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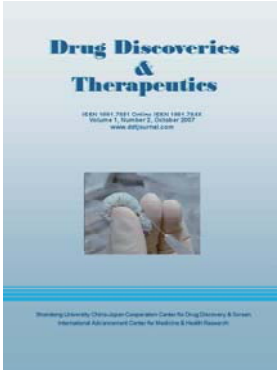

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China's efforts to shed its title of "Leader in liver disease"

Xun Li, Wen-Fang Xu

According to *Xinhua News*, Chen Zhu, China's Minister of Health, mentioned at the 2007 Annual Conference of the China Association for Science and Technology (CAST) that an action plan for the diagnosis and treatment of hepatitis B could control hepatitis B virus (HBV) infection to below 1% by 2050. This plan is one of the Health Ministry's goals for middle-long-term development planning in medical science and technology that China is endeavoring to reach (*http://hbv.39.net/079/18/127612.html, available as of September 18, 2007*).

The Ministry's strategy involves a series of action plans for other areas like HIV, tuberculosis, malignant tumor control, and mental health, but chronic HBV therapy is more important and more urgent. HBV infection is a leading cause of illness and death in China. Approximately 60% of the population has a history of HBV infection, and 9.8% of persons in China are chronically infected with HBV and at risk for premature death from liver disease. Each year, an estimated 263,000 persons in China die from HBV-related liver cancer or cirrhosis, accounting for 37~50% of HBV-related deaths worldwide (*Available as a report from the Centers for Disease Control and Prevention (CDC), 2007;56:441-445. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5618a2.htm, May 11, 2007*).

Besides China, HBV is highly prevalent in approximately 45% of the global population and is found in the Far East, parts of the Middle East, sub-Saharan Africa, parts of South America, and the Amazon basin, where at least 8% of the population are HBV chronic carriers (hepatitis B surface antigen [HBsAg] positivity rates > 8%) (Figure 1) (*Int J Med Sci 2005;2:50-57*).

China seems to have become a "Leader in liver disease." Annually, more than 1,000 billion RMB is spent on HBV therapy and prevention, while the resulting indirect economic losses are inestimable. The reasons for the high rates of chronic HBV infection in China are complex.

First, HBV infection has broad clinical manifestations, including asymptomatic carriers, acute hepatitis, and chronic (lifelong) hepatitis, due to different immune reactions by the host. However, little is currently known about the mechanisms for HBV's unremitting infection and long-term nonprogressive HBV infection of asymptomatic HBV carriers. Although a safe and effective vaccine against HBV has been available since 1982, there are still approximately 5~10% nonresponders to the hepatitis B vaccine. Moreover, little is known about the possible immunogenetic

