinosis.

In a double-blind, placebo-controlled trial, 27 elderly volunteers (aged 65 or older) were administrated BB536 for 6 weeks and vaccinated with the influenza vaccine at week 3. After assessment for levels of antibody titers to influenza vaccine at the week 6, the subjects were divided into two groups given either BB536 or placebo for another 14 weeks. It was found that the occurrence of influenza virus infection and fever during the 14 weeks was significantly lower in BB536 group compared to placebo group. NK activity and the bactericidal activity of neutrophils were enhanced by BB536.

Japanese cedar pollinosis (JCPsis), an immunoglobulin E (IgE)-mediated type I allergy caused by exposure to Japanese cedar pollen (JCP), represents a public health issue affecting over 20% of the Japanese population. In a randomized, double-blind, placebo-controlled trial, 44 JCPsis subjects received BB536 or placebo for 13 weeks during the pollen season. BB536 intake was associated with a significant reduction in subjective symptom scores. BB536 intake suppressed a Th2-skewed immune response occurring along with pollen dispersion. It was found that some intestinal bacteria such as the *Bacteroides fragilis* group fluctuated significantly during the pollen seasons and BB536 intake suppressed the fluctuation.

These results demonstrated the immuno-modulating effects of BB536 and efficacy in the prevention of influenza virus infections and in the prevention and treatment allergic disorders.
Production of anti-influenza PR8-scFv using a phage display

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2 Dept. of Infectious Pathology, National Institute of Infectious Diseases.

Introduction The influenza virus alters the antigenic properties of its surface hemagglutinin (HA) so as to avoid being affected by preexisting immunity. In recent years, highly pathogenic avian influenza has caused death in humans as well as in birds. Therefore, development of drugs effective against influenza is expected because of the limited drugs that can be used for influenza treatment. Phage display technology has been developed so that antibody variable domains VH and VL can be easily combined, thus making the production of antibody molecules possible. In our previous study, anti-influenza A/PR/8/34(PR8)-Fab was produced from a phage display library and displayed inhibitory action on virus infection. In this study, we attempted to use a phage display to produce single-chain antibodies from an anti-PR8 hybridoma in order to examine the clinical applicability of an anti-influenza single-chain antibody (scFv).

Methods mRNA was extracted from an anti-PR8 HA hybridoma, a fusion of myeloma cells with spleen cells from PR8-immunized mice. After cDNA was formed through reverse transcription, the genes that encode VH and VL were amplified by PCR and a VL-LINKER-VH probe was made using assembly primers. This was then incorporated into the phagemid vector pCANTAB 5E and transformations were carried out in JM109 E.Coli strains. Gene-containing strains were infected with an M13K07 phage, and phage antibodies that were anti-PR8-scFv were produced. ELISA was used to screen phage antibodies. The DNA sequence of positive clones was examined.

Results Binding probes were made by combining VH and VL because only VL existed after the amplification of the VL probe from the anti-PR8 hybridoma. According to the results from transformation and ELISA, 2 clones from phage antibodies showed binding affinity to antigen PR8-HA. Further examination of both clones led to the confirmation of a high degree of homology in their DNA sequences.

Perspective This study succeeded in making 2 clones of anti PR8-scFv from an anti-PR8 hybridoma. Therefore, virus inhibition by anti-PR8-Fab (produced in a previous study) is anticipated in MDCK cells, as is anti-viral activation by the recently produced scFv. We will continue research to make scFv antibody phages and we will examine their effects on defenses in the body and also study their affinity to various strains other than PR8.
Emerging infectious diseases and anti-viral drugs: Urgent need to develop effective drugs which cause less resistant virus

Nobuyuki Kobayashi\textsuperscript{1,2}

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  \item \textsuperscript{2} Central Research Center, AVSS Corporation 1-22, Wakaba-machi. Nagasaki 852-8137, Japan.
\end{itemize}

Viral diseases caused by pathogenic virus infections which have high morbidity and mortality rates are still the leading cause of death in humans worldwide. Although effective vaccines have led or might lead to the eradication of important viral pathogens such as smallpox, polio, and mumps, other viral diseases such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) have proven difficulty to combat using the conventional vaccine approach. However, unlike anti-bacterial drugs, the development of anti-viral drugs are still in the middle of progress. In past 50 years we have encountered with many emerging infectious diseases but we have not succeeded in eliminating these emerging infectious agents with anti-viral agents. More efforts should be addressed to develop anti-viral agents. I would like call researchers as well as pharmaceutical companies to join with us to develop anti-viral drugs.

Today, I would like to overview the present development of anti-viral drugs and the importance of setting up anti-viral screening system I would also briefly talk on our present approach to develop anti-influenza viral drugs which cause less resistant viruses.
Design, synthesis and antiviral evaluation of novel heterocyclic compounds as HIV-1 NNRTIs

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The acquired immune deficiency syndrome (AIDS) continues to be a major health problem worldwide with approximately 40 million people infected with the human immunodeficiency virus (HIV). Although the introduction of highly active anti-retroviral therapy (HAART) has dramatically decreased the morbidity and mortality from the infection by HIV, however, the AIDS prevalence remains one of the world's most serious health problems.

In the research of anti-AIDS agents, non-nucleoside inhibitors of reverse transcriptase (NNRTIs) have gained a definitive and important place due to their unique antiviral potency, high specificity and low toxicity. Currently marketed NNRTIs include efavirenz, nevirapine, delavirdine, and etravirine. Efavirenz and nevirapine have good pharmacokinetic profiles and effectively inhibit replication of the wild-type virus, but they are less effective against several commonly observed mutant viruses, such as Y181C, Y188C, K103N, and L100 A. Etravirine shows improved potency against many NNRTI-resistant viruses, but must be administered twice daily and is approved for use only in patients infected with HIV-1 strains resistant to an NNRTI and other antiretroviral agents. Therefore, it is a need for new NNRTIs that are active against virus strains resistant to current NNRTIs and have good pharmacokinetic properties suitable for once daily dosing.

1. **Substituted 1,1,3-trioxo-2H,4H-thieno[3,4-e][1,2,4]thiadiazine derivatives (TTDs)**

Substituted 1,1,3-trioxo-2H,4H-thieno[3,4-e][1,2,4]thiadiazine derivatives (TTDs) represent a new class of specific HIV-1 NNRTIs, and have been found to effectively inhibit the replication of a variety of HIV-1 strains at the reverse transcription steps, including strains that are resistant to AZT, but not against HIV-2 (ROD).

In an effort to elucidate the structure-activity relationships (SARs), a variety of new analogues of 2,4-disubstituted-1,1,3-trioxo-2H,4H-thieno[3,4-e][1,2,4]thiadiazines were synthesized and evaluated for their activity against HIV-1 (HIVB) replication in MT-4 cell culture. The most active compound with the highest selective index was the N2-3-F-benzyl, N4-2-Cl-benzyl derivative (14o). As a new lead compound, it displayed an EC50 of 0.19 μM, an EC90 of 0.39 μM, a CC50 of 133.2 μM, and an SI of 704 against HIV-1 replication. In another series, compound 12t [2-(3,5-dichloro)benzyl, 4-cyanomethyl derivative] showed the most potent activity, exhibiting an EC50 of 0.18 μM, EC90 of 0.49 μM, CC50 of 126.3 μM and SI of 700 against HIV-1 replication. Both compounds 14o and 12t as lead compounds were thought to be further developed. 3D conformational structures (drawn by SuperChem 5.0) of the new lead compounds, possessing two π system moieties: benzyl and cyanomethyl/or benzyl, and one carbonyl group between them, seem to be strongly related to the reproduction of the structural elements described in the Schafer's 3D model.

![Figure 1. The newly discovered bioactive molecules from TTD series.](image)

2. **Substituted 7-methylpyrazolo[4,5-e][1,2,4]thiadiazines (PTDs) series**

![Figure 2. The newly synthesized N2/N4-monosubstituted and N2,N4-disubstituted PTDs.](image)
Recently, aimed at the discovery of new HIV-1 NNRTIs, we undertook a study of the 2,4-disubstituted-7-methyl-1,1,3-trioxo-2H,4H-pyrazolo[4,5-e][1,2,4]thiadiazines (PTDs) series, because of the known bioisosterism between TTDs and PTDs. We had reported the regioselective method and one-pot reaction for synthesis of \( N_2/N_4 \)-monosubstituted and \( N_2,N_4 \)-disubstituted PTDs. However, their anti-HIV screening results were not encouraging.

3. \( N_1,N_3 \)-disubstituted-thieno[2,3-e][2,1,3]thiadiazin-4-one 2,2-dioxides (TTDDs) and pyrazolo[4,5-e][2,1,3]thiadiazin-4-one 2,2-dioxides (PTDDs)

There are several reports indicating that 1,3-disubstituted-2,1,3-benzothiadiazin-4-one 2,2-dioxides (BTDs) is a type of novel HIV-1 NNRTIs which exhibit potent anti-HIV-1 RT activity. Some lead compounds of BTDs were found to inhibit the HIV-1 at low concentration with good selectivities. Based on the general crystal structure of the HIV-1 RT complexed with NNRTIs, which is like a “butter-fly” type, and furthermore, TTDs and BTDs are served as templates, and according to the general principle of bioisosteric replacement in medicinal chemistry, we designed and synthesized a series of novel \( N_1,N_3 \)-disubstituted-thieno[2,3-e][2,1,3]thiadiazin-4-one 2,2-dioxides (TTDDs) and \( N_1,N_3 \)-disubstituted pyrazolo[4,5-e][2,1,3]thiadiazin-4-one 2,2-dioxides (PTDDs).

The anti-HIV-1 activities of TTDDs series were evaluated by inhibition of HIV-1(IIIB)-induced cytopathogenicity in MT-4 cell culture. The most active and selective compound was \( 1-(3\text{-}\text{Cyano})\text{benzyl}-3\text{-}\text{benzyl}\text{-thieno[3,2-c][1,2,6]} \text{thiadiazin-4(3H)}\text{-one 2,2-dioxide} \) with an EC\(_{50}\) value of 4.0 \(\mu\)M and SI > 76.

The preliminary activity and cytotoxicity screening of the newly designed and synthesized PTDDs derivatives were tested in MT-4 cells for inhibition of HIV-1 and in HEL cells for inhibition of HCMV. The screening results indicated that these compounds did not exhibit inhibitory activity against HIV-1, but several compounds exhibited certain anti-HCMV activity. Among the evaluated compounds, \( N_1(2,4\text{-Dichlorobenzyl})-N_3\text{-benzyl-one} \) emerged as the most active HCMV inhibitor with EC\(_{50}\) = 4 \(\mu\)M and CC\(_{50}\) = 41.7 \(\mu\)M.

4. 1,2,3-thiadiazole thioacetanilides (TTA) and imidazole thioacetanilides (ITA)

Recently, from high-throughput screening (HTS) of compound libraries, several interesting sulfanyltriazole and sulfanyltetrazole-typed leads were identified as novel and potent HIV-1 NNRTIs, which have a simple, yet distinctively
different chemical structure from the other HIV-1 NNRTIs reported in the literature.

In order to further delineate the importance of the five-membered heterocycle, a series of novel \(N\)-substituted phenyl-2-(4-aryl)-1,2,3-thiadiazol-5-ylthio)acetamide (TTA) and \(N\)-substituted phenyl-2-(1-aryl-1H-imidazol-2-ylthio)acetamide (ITA) derivatives were designed and synthesized based on the general principle of bioisosterism in medicinal chemistry.

The preliminary activity and cytotoxicity screening of TTA and ITA series were tested in MT-4 cells for inhibition of HIV-1 (strain IIIB) and HIV-2 (strain ROD). The screening results indicated that many compounds exhibited potent inhibition activity against HIV-1 and none of the compounds exhibited inhibitory activity against HIV-2. Among the evaluated TTA series, \(7f2\) (EC\(_{50}\) = 0.059 \(\mu\)M, CC\(_{50}\) > 283.25 \(\mu\)M, SI > 4883), \(7f6\) (EC\(_{50}\) = 0.099 \(\mu\)M, CC\(_{50}\) > 228.41 \(\mu\)M, SI > 2298), \(7f4\) (EC\(_{50}\) = 0.149 \(\mu\)M, CC\(_{50}\) ≥ 146.88 \(\mu\)M, SI ≥ 981), \(7f5\) (EC\(_{50}\) = 0.204 \(\mu\)M, CC\(_{50}\) > 255.50 \(\mu\)M, SI > 1265), and \(7f1\) (EC\(_{50}\) = 0.118 \(\mu\)M, CC\(_{50}\) = 111.43 \(\mu\)M, SI = 943) emerged as the most active HIV-1 inhibitors in 1,2,3-thiadiazole thioacetanilides series, which provide useful information for further investigation of 1,2,3-thiadiazole thioacetanilides. Among the evaluated ITA series, \(5h1\) emerged as most active derivatives with EC\(_{50}\) = 0.2 \(\mu\)M and CC\(_{50}\) = 35.24 \(\mu\)M. Moreover, the anti-HIV activity against an NNRTI-resistant strain of the newly synthesized derivatives is in progress.

Acknowledgements
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On the basis of experience on antibiotics research, we have developed screening systems for biologically active compounds from microbial products. To find novel and useful compounds from culture extracts, we have focused on phenotypic assays which might have characteristic events like mitogen-induced lymphocyte proliferation and typical morphological change of fungi.

As a consequence, two compounds, Tacrolimus (immunosuppressant, FR900506) and Micafungin (antifungal, a derivative of FR901379) have been developed and accepted as clinically useful.

The landscape of antiviral research from natural products seems to be somewhat different from our previous fields from the aspects of virus replication cycle utilizing various cell machineries and the pathogenesis in specific host.

Observations of cytopathic effect (CPE) triggered by a specific virus strain combined with an appropriate host cell has been employed on screening antiviral compounds from library source. As a result, many glycoprotein processing inhibitors have been discovered, including functional modulators of ER or Golgi apparatus. Also by using recombinant cells expressing a particular viral glycoprotein like HIV gp120, some unique inhibitors acting on CD4-gp120 fusion event have been identified.

As in vitro cell culture system is not available in certain viruses, unique cell-based assay systems have been recently established, for example HepG2.2.15 for hepatitis B virus and replicon systems for hepatitis C virus. These systems with quantitative PCR have been well defined and include characteristic viral replication steps. Not only from biochemical procedure, with such methods, novel interaction of viral protein with host protein could be revealed from the studies on mechanism and action of compounds derived from screening outcome.

Apart from drug design based on well characterized targets, applying well-established cell-based assays preserving intact intracellular environment could provide us crucial clue to the new strategy and target for creating useful antiviral drugs.
Viral factors that determine the natural course of chronic hepatitis B viral infection

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The natural history of chronic hepatitis B virus (HBV) infection is divided into immune tolerant, immune clearance and non-replicative phases. Most patients with chronic hepatitis B enter non-replicative phase after immune-clearance phase. On the contrary, about 5% of patients with chronic HBV infection suffer advanced liver disease before entering the non-replicative phase. The discrimination of these two groups is very important.

HBV genotype is a useful marker for discriminating the two groups. In eastern Asian countries, genotype C is associated with more severe liver disease than genotype B. As regards antiviral treatment, genotype C is associated with a lower response rate to interferon and lamivudine.

Nucleic/amino acid substitutions in the precore and basic core promoter regions are important for predicting final outcome. The substitutions modify transcription and translation of pregenomic and preC mRNA and have a close relation to clinical outcome.

Besides these factors, we recently found that the number and position of substituted amino acids in the core region may be related to final outcome of chronic HBV infection. The average numbers of substituted amino acids in the core region decreased with the progression of disease. The majority of substituted amino acids are located in the immunological epitopes (CD4, CD8 and CTL epitopes).

These viral markers may be useful for discriminating patients who may develop advanced liver disease and need antiviral treatment.
Effect of andrographolide derivatives having $\alpha$-glucosidase inhibition, on HBsAg, HBeAg secretion in HepG2 2.2.15 cells

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Andrographis paniculata is a plant indigenous to Southeast Asian countries that has been used as an official herbal medicine in China for many years. Extracts of the plant and their constituents have been reported to exhibit a wide spectrum of biological activities of therapeutic importance including antibacterial, antiviral, anti-inflammatory, antimalarial, immunostimulant, hepatoprotective, antithrombotic, anticancer and hypotensive properties. The plant extract is known to contain diterpenes, flavonoids and stigmasterols. Diterpenoid chemicals are the primary constituents present in the extracts of A. paniculata, where andrographolide (1), a bicyclic diterpenoid lactone, is the major constituent. Extensive research of the last few decades has revealed that andrographolide is useful as an anti-inflammatory drug for the treatment of laryngitis, diarrhea, and rheumatoid arthritis. Andrographolide is very potential as antiviral, antithrombotic, anticancer and hypoglycaemic agent, too. Recently, andrographolide, neoandrographolide and 14-deoxy-11,12-didehydroandrographolide, ent-labdene diterpenes isolated from A. paniculata were reported to show viricidal activity against herpes simplex virus 1 (HSV-1) and andrographolide, 4-deoxy-11,12-didehydro andrographolide were also reported to show anti-HIV (Human Immunodeficiency Viruses) activity. However, there is not any report about anti-HBV (Hepatitis B Virus) activity for andrographolide and its derivatives till now.