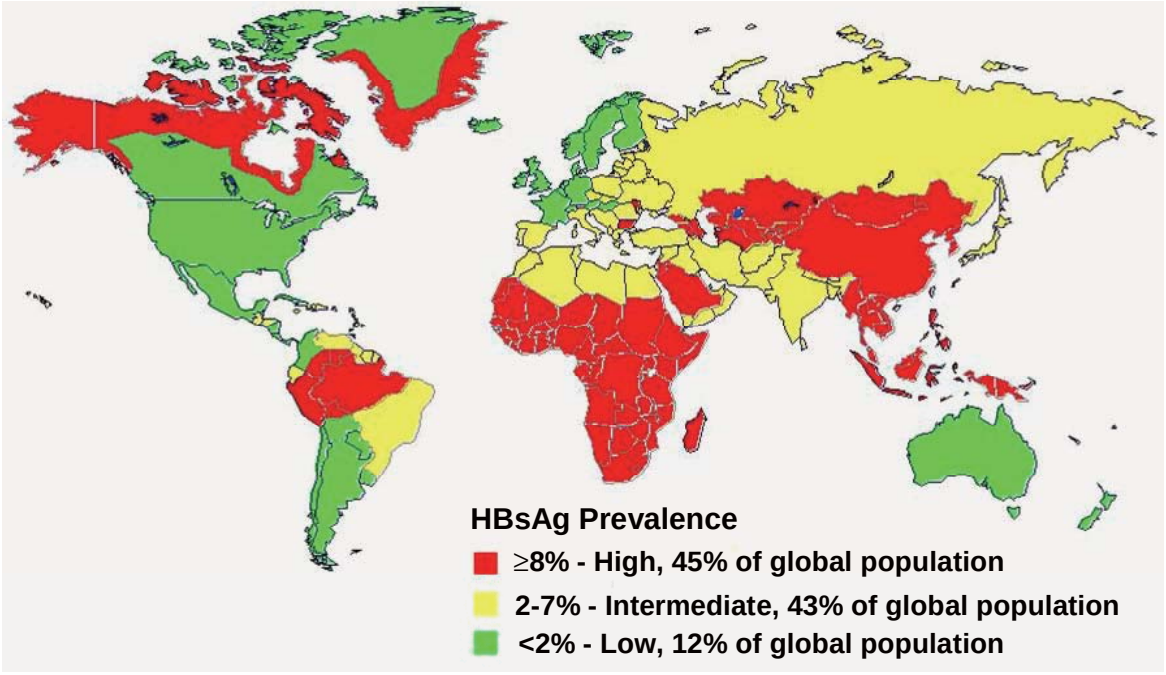

Figure 1. Geographic distribution of chronic HBV infection (*Int J Med Sci 2005;2:50-57*).

mechanisms of HBV-infected individuals developing cirrhosis and hepatocellular carcinoma, which are considered to be the biggest bottleneck for HBV therapy.

Second, some social factors may explain the high rates of HBV infection. The public often pales at the mention of HBV infection, but they have incorrect perceptions or little knowledge about how hepatitis B is transmitted, resulting in inadequate self-prevention, unfounded prejudices, or unfair treatment of the chronically infected. Worse, even many physicians are not aware of the risk, the association between hepatitis B and liver cancer, the importance of HBV vaccination to prevent infection, and the need for carriers to have regular liver cancer screening. The latter is important because a carrier is an infected individual who does not develop the disease but can transmit the virus to others. Research has proven that the hepatitis B virus is mainly transmitted through body fluids like blood, semen, vaginal or menstrual secretions, serum, and wound exudates, and the virus has also been found in saliva, amniotic fluid, tears, urine, feces, sweat, and mother's milk (*Int J Med Sci 2005;2:50-57*). Thus, people should actively acquire HBV-related knowledge, perform good hygiene, and heighten individual awareness of prevention to contain the transmission of HBV, which is of great importance to everyone.

Finally, poor living habits are another important factor. In most rural areas in China, and especially in the poverty-stricken areas inhabited by smaller ethnic groups, people still lead poor lifestyles and have poor health habits, leading to their decreased immunity to HBV. This, to a certain extent, may be attributed to the inadequate public health advertising and financial input of the Government. Except in some large general hospitals, the sanitary conditions of most hospitals and rural health clinics still need to be improved, including a system of social relief and assistance. Another important aspect is attributed to people's lack of awareness concerning regular physical examinations. Many chronically infected individuals may not know that they have been infected because they feel perfectly healthy. By the time symptoms develop, however, action will be too late.

The public is glad to see that a series of measures have been taken by Chinese authorities to provide effective HBV treatment and prevention. For instance, the "Wang Bao-En hepatic fibrosis research fund" was established by the China Foundation for Hepatitis Prevention and Control (CFHPC) on January 30, 2007 for the financial support of HBV research (*Xinhua News, http://news.xinhuanet.com/health/2007-02/02/content_5687887.htm, available as of February 2, 2007*). Similarly, the "Vaccination against Hepatitis B & Health Education Program," supported by the CFHPC, the Asian Liver Center at Stanford University, and several philanthropic foundations in Hong Kong, was formally inaugurated on August 31, 2007 to provide students of Qinghai Province with free and full-range protection with hepatitis B vaccination (*CFHPC News, http://www.cfhpc.net/CN/News/Detail.asp?gCatalogID=3&SystemID=792, available as of August 31, 2007*).

The PRC is currently forming exceptional scientific teams, both from clinical and research institutes, to study the integrity, development, and natural history of HBV as well as mechanisms for unremitting HBV infection in terms of aspects such as the virus, host, and environment. In the meantime, researchers are endeavoring to develop novel anti-hepatitis virus drugs pursuant to the "Guideline for Prevention and Treatment of Chronic Hepatitis B" enacted at the end of 2004.