As we known, $\alpha$-glucosidase inhibitor not only have potential to be developed as anti-diabetes drug, but also have potential to be developed as anti-virus agent. In our previous work, a series of derivatives of andrographolides with inhibitory activities against $\alpha$-glucosidase were synthesized firstly. Here, the anti-HBV effect of derivates of andrographolide was studied by using HepG2 2.2.15 cells transfected with HBV DNA. HBsAg, HBeAg in supernatants were determined by ELISA. At the same time MTT method was applied for the detection of cytotoxicity of compounds, selecting Lamivudine (3TC) as control medicine. When HepG2 2.2.15 cells was treated by compounds at low cytotoxic concentration, the inhibitory effects of compounds on HBsAg, HBeAg were gradually enhanced with the increase of 2. This shows that 2 (an $\alpha$-glucosidase inhibitor) possesses the effect of anti-HBV in vitro, which mechanism is possible with its high inhibitory activity to $\alpha$-glucosidase.

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Current and future antiviral therapy for influenza

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Influenza is still a serious infectious disease that cannot be completely controlled. This is because the influenza virus alters the antigenic properties of its surface HA so as to avoid being affected by preexisting immunity. Moreover, avian influenza viruses such as the H5N1 virus that recently emerged in Southeast Asia can cause severe disease in humans (highly pathogenic avian influenza). The development of effective vaccines is necessary for the prevention of such influenza outbreaks. However, effective protection by vaccination cannot be expected in the event of a major change in the antigenicity of influenza. Therefore, new drug development to treat its acute stage is essential. This review summarizes recent vaccine development and antivirals for influenza.

Vaccines
To control influenza, trivalent inactivated vaccines containing strains of the three influenza viruses are licensed for parenteral administration and used in many countries. However, such vaccines have been found to be less effective against heterologous drift virus infection. In contrast, immunity acquired by natural infection provides more cross-protection against heterologous strains than that induced by vaccination. This is because secretory IgA Ab induced by infection can produced in the upper respiratory tract and widely protect against influenza challenge. Although intranasal immunization can induce IgA Ab in upper respiratory mucosa, this is not easily achieved. We have demonstrated that i.n. inoculation of influenza vaccine together with various mucosal adjuvants is effective in this regard. Here, we review intranasal vaccination and discuss the necessity for advanced mucosal adjuvants.

Targets for antiviral therapy
- Various medicines
Licensed anti-influenza drugs (the M2 ion channel blockers amantadine and rimantadine and the neuraminidase inhibitors oseltamivir and zanamivir) are helpful in treating uncomplicated influenza infection. However, influenza resistant to these drugs has already been noted. Some reports have indicated that the modified or combined usage of these drugs was effective, so such new uses will be discussed here.
- Antibody therapies
Treatment with anti-influenza virus antibodies could potentially be of benefit in severe influenza, preventing the binding of virions to target cells and marking infected cells for destruction by complement or T cells. That said, no immunoglobulin product is in clinical use. In a recent study, however, we found that anti-influenza Fab produced by phage display was able to inhibit cell binding by the influenza virus. Moreover, an anti-influenza scFv was produced. Therefore, the potential of antibody therapies will also be reviewed.
Establishment of an HIV-based pseudotyping system as a safe model for screening inhibitors on bird flu H5N1 entry

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Avian influenza virus H5N1 is a major concern as a potential global pandemic. It is thought that multiple key events must take place before efficient human-to-human transmission. The first step in overcoming host restriction is viral entry which is mediated by HA, responsible for both viral attachment and viral/host membrane fusion. HA binds to receptors containing glycan with terminal sialic acid (SA). This “bird flu” poses an increasing pandemic threat since there is no effective vaccine against this virus for humans. Furthermore, appearance of oseltamivir-resistant strains of H5N1 indicates that novel therapeutic treatments are urgently needed. Our major focus is to discover anti-H5N1 entry inhibitors and to develop entry therapeutics for H5N1 and other potential pandemic influenza viruses. Due to the safety concerns, research work using “live” H5N1 is carried out in the enhanced BSL-3 facilities. In this research, we have established an efficient HIV-based pseudotyping system which will be used for screening potential anti-H5N1 entry inhibitors with the BSL-2 facilities. By using this pharmacological compound screening system, we have found several “hits” which can specifically inhibit H5N1 high pathetic avian influenza viral entry.
Strategy of discovery for novel antibiotics using silkworm infection model

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Most of drug candidates obtained by in vitro screening have inappropriate properties as medicines because of problems of toxicity and pharmaco-dynamics in human bodies. Preclinical tests using animal models are essential for evaluation of therapeutic effects of drug candidates for further development. Mammalian models have been used to examine pharmaco-dynamics of chemicals. Not only high cost, but also a problem for ethical issues to sacrifice mammalian animals cause disruption of drug development. Use of invertebrate animals is expected to overcome these problems. We are proposing use of silkworms, bombyx mori, as model animals to evaluate the properties of drug candidates. Silkworm has fat body, which function is responsible for drug metabolism, as well as livers in mammalian animals. There is a number of cytochrome P450s and sulfur or glucose conjugation enzymes, which involved in detoxication of drugs, in the fat body.

In this symposium, we report that pathogenic microorganisms such as Staphylococcus aureus and Candida albicans, killed silkworm, and those antibiotics clinically used showed therapeutic effects. Effective doses (ED50) of antibiotics in silkworm infection model were similar to those in mammalian models. We also show that similar results could be obtained regarding to the availability of antibiotics by oral administration and distribution, drug metabolism, between silkworms and mammalian animals.
Potent neuraminidase inhibitors and anti-inflammatory substances from *Chaenomeles speciosa*

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**Aim** Applying the assay method of neuraminidase (NA) activity established for high throughput screening to find potent inhibitors of influenza virus NA from *Chaenomeles speciosa* and observing their anti-inflammatory activity in the murine macrophage cell line RAW264.7.

**Methods** Some compounds were isolated from *C. speciosa*, a traditional Chinese herb for the treatment of rheumatism. A high throughput screening model of NA inhibitor was applied to test the isolates. Then, the inhibitory effects of the substances on the proliferation of RAW264.7, the release of interleukin-6, tumor necrosis factor-alpha (TNF-α) and nitric oxide production in lipopolysaccharide (LPS) activated macrophages were examined.

**Results** Phytochemical investigation on the title plant led to the isolation of 18 compounds, including a novel triterpenoidal peroxide, their structures were determined on the basis of spectral and chemical evidences. Among them, protocatechuic acid (1) and methyl 3-hydroxylbutanedioic ester (2) displayed higher inhibitory activities on NA with IC₅₀ values of 1.27 μg/mL and 1.90 μg/mL, respectively. Compounds 1, 2 and rososide (3) could inhibit the production of TNF-α by 22.73%, 33.14% and 37.19% at 5 μg/mL, *P* < 0.05 compared with the control. Compound 2 was found to show inhibitory effects on the release of IL-6 in RAW264.7 with the inhibitory rate 39.79% (*P* < 0.05).

**Conclusion** The structure of compound 2 is quite different from those of known NA inhibitor, the anti-inflammatory effect of compound 3 was firstly disclosed in this study. Avian influenza is usually accompanied by virus invasion followed the occurrence of serious inflammation, the dual effects of the isolates may play a cocktail wine-like role in the treatment of avian influenza, and *C. speciosa* may be a potent source of anti-viral and anti-inflammatory agents.

**Keywords:** Neuraminidase inhibitor, Anti-inflammation, High throughput screening

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High-throughput screening assay for hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon

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Hepatitis C virus (HCV) is a major etiological agent of hepatitis; 3% of the world population have been infected with the virus. Most patients develop chronic hepatitis, and persistent infection often leads to liver cirrhosis or hepatocellular carcinoma. To date, no vaccine against HCV has been developed, and current therapies are relatively ineffective and have significant side effects. Therefore, the development of anti-HCV drugs with greater efficacy and safety is desired.

Recently, the HCV NS3 helicase was found to be a promising target for anti-HCV drugs because the helicase activity is essential for viral replication, presumably unwinding double-stranded replication intermediates and secondary structures, allowing RNA amplification. Inhibition of the helicase activity has the potential to terminate the proliferation of the virus. Therefore, simple and high-throughput screening system of potential NS3 helicase inhibitors is required.

Here, we have developed a novel high-throughput screening assay that is based on a fluorescence-quenching phenomenon by photo-induced electron transfer between fluorescent dyes and guanine bases. We prepared double-stranded oligonucleotides with a 5′-fluorescent-dye (BODIPY FL)-labeled strand hybridized with a complementary strand, the 3′-end of which has guanine bases. When the double-stranded oligonucleotides are unwound by the helicase, the dye emits fluorescence due to its release from the guanine bases.

First, we examined the ATP dependency of the HCV NS3 with the assay. The results show that BODIPY FL-labeled oligonucleotides emitted fluorescence in the presence of ATP, but not in the absence of ATP. These results indicate that the observed fluorescence emission arises from the unwinding of the DNA substrate by the helicase activity of NS3, and that no contaminants in the HCV NS3 preparation contribute to the unwinding activity. Next, to demonstrate the potential of the new assay to detect the inhibition of helicase activity, NS3 was incubated with various concentrations of KCl, NaCl, and ATP-γ-S. The increases in the concentrations of KCl, NaCl, and ATP-γ-S significantly decreased the level of helicase activity. Moreover, we applied the new assay to screen for an inhibitory effect of culture supernatant of microorganisms on the helicase activity. Among 24 randomly selected culture supernatant, 4 samples showed significantly low fluorescence intensities, possibly indicating the presence of uncertain inhibitor substances for the helicase activity.

In conclusion, our results demonstrate that the new assay is suitable for quantitative assay of HCV NS3 helicase activity and useful for high-throughput screening of the inhibitors. This method will accelerate the finding of the inhibitors of HCV NS3 helicase.
A novel conjugate of low-molecular-weight heparin and Cu,Zn-superoxide dismutase: study on its mechanism in preventing brain reperfusion injury after ischemia in gerbils

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Background and purpose Low-molecular-weight heparin (LMWH) and Cu,Zn-superoxide dismutase (SOD) were extensively investigated on preventing brain reperfusion injury after ischemia (BRII) in the past few years and both exhibited some advantages as well as limits in practice. To explore whether chemical modification for LMWH and SOD can lead to improved bioactivity, in our present study, we examined the efficacy of LMWH conjugated SOD (LMWH–SOD) prepared in our laboratory on BRII in gerbils.

Methods Ischemia/reperfusion was performed for 5 min by clamping the bilateral common carotid arteries of gerbils. LMWH–SOD, SOD and LMWH+SOD were administered intravenously to corresponding animals just before ischemia. 24 h after reperfusion, serum malondialdehyde (MDA) content and SOD activity were measured, the expression of intercellular adhesion molecule-1 (ICAM-1) was examined by immunohistochemistry, and the brain sections were processed for Nissl staining and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling method respectively.

Results LMWH–SOD significantly lowered MDA content ($P < 0.001$, versus SOD and LMWH+SOD) and elevated SOD activity ($P < 0.05$, versus SOD and LMWH+SOD) in the serum of BRII gerbils. Immunohistochemical results showed that ICAM-1 positive staining was lighter, pyramidal cells of hippocampal CA1 region were more regular and the changes in cell edema were minor, and apoptosis of hippocampal cells was milder in LMWH–SOD treated animals than in SOD or LMWH+SOD treated animals, untreated BRII animals and sham-operated animals.

Conclusion These results suggest that the novel LMWH–SOD conjugate can inhibit upregulation of ICAM-1 and prevent neuronal cell apoptosis in BRII gerbils, and consequently provide evidence that the LMWH–SOD has anti-inflammatory and neuroprotective effects in BRII.

Keywords: Superoxide dismutase, Low-molecular-weight heparin, Conjugate, Cerebral ischemia, Gerbils

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A novel gene *fudoh* in SCCmec region regulates the colony spreading ability and virulence in *Staphylococcus aureus*

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**Background** *Staphylococcus aureus* has the ability to spread on the surface of soft agar plates. In this study, we compared the colony spreading ability between clinically isolated methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) and identified a novel gene that affects the spreading ability and virulence of *S. aureus*.

**Methods** Overnight culture of *S. aureus* cells was spotted onto soft agar (0.24%) plates.

**Results** We found that all MSSA strains (10/10) spread, whereas most MRSA strains (73%, 29/40) carrying type-II SCCmec spread very little. Deletion of entire type-II SCCmec region from these MRSA strains restored the spreading ability. Introduction of the novel gene named *fudoh* in type-II SCCmec into Newman strain suppressed the spreading ability. MRSA strains with the high spreading ability (27%, 11/40) had no *fudoh* gene, or a point mutated *fudoh* gene with K29R substitution, which did not suppress the spreading ability in Newman. Newman strain transformed with the *fudoh* gene decreased the exotoxin production and attenuated virulence in mice. Most community-acquired MRSA strains, a cause of severe infections, carried type-IV SCCmec encoding no *fudoh* gene and showed high spreading ability.

**Conclusion** These results suggest that the *fudoh* gene in type-II SCCmec region regulates the colony spreading ability and the exotoxin production, and is involved in the pathogenesis of *S. aureus*. 
It is well-known that carbohydrates play an important role in numerous critical biological processes. Particularly, some oligosaccharide in tumor-cell surface, such as sialyl Lewis A (sLea) and sialyl Lewis X (sLex), have been confirmed to be related with development and progress of many types of cancers, and could be used as a tumor marker for colon cancer now.

Boronic acid has been used for carbohydrate sensors due to its strong interaction with diol, which shows the potential applications including glucose concentration determination, cell labeling and targeting based on carbohydrate biomarkers as *in vitro* diagnostic tools, and biomarker-directed cellular imaging. Our group has been interested in water soluble boronic acids as fluorescent sensors for saccharide. This presentation will discuss design, synthesis and evaluation of the diboronic acid compounds for the recognition of sLea and sLex.
Molecular characterization of the biosynthetic enzyme for the biotechnological production of tetrahydrocannabinol, the active constituent of marijuana

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To date, more than 60 cannabinoids have been isolated from marijuana or fresh Cannabis sativa plants. Among them, tetrahydrocannabinol (THC) is the well-known psychoactive cannabinoid. Recent studies have demonstrated that this cannabinoid exerts a variety of therapeutic activities, and therefore, THC has attracted a great deal of attention as a promising medicine for treating various diseases. In some countries, THC has been approved as a medicine for suppressing nausea and vomiting caused by cancer chemotherapy, and more recently, Sativex, a Cannabis-based preparation containing THC, was licensed in Canada as a neuropathic pain reliever for adult patients with multiple sclerosis. The demand for THC has been increasing, however, asymmetric synthesis of this cannabinoid requires very intricate procedures. In addition, it is not easy to isolate THC because marijuana contains a complicated mixture of various cannabinoids.

Our previous study has demonstrated that THC generates non-enzymatically from the acidic precursor, tetrahydrocannabinolic acid (THCA), and further, THCA is biosynthesized by THCA synthase that catalyzes oxidative cyclization of the substrate cannabigerolic acid (CBGA). Here I report the molecular characterization and heterologous expression of THCA synthase.

The cloning of cDNA encoding THCA synthase was carried out by RT-PCR. The cDNA consisted of a 1635-nucleotide open reading frame encoding 545 amino acid residues. Interestingly, THCA synthase showed high homology (40.2% identity) to berberine bridge enzyme, a covalently flavinylated oxidase involved in alkaloid biosynthesis. In addition, the sequence motif (Arg-Ser-Gly-Gly-His), which is characteristic of FAD-binding sites in flavoproteins, was confirmed present in the primary sequence of THCA synthase.

To characterize the structural and functional properties of the enzyme, I expressed the recombinant THCA synthase using a baculovirus-insect cell system. The recombinant enzyme was secreted from insect cells, and was readily purified to homogeneity. Various spectroscopic properties have demonstrated that THCA synthase has a covalently attached FAD cofactor with a molar ratio of FAD to protein at 1:1. In addition, the site of flavin attachment was confirmed to be His-114 by site-directed mutagenesis. Further, functional analyses have indicated that THCA synthase is an oxidase-type enzyme, since the reaction absolutely requires molecular oxygen, and produces hydrogen peroxide proportional to THCA. THCA synthase is the first cannabinoid synthase that has been cloned and characterized.

For the biotechnological production, I attempted heterologous expression of THCA synthase in a yeast Pichia pastoris, since Pichia is a fermentation- and cost-friendly host organism for secreted protein expression. As expected, the transgenic Pichia could secrete a catalytically active THCA synthase. Interestingly, various supplements to the medium, such as riboflavin, high concentration of methanol, and casamino acids, considerably increased the expression level of the recombinant enzyme. The culture supernatant containing THCA synthase could convert CBGA into THCA with a conversion rate of ~98%, and a productivity of ~33 mg/L. Further studies are now underway for the practical production of THC in near future.
Galloyl cyclic-imide derivative CH1104I inhibits tumor invasion via suppressing matrix metalloproteinase activity

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Matrix metalloproteinase-2 (MMP-2) and MMP-9 have been associated with the ability of tumor cells to metastasize due to their capacity to degrade type IV collagen, the main component of basement membrane, and to their elevated expression in malignant tumors. (S)-methyl 6-(benzyloxycarbonylamino)-2-(2-(S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)hexanoate (CH1104I) is a galloyl cyclic-imide derivative designed to fit and extend into the S1' active pocket of MMP-2 and MMP-9. We aimed to evaluate the efficacy of CH1104I as a candidate compound for anti-invasion and anti-metastasis of tumor cells. CH1104I significantly blocked gelatinase activity as evidenced by a decrease in the degradation of succinylated gelatin. Gelatin zymography analysis showed that the compound (7-210 μM) inhibited the activity of MMP-2 and MMP-9 produced by human ovarian carcinoma SKOV3 cells. Inhibition of MMP-2 and MMP-9 expression was also observed using the assays of immunocytochemical staining and Western blot analysis. The results showed that CH1104I suppressed the expression ofzymogens and active-MMP-2 and MMP-9. The effects of CH1104I on invasion and migration of SKOV3 cells were then measured. CH1104I displayed an inhibitory effect on the penetration of SKOV3 cells through Matrigel-coated membrane in transwell chamber. Furthermore, Lewis lung carcinoma (LLC) model was employed to evaluate the efficacy of CH1104I in vivo. A significant inhibition of pulmonary metastasis of carcinoma cells was observed in CH1104I-administrated mice (25-100 mg/kg). These results suggest that CH1104I is a potential MMP-2 and MMP-9 inhibitor that may effectively suppress tumor invasion and metastasis.

Keywords: Galloyl cyclic-imide derivative, CH1104I, MMP-2, MMP-9, Invasion and metastasis

Acknowledgements
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Neuroprotection by inhibition of GAPDH-MAO B mediated cell death induced by ethanol

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Introduction The anti Parkinson drugs, deprenyl (selegiline) and Azilect (rasagiline) are inhibitors for monoamine oxidase B (MAO B). They are also effectively used for the treatment of several neuropsychiatric and neurodegenerative diseases. However, a large body of research has now shown that selegiline and rasagiline can also increase neuronal survival by interfering with apoptosis signaling pathways mediated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Thus, the neuroprotective effect of MAO B inhibitors has been thought to be independent of MAO B inhibition.

GAPDH is an important enzyme in the glycolysis and gluconeogenesis pathways. Recently, it has been reported that GAPDH also plays multiple roles in numerous intracellular activities including the initiation of cell death by translocating into the nucleus as a transcription factor. More interestingly, GAPDH is a target for the inhibitors of MAO B, selegiline and rasagiline.

MAO B is an enzyme that degrades a number of biogenic amines (such as neurotransmitters) and generates hydrogen peroxide (H2O2) which causes toxicity to cells and neurons. Therefore aberrant increase of MAO B activity in the elderly has been implicated in neurodegenerative diseases. The 5'-flanking sequence (the promoter) of the MAO B gene contains a maximal activity region which is located between -246 to -99 bp (a core promoter region). This core promoter region consists of two clusters of overlapping Sp1-binding sites. The transcription factors can bind to Sp1-binding sites in this region and activate MAO B promoter activity.

This study sought to determine whether GAPDH has a relation with MAO B using brain cell lines and prefrontal cortex of subjects with alcohol dependence, and also investigate whether MAO B inhibitors have neuroprotective activity in ethanol-induced brain cell death.

Methods The following techniques have been used in our study: cell culture of human neuroblastoma SH-SY5Y and glioblastoma U-118 MG, generation of GAPDH-stably expressed cell line, MAO B catalytic activity assay, MTT cell viability assay, real-time RT-PCR, Western Blot and immunofluorescence.

Results The expression of both GAPDH and MAO B are increased in brain cells upon ethanol-induced cell death, and also elevated in the prefrontal cortex of human alcohol dependent subjects compared to normal control subjects. Over-expression of GAPDH enhances ethanol-induced cell death, and also increases the ethanol-induced activation of MAO B. In contrast, the MAO B inhibitors rasagiline and selegline or siRNA-mediated GAPDH gene knockdown decreases the ethanol-induced MAO B and reduces cell death. Furthermore, GAPDH physically interacts with MAO B transcription factor, and this interaction is increased in the nucleus by ethanol but reduced by MAO B inhibitors.

Summary Ethanol-induced cell death, attenuated by MAO B inhibitors, may result from disrupting the movement of GAPDH with the transcriptional activator into the nucleus and secondly inhibit MAO B gene expression. Thus, an inhibitor targeting both GAPDH and MAO B may be a new approach for screening the more powerful drug and therapeutically useful in combating the harmful effects of neurobiological diseases including alcohol-use disorders and Parkinson's disease.

Acknowledgement This study was supported by Public Health Service Grants P20 RR17701 and MH67996, a NARSAD Young Investigator Award and by an Intramural Research Support grant from The University of Mississippi Medical Center. We acknowledge the invaluable contributions made by the families consenting to donate brain tissue and be interviewed. We also thank the Cuyahoga County Coroner and staff, Cleveland, Ohio, for their willing assistance. Gouri Mahajan in preparing tissue samples and Hailin Zheng for synthesizing rasagiline are also acknowledged.
Effect of Ginsenoside Rg1 on learning and memory ability and inflammatory cytokines in senescence accelerated mouse

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Objective The aim of this investigation was to determine the effect of Ginsenoside Rg1 (Rg1) on learning and memory ability and inflammatory cytokines in the senescence-accelerated mouse.

Methods The 9-month-old senescence accelerated mice (SAM) were divided into four groups: SAM-resistance/1 (SAMR1) control, SAM-prone/8 (SAMP8) group, SAMP8 treated with Rg1 10 and 30 mg/kg for 65 days. The learning and memory ability of animals were evaluated by square water maze test. The levels of IL-1β, TNFα in the serum were measured using enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's protocol.

Results Compared with same age SAMR1, the learning and memory ability of SAMP8 were significantly lower ($P < 0.01$). Rg1 10 and 30 mg/kg group were significantly better than SAMP8 group ($P < 0.01$). The learning and memory ability of animals were evaluated by square water maze test. The levels of IL-1β, TNFα in the serum were measured using enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's protocol.

Conclusion Rg1 may improve the ability of learning and memory and inhibit the inflammatory cytokines release in SAMP8.
Effects of peroxisome proliferator-activated receptor gamma agonist on the neuropathic pain induced by partial sciatic nerve ligation in mice

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The symptoms of neuropathic pain consist of hyperalgesia (excessive pain caused by stimulus that is usually nociceptive) and allodynia (a pain caused by a stimulus that is not usually nociceptive). Only large dose of opioid analgesics, such as morphine, can prevent the neuropathic pain, indicating the resistance to opioid analgesics. Recently, various key mediators of neuropathic pain following peripheral nerve injury have been clarified in animal model. And it is reported that the inflammation in the injured paripheral nerous system plays an important role in the development and the maintenance of the neuropathic pain.

On the other hand, peroxisome proliferator-activated receptor (PPAR) and retinoid X receptor are ligand-activated transcription factors of the nuclear hormone receptor superfamily. PPAR exists as three isoforms (α, β/δ and γ) that control many cellular functions including lipid metabolism, glucose absorption, and cell growth and differentiation. Recently, PPAR γ agonists were shown to prevent neuronal inflammation after focal cerebral ischemia in rodents. However, there is no report showing the relationship between neuropatic pain and PPAR γ agonists.

In this experiment, we studied the effects of PPAR γ agonist (pioglitazone) on the tactile allodynia in a neuropathic pain model, which is made by the unilateral sciatic nerve ligation (PSL) in mice. Moreover, we observed the expression of PPAR γ in dorsal root ganglia (DRG) and the sciatic nerve by western blotting and immunohistochemistry, and determined the role of inflammatory cytokines in PSL-induced tactile alldinia.

ICR male mice weighing 15-20 g were used. The tactile allodynia was evaluated by withdrawal responses to stimuli with von Frey filament. Pioglitazone was administered p.o. once a day for 7 days. PSL resulted in the tactile allodynia in the ipsilateral hind paw after day 3 of PSL. Daily treatment with pioglitazone (1-25 mg/kg) on day 0-6 ameliorated the tactile alldinia on day 7 and day 14, dose-dependently. Although PSL-induced tactile allodynia was not affected by the treatment with pioglitazone for 7 days before PSL, the amelioration of the allodynia was observed on day 14 by the treatment with pioglitazone on day 7-13, indicating that pioglitazone inhibits the development and the maintenance of PSL-induced tactile alldinia.

PPAR γ was detected in DRG in naïve mice. The expression of PPAR γ was decreased by PSL on day 7. On the other hand, the expression of PPAR γ was also observed in the sciatic nerve, and was increased by PSL on day 7. These changes in the PPAR γ expression were reversed by the treatment with pioglitazone on day 0-6.

In DRG and the sciatic nerve, the expression of inflammatory cytokines, i.e., interleukin-6 (IL-6) and tumor necrosis factor-α, were increased by PSL on day 7, and these increased cytokines were restored by the treatment with pioglitazone on day 0-6. Moreover, the injections of neutralizing antibodies of IL-6 around the injured sciatic nerve (on day 0, 2, 4 and 6) were suppressed the development of the tactile alldinia.

These results suggest that PPAR γ agonists may be a useful drug for the medications of neuropathic pain.
Advanced glycation end products serve as ligands for lectin-like oxidized low density lipoprotein receptor-1 (LOX-1): biochemical and binding characterizations assay

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Advanced glycation end products (AGEs) are a class of complex heterogeneous compounds which accumulate with age and is known to be involved in the pathogenesis of several diseases from diabetes to atherosclerosis. AGEs serve as ligands for multiple receptors including SR-A, CD36 and SR-B1. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) plays an important role in both atherosclerosis and is found to be an endothelial cell receptor for AGEs. To explore the binding characterization of AGEs to LOX-1, AGEs were prepared by three different reducing sugars (D-glucose, D-fructose, and D-ribose) and the biochemical characterization including, free amino groups, free amine content, fructosamine residues, carbonyl content, fluorescence and absorbance were determined. The binding activity was determined by FITC labeled AGEs using Chinese hamster ovary-K1 cells stably transfected with human LOX-1 gene. The obtained AGEs showed significant differences in the extent of side chain modifications, carbonyl content, fluorescence and absorption models. All of the AGEs showed specific and saturable binding to hLOX-1-CHO-K1 cells. Furthermore, dose-dependent binding processes were observed. However, the maximal cellular binding of AGEs differs between the sugars (glucose > ribose > fructose). In addition, oxidized low-density lipoprotein (ox-LDL) could significantly inhibit the binding of AGEs to LOX-1 with different inhibitory efficiency. LOX-1 serves as receptor for AGEs which may give some insight into the role of LOX-1 in the pathogenesis of diabetes and related disorders.

Keywords: Advanced glycation end products, Lectin-like low-density lipoprotein receptor-1, AGEs characterization, Binding activity
Theoretical studies on the point mutations in the active site of protein tyrosine phosphatase 1B

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In recent years, close attention has been paid to the protein tyrosine phosphatase 1B as a novel drug target. In this study, we investigate the importance of six residues contributed to catalysis in PTP1B by means of point mutation. And it is hope that an improved understanding of the structure of PTP1B will allow for the design of better inhibitors for PTP1B.

Methods Theoretical studies were carried out using molecular mechanics with an AMBER99 force field from the HyperChem7.0 program, six residues in the active site of PTP1B were mutated respectively and the complex structures were optimized with energy minimization using Polak-Ribiere conjugate gradient method. The electrostatic interaction energies, van der Waals interaction energies, hydrogen bonds, dihedral angles were calculated as descriptors to identify the interaction change between the substrate and enzyme after mutation.

Results The results show that S215K mutation lead to increased interaction energy between the catalytic residues and substrate, indicating that the catalytic activity of the enzyme increases after mutation. S215F, S215T, D181K, D181I, D181T, Q259E, Q259F, Q259T, G262K and Y46I mutations cause increase in total interaction energies between the enzyme and substrate, indicating that these mutations increase the binding affinity for substrate. Y46K, Y46E and Y46F mutations not only result in increased interaction energies between the catalytic residues and substrate, but also cause increased total interaction energy between the enzyme and substrate. It indicates that these mutations lead to increased catalytic activity and binding affinity for the substrate.

Conclusion The point mutations have little influence on the shape of the active site in PTP1B. However, the mutations of Ser215 have great influence on the catalytic ability of the enzyme. The mutations of Arg221 lead to decreased binding affinity of the active site. Except D181E, other mutations of Asp181 enhance the binding affinity of WPD loop. Most mutations of Gly259 and Gln262 enhance the binding affinity of the enzyme.

Keywords: PTP1B, Point mutation, PTP1B inhibitors, molecular mechanics, Optimize, Electrostatic interaction energy
Evaluation of anti-diabetic drugs using silkworm, *Bombyx mori*

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**Introduction** Diabetes is a disease that shows chronic hyperglycemia and glucose intolerance. Hypoglycemic drugs, such as insulin, are useful to diabetic therapy. However, hypoglycemic drugs causes drug resistance and several diseases by side effect. Therefore, development of a novel hypoglycemic drug is desired.

Almost of researchers uses pathology mimic animal model using mouse or rat for elucidation of lifestyle-related diseases, such as diabetes. However, it is designated ethical problem to sacrifice of large amount of mammal. In this report, we attempt to establish a novel invertebrate diabetic model using silkworm.

**Result and discussion** Hemolymph sugar concentration of silkworm was increased by intake of high glucose diet, immediately. Silkworm shows growth defect by continued intake of glucose diet. In high hemolymph sugar silkworm, hemolymph sugar was decreased and the growth defect was suppressed by injection of human insulin into hemolymph. Akt phosphorylation and glucose content in silkworm fat body, which has function of both liver and adipose tissue in mammal, was increased by treated with human insulin *in vitro*. These enhance effects were suppressed by addition of PI3 kinase inhibitor, wortmannin. On the other hand, Hemolymph sugar of silkworm was decreased by AICAR, AMP kinase stimulator. These results suggest that stimulation of insulin pathway or AMPK pathway lead to hemolymph sugar reduction in silkworm. Furthermore, we identified hypoglycemic compound A, from Chinese herbal drug, jiou, using silkworm diabetic model. The compound A has hypoglycemic activity against mouse model. These results suggest that silkworm diabetic model is useful to identify hypoglycemic compound. This is first report in our knowledge that hypoglycemic agent is identified using invertebrate diabetic model.
Chemical function based pharmacophore generation of dual AT₁ and ET₆ selective receptor antagonists

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Since hypertension is a major sign of future cardiovascular disease, there exists one of the largest unmet medical needs for an antihypertensive drug that is effective across a wide variety of patients as a monotherapy (1).

Angiotensin II (AngII) and endothelin-1 (ET-1) are both potent endogenous vasoconstrictors (2), therefore the latest research suggested that disruption of the effects of angiotensin II (AngII) and endothelin-1 (ET-1) has shown promise for treating hypertension (3-5). Thus, it is anticipated that dual AT₁ and ET₆ receptor antagonists (DARAs) in humans could be more effective than current standard therapies for treating hypertension and other cardiovascular diseases.

Chemical feature based pharmacophore model may serve as a guide in the design of selective antagonists. Pharmacophore models for AT₁ receptor antagonists and ET₆ receptor antagonists have been reported. To the best of our knowledge there is no report in pharmacophore for DARAs. 3D pharmacophore models were built from a set of 6 dual AT₁ and ET₆ receptor antagonists by us. Among the 10 common-featured models generated by program Catalyst/HipHop, the forth hypothesis (Hypo-DARA-4) was considered to be important in evaluating the DARAs activity, which consists of two hydrogen bond acceptors, two hydrophobic aliphatic, one ring aromatic, one hydrophobic aromatic and one negative ionizable feature. Also, 3D pharmacophore hypotheses for AT₁ receptor antagonists and ET₆ receptor antagonists were built, respectively. Something in common among the hypotheses of AT₁ receptor antagonists, ET₆ receptor antagonists and DARAs was found. Additionally, docking method was used to predict the binding modes of the highest active compound (DARA-3) of the training set in the AT₁ and ET₆ receptors. Structural features relevant to the interactions of DARA-3 with the amino acid residues in the active sites were discussed.

Keywords: Dual AT₁ and ET₆ receptor, Antagonist, Pharmacophore

References
Synthesis of 2α,3α-epoxy-16β-pyrrolidino-5α-androstane-17-ol

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Neuromuscular blocking agents (NMBs) were first used in clinical anesthesia in 1942. Nowadays, the research of steroidal NMBs has been a highlight because of their highly potent and selective, less side effects and different action time.

Attention has been focusing on the method of attaching 16β-azacycle to steroidal skeleton since pancuronium bromide went on the market in 1968. From then on, a number of methods have been developed\textsuperscript{1-4}, however, the yield remains low. A latest patent\textsuperscript{5} has reported the intermediate of 2α,3α-epoxy-16α-bromo-5α-androstane-17-one 3 from which 16β-azacyclosteroidal derivates was obtained via nucleophilic substitution. 16β-Azacyclo steroids were key intermediates for preparing steroidal NMBs. In this work, compound 5 is a crucial intermediate (scheme 1) for the synthesis of rocuronium.

2 has been directly obtained from 1 via elimination reaction catalyzed by p-toluenesulfonic acid absorbed on silica gel in benzene. Then, 16α-bromination occurs by reacting 2 with CuBr\textsubscript{2} in methanol to produce 3. The epoxidation of 3 has been optimized by using water-dichloromethane as a solvent and using sodium carbonate as a catalyst to raise the yield of 4. 5 is synthesized from 4 through substitution by pyrrolidine and then reduction with sodium borohydride. Compared to the procedures reported in the literatures mentioned before, these optimized conditions have greatly increased the overall yield.

**Scheme 1**

\begin{align*}
\text{HO} & \text{(1)} & \overset{a}{\longrightarrow} & \text{O} & \text{(2)} & \overset{b}{\longrightarrow} & \text{O} & \text{Br} & \text{(3)} \\
\text{O} & \text{Br} & \text{(4)} & \overset{c}{\longrightarrow} & \text{O} & \text{Br} & \text{OH} & \text{N} & \text{(5)}
\end{align*}

\text{a)TsOH-Si/benzene ; b)CuBr}_2/\text{MeOH; c)m-CPBA/H}_2\text{O-CH}_2\text{Cl}_2 ; d)\text{pyrrolidine/CH}_3\text{CN; e)NaBH}_4/\text{MeOH-CH}_2\text{Cl}_2}

**Keywords:** Neuromuscular blocking agents, 2α,3α-epoxy-16α-bromo-5α-androstane-17-one, Steroids, Rocuronium

**References**

An improved procedure for synthesis of leonurine

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Leonurine is an important component in Leonurus japonicus Houtt which can be used to activate blood circulation to disperse blood stasis and inhibit blood platelet aggregation. Similarly, the Leonurine possesses significant biochemical and medicinal properties. A number of methods to synthesize this compound have been reported. However, it is difficult to find a convenient material and get a good yield. Through our search, the material 3, 4, 5-trihydroxybenzoic acid was used, which is easy and cheap to get. Also, a new catalyst has been used in synthesis and get a better yield. The total yield was 27%.

Scheme 1

References
Design and synthesis of folate-targeted and PEG-modified liposomes as drug deliver

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Liposome is a bind of lipid micelles with two-double membrane always made by men. As drugs carrier, it has many advantages, such as protecting drugs, increasing drugs effect, minimizing toxicity of drugs, increasing drug targeting etc. Recent studies have suggest that folic acid was covalently conjugated to liposomes attempt to target the folate receptors to tumors.

The design of liposomes based on surface modification with PEG have resulted in new formulations of small (<100 nm), long-circulating vesicles. In the preparation of liosomes, we synthesized by three steps. First, folic ester 1 was initially prepared, then it treat with polyethylene glycol (PEG) bis-amine. Finally, hydrogenated soybean phosphatidylcholine (HSPC), cholesterol, distearoylphosphatidylethanolamine (DSPE) and 2 mixed with a certain percentage to prepare the liposomes. The products 1 and 2 were identified by 1H NMR and the further study on drug delivery efficiency is in process.

Scheme 1

a: DCC, DMSO, NEt₃, rt   b: DMSO, NH₂-PEG-NH₂   c: HSPC, DSPE, cholesterol

References
Synthesis and evaluation of quinoxalinone derivatives as potent modulators of multidrug resistance

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P-glycoprotein-mediated drug efflux from cells is believed to be an important mechanism in multidrug resistance (MDR) in cancer chemotherapy. It holds great promise for overcoming MDR to identify and develop P-glycoprotein inhibitors with high potency and low cytotoxicity. A series of quinoxalinone derivatives were synthesized and evaluated to their anti-proliferative effect and MDR reversal activity in vitro assay systems. Biological assays demonstrated that the compounds were, in general, endowed with good activity as P-glycoprotein inhibitors. Among them, two compounds gave the highest MDR reversal activity without significant cytotoxicity and displayed potent P-glycoprotein inhibition activities. Thereby, they may be worthy of further research as potential adjunctive agents for tumor chemotherapy.

Keywords: Quinoxalinone derivatives, P-glycoprotein inhibitor, Multidrug resistance (MDR)

References

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Antioxidant activity of meat treated with extracts from edible lotus (*Nelumbo nuficera*)

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Lotus (*Nelumbo nuficera*) is widely used, not only as an ornamental plant, but also for a dietary staple in Eastern Asia, particularly in China. For the purpose of searching antioxidant agents from natural resources against meat oxidation, we found that the meat treated with extracts from the lotus root knot and lotus leaf had potent inhibitory activity against oxidation.

**Methods** Fresh porcine and bovine meat samples were divided into three treatment groups and were homogenized with either lotus rhizomes knot extract (LRK) or lotus leaves extract (LL). Samples in the control (CONTROL) treatment had no extract added. Meat samples were then stored at 4°C for 24 h before assays for antioxidant activity (*i.e.* day 1) were performed. Consequently, each sample was split into two 3 g portions of which one remained in the raw state and the other received heat treatment (at 85°C for 30 min) and is referred herein as “cooked”. Both raw and cooked samples were subsequently stored at 4°C and assayed three more times (*i.e.* at 3, 6 and 10 days of storage) for antioxidant activity using four different assay methods, namely thiobarbituric acid (TBA) assay, diphenylpicrylhydrazyl (DPPH), azinobisethylbenzthiazolinesulfonic acid (ABTS) radical scavenging assay and reducing power assay. And the main polyphenolic compounds of two extracts have been also investigated.

**Results** The results of this study using four different assay methods demonstrate that the extract treatments, LRK and LL, irrespective of sample state (raw or cooked), gave generally higher, (in most cases significant), antioxidant activity, compared to the CONTROL. The treatments LRK and LL therefore resulted in lower oxidation of porcine and bovine meat upon storage at 4°C, with the LRK treatment being the most potent using the TBA assay. Interestingly, while the results from the TBA assay, the greater antioxidant activity seen for the LL treatment was showed in DPPH, ABTS radical scavenging assay and reducing power assay.

**Conclusion** The extracts of LRK and LL, which from the residual parts of edible lotus, are both effective antioxidants. These findings led us to conclude that natural residual resources are important leads for development of various antioxidant agents.
Tissue tropism of Kakugo virus, a novel insect picorna-like virus identified from the brains of the aggressive worker honeybees Apis mellifera L.

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The European honeybee Apis mellifera L. is a eusocial insect, and the female adults differentiate into a reproductive queen and thousands of sterile workers. The queen is engaged in reproduction, while the workers are engaged in various tasks required to maintain colony activity, which leads to effective propagation of the colony as a 'super-organism'. In addition, workers show division of labors such as nursing, defense of the hive from their natural enemies, and foraging. We previously identified a novel insect picorna-like virus, termed Kakugo virus (KV), from the brains of aggressive workers that had counterattacked giant hornets, suggesting that KV infection might affect the worker behaviors. It remains to be solved, however, how KV infects the honeybee workers and how KV infection affects their physiology, brain function and/or behaviors.

With the aim to analyze the neural and physiological influence of KV infection, we used in situ hybridization to examine tissue tropism of KV in workers experimentally infected with KV. In the brains, KV was detected in a restricted part of the brains at early stage of the infection. As the KV infection progresses, KV became to be detected in various brain regions including the mushroom bodies (higher order center), optic lobes (visual center) and neurons connected to the ocelli, suggesting that KV widely infects the brains and the possible effect on brain function alters depending on the time after infection. KV was also detected from the hypopharyngeal gland (exocrine gland in the head of the workers) and the fat body. The hypopharyngeal gland synthesizes and secretes royal jelly as a food for queen and brood, and an enzyme that converts nectar into honey. Thus KV infection in the gland might prompt oral transmission of KV. Since the fat body is involved in nutrient transport/metabolism, hemolymph protein synthesis/storage, and immunity, KV infection in the fat body may affect various physiological states of the bees. The honeybee-KV relationship could be a good model for the further understanding of host-virus interactions from the view of comparative virology.
Risk factors in environment and behavior for human infection with influenza A H5N1 in rural China

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Since 2003, outbreaks of highly pathogenic avian influenza A (H5N1) virus with sporadic transmission from birds to human worldwide have raised a considerable concern of the potential pandemic. In China, most human cases of avian influenza were rural residents and were reported with a contact or an intake of sick poultries, suggesting rural residents have relevant risks to be exposed to the fatal virus. This study aimed to determine risk factors in living environment, economic condition and hygienic behavior for human infection with influenza A subtype H5N1, to understand villagers' knowledge and attitude towards the disease prevention and the potential pandemic, and to examine the performance of local surveillance system and the effect of preventive interventions. A cross-sectional study conducted in Shandong Province, Anhui Province and Inner Mongolia Autonomous Region during September 2007 to January 2008. The target population was rural residents aged 18 and above. It was selected by multi-stage sampling. We interviewed 1379 participants by using a semi-structured questionnaire. Officers from primary healthcare settings and the local Health Agency were also interviewed. As the results, we identified the frequent and inevitable contact between rural residents and poultries in living environment of rural residents. The sanitary conditions, especially waste dumping, toilet, and water supply need to be improved. There still remain some unsafe and inappropriate practices on poultries handling such as food preparing and treatment of dead poultries. The level of rural residents' knowledge on avian influenza and disease prevention was low in general. Moreover, we found risk behaviors such as intake of sick and died poultries, action of the disease prevention, and willingness to report the epidemic associated with it, suggesting intervention focusing on rural residents urgently need to be improved in future.
A rapid identification of *Radix inulae* and its active component alantolactone in the Tibetan medicine Manuxitang

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Traditional Chinese medicines (TCM) are usually prepared by decocting multiple materials, which causes difficulty in identifying their active components and achieving quality control. There is an urgent need for systematic scientific standards to evaluate their safety and efficacy and to control their quality based on scientific research and evidence. One of the most popular Tibetan medicines, which is known by the Tibetan name Manuxitang, has been widely used in clinical practice for more than 1,000 years. Although the principal component of Manuxitang was known as *Radix inulae*, identification and quantitative determination were not included in the standards for Manuxitang. This study attempted to establish a rapid method of identifying the principal drug *Radix inulae* and its active component alantolactone (AL) in Manuxitang. On thin layer chromatography (TLC) analyses, *Radix inulae* and AL in a Manuxitang decoction were both successfully identified. In gas chromatography (GC) analyses, AL was separated and quantitatively determined in the range of 0.1-1.0 μg/mL ($r = 0.9998$). The precision was 1.20% ($n = 6$) with an average relative standard deviation (RSD) of 1.74%. Recovery was in the range of 93.5-98.5% with RSD value of 1.85%. These results suggested that the proposed technique using TLC and GC can be used as a simple and convenient method for determining AL in *Radix inulae*. Moreover, this technique might be suitable for the quality control of TCM containing *Radix inulae*.
Identification of Tibetan medicine Manuxitang and quantification of its active components, alantolactone and isoalantolactone

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Objectives This study aimed to establish a more reliable method to control the quality of Tibetan medicine Manuxitang.

Methods The four raw herbs in Manuxitang including Radix Inulae, Tinosporae sinensis Caulis, Rubus niveus Thunb, Zingiber officinale Posc were identified by thin layer chromatography (TLC). Two active sesquiterpene lactones, alantolactone (AL) and isoalantolactone (IAL) in “monarch” drug Radix Inulae were determined by gas chromatography.

Results The four herbs in Manuxitang were effectively identified by TLC with the spots clear and no interference. AL and IAL were both quantitatively determined by gas chromatography. Good linear calibration curves were obtained over the entire range of concentrations studied. The equations were as follows: for AL, $Y = 9.0 \times 10^7 X + 2.0 \times 10^6$ in the range of 0.1~1.0 $\mu$g/mL with $r = 0.9998$; and for IAL, $Y = 6.0 \times 10^7 X + 6.0 \times 10^6$ in the range of 0.2~1.0 $\mu$g/mL with $r = 0.9999$.

Conclusion The methods established were simple, accurate and specific and could be applied to the quality control of Manuxitang.

Keywords: Manuxitang, Thin layer chromatography (TLC), Gas chromatography, Tibetan medicine
Development of benzofurazan derivatization reagents for LC-MS/MS and application to the analysis of bio-markers of peroxisomal disorders

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The development of sensitive and selective determination methods of bio-markers is essential for the diagnosis. Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been utilized for this purpose. However, majority of biological markers compounds are not suitable for LC-MS/MS analysis, and thus, chemical derivatization is often needed to enhance the sensitivity or selectivity. Recently, we have synthesized benzofurazan derivatization reagents for LC-MS/MS, DAABD-AE (4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole) for carboxylic acids (1-3) and DAABD-MHz (4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-N-methylhydrazino-2,1,3-benzoxadiazole) for carbonyl compounds (4). In this paper, we present a rapid, sensitive and selective analysis of pristanic acid, phytanic acid and very long chain fatty acids (VLCFAs) bio-markers of peroxisomal disorders using DAABD-AE as a derivatization reagent.

Analytes in plasma samples were hydrolyzed, extracted and derivatized with DAABD-AE in the presence of the condensation reagents. The generated derivatives were separated on a reversed phase column and detected by positive ion electrospray ionization-tandem mass spectrometry in the selected reaction monitoring (SRM) mode. The derivatives afforded intense (M+H)+ ions by MS and efficiently generated product ion at m/z 151 originated from (dimethylamino) ethylaminosulfonyl moiety by MS/MS. The detection limits were of fmol levels. The calibration curves were linear over the ranges that cover physiological and pathological concentrations. The injection-to-injection time was five minutes. This method was successfully applied to the determination of pristanic acid, phytanic acid and VLCFAs and the results were clinically used for the detection of at least nine peroxisomal disorders (5).

References
Geniposide, a novel agonist for glucagon-like peptide 1 receptor, shows neurotrophic and neuroprotective characteristics in PC12 cells

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AD is by far the most common neurodegenerative disease with an extensive neuron loss. Recently, a new AD interventive strategy consisting of the use of glucagons-like peptide-1 was described by Perry and Greig. The stimulation of neuronal GLP-1 receptors plays an important role in regulating neuronal plasticity and cell survival.

In our previous work, with the model of high throughput screen for GLP-1 receptor agonists, we identified that geniposide, isolated from Gardenia jasminoides Ellis, is a novel selective agonist for GLP-1 receptor, which shows neurotrophic characteristics to induce the neuronal differentiation of PC12 cells. We also identified that geniposide increased the expression of anti-apoptotic proteins, including Bcl-2 and heme oxygenase-1 (HO-1), to antagonize the oxidative damage in PC12 cells induced by hydrogen peroxide. We also probe the possible signal pathway after geniposide activated GLP-1R to prevent PC12 cells from oxidative damage. The results showed that PI3K and PKA, including MAPK, played important roles in this progress. Furthermore, with RNAi on the expression of GLP-1R, we found that the neurotrophic and neuroprotective abilities were decreased significantly. All these results showed that activation of GLP-1R by geniposide would provide benefits in a number of related and unrelated disorders prevalent in aging, including AD.
Antitumor mechanism of a new diorganotin(IV) complex di-n-butyl-di-(4-chlorobenzohydroxamato)tin(IV)

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Abstract
Di-n-butyl-di-(4-chlorobenzohydroxamato)tin(IV) (DBDCT) is a new diorganotin complex which exhibits strong cytotoxic activity toward a series of human tumor cell lines and displays a high in vivo activity against H22 liver and BGC-823 gastric tumors. In order to investigate its antitumor mechanism, in this paper, the fluorescent staining, light and electron microscope were first used to examine the nuclear morphology characteristic of SGC-7901 apoptosis cells treated with DBDCT, and several apoptotic bodies were observed. In the agarose gel electrophoresis, DNA ladder-shaped strap was also clearly observed. There were hypodiploid apoptotic peaks pre-G0-G1 phase of cell cycle from DNA figure by flow cytometry (FCM). Significant statistics difference ($P < 0.05$) was showed in apoptosis rates of SGC-7901 cells treated with DBDCT for different times with cisplatin as positive contrast drug by annexin V-FITC method. It was clearly showed that DBDCT could kill SGC-7901 tumour cells through arresting it in the G2/M-phase and S-phase of cell cycle. The results of immunohistochemical method showed that p21 positive cells increased obviously, however, PCNA positive cells significantly decreased with the increased concentration of DBDCT ($P < 0.01$). The results of RT-PCR indicated that the express of p21, p53 and bax mRNA in the experimental groups were obviously higher than these of the blank groups ($P < 0.01$), nevertheless, the express of Bcl-2 and Bcl-2/Bax in the experimental groups were obviously lower ($P < 0.001$) than these of the blank groups with the concentration and time increased. The enzymatic activities of caspase-3, caspase-8 and caspase-9 of SGC-7901 cells treated with different doses for different times increased significantly ($P < 0.01$). The results confirmed that SGC-7901 cells apoptosis induced by DBDCT was concerned with mitochondria and death receptor pathway. The mitochondria apoptosis pathway was also ascertained via caspase-8 inhibitor Z-LEHD-FMK which could not inhibit the apoptosis induced by DBDCT. Treated with DBDCT in SGC-7901 cells, the concentration of Ca$^{2+}$ was significantly heightened which was detected by a new fluorochrome 3-AM, the mitochondria transmembrane potential strikingly decreased which was detected by Rhodamine123. Meanwhile, DBDCT could stimulate the tumor cells to produce ROS. The results of western blot analysis indicated that the express of Bcl-2 protein was down-regulated and the express of Bax, Cyt-c and Caspase-3 was up-regulated The ratio of Bcl-2/Bax decrease from 1.3 to 0.52. Hence, the mitochondria caspases signal transduction pathway was further probed.

Keywords: Diorganotin(IV), Antitumor activity, Action mechanism, Apoptosis pathway
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