As Health Minister Chen Zhu said, "China must cast its title of 'Leader in liver disease' into the Pacific Ocean because," he added, "we already have an extremely effective vaccine against HBV." (Xun Li, Wen-Fang Xu: *Shandong University, Jinan, China. e-mail: tjulx2004@sdu.edu.cn*)

Study of drug resistance among 78 antiretroviral treatment-naïve patients with HIV-1 subtype B infection in central China

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ABSTRACT: To study the prevalence of drug resistance mutations among HAART (highly active anti-retroviral therapy) naïve subjects with HIV-1 subtype B infection, evaluate the correlation between major mutations and viral loads. Additionally, to investigate the primary resistance spectrum in the central plains of China and provide some guidance for the choice of antiretroviral drugs (ARV). Drug resistance mutations and viral loads were measured in 78 treatment-naïve patients with HIV infection and the results were analyzed with descriptive statistical and multiple statistical analysis. The most common mutations were L63P, V77I and I93L, which belong to minor mutations of the proteinase gene, and none of which had any relation to viral loads. The major mutations, which were mainly K103N and Q151M, were less frequent in China than those in other countries. There was a certain correlation between viral loads and I93IL according to stepwise regression analysis. The incidence of primary mutations among HAART naïve patients was lower in China's central plains than that in other countries, and the most common mutations had no relation to viral loads. Though major mutations affecting choice of ARV are not common in China, they deserve further attention.

Key Words: HIV-1 subtype B, drug resistance, viral load

Introduction

Since the advent of highly active anti-retroviral therapy (HAART), the prognosis for AIDS has significantly improved. However, its appearance was followed by a series of problems, chief among them drug resistance brought about by HAART, that could greatly impact the effects of this therapy and cause difficulty in choosing therapy regimens. The study aimed to analyse the primary drug-resistances among treatment-naïve patients infected with HIV in central plain of China, to detect the prevalence of resistance mutations and the relation to HIV viral load so as to guide the choice of HAART.

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Patients and Methods

Patient selection

Subjects were 78 patients infected with HIV during 1992 to 1994 through commercial blood donation who were all from central China. Genotype test analysis showed that all of the patients were infected with HIV-1 type B. All subjects had received no ARVs previously, had a $CD4^+$ T lymphocyte count of more than 200 cells/μL, and did not have AIDS-associated diseases. Patient samples were collected and analyzed in June 2006.

Detection methods

200 μL of anticoagulated whole blood specimen were collected from each subject. Viral genome RNA was extracted with a QIAamp Viral RNA Mini Kit and amplified by reverse transcription PCR and nested-PCR (as shown in Table 1, two pairs of primers were designed). The products of PCR were purified and then genotyped with a Trugene HIV Genotyping Kit. HIV viral loads were detected by nucleic acid sequence-based amplification (NASBA).

Data statistics

Resulting mutations and HIV viral loads were analyzed with the SPSS13.0 statistical package; this work included descriptive and multiple statistical analysis.

Results

1. *Prevalence of HIV drug resistance*

Different gene mutations of RTIs and PIs were detected among the 78 patients. All of the mutations and their frequencies are summarized in Table 2.

2. *Classification of mutations*

Table 1. The sequences of two pairs of primers

	Sequences	Gene location
Outer-primers	5'-CCTAGGAAAAAGGGCTGTTGGAAATGTGG-3' 5'-AACTTCTGTATGTCATTGACAGTCCA-3'	2011～2039 3303～3328
Inter-primers	5'-ACTGAGAGACAGGCTAATTTTTTAGGGA-3' 5'-CATTTATCAGGATGGAGTTCATA-3'	2068～2095 3243～3265

Table 2. Gene mutations and their frequencies in 78 patients with HIV infection

Gene mutation	Frequency	Percentage	Gene mutation	Frequency	Percentage
PI mutations:					
A62AV	1	1.3	L63LP	2	2.6
A71AT	4	5.1	L63P	64	82.1
A71AV	3	3.8	L63PS	2	2.6
A71T	5	6.4	L63S	2	2.6
A71V	5	6.4	L63T	2	2.6
D60DE	3	3.8	L90M	1	1.3
D60E	1	1.3	M36I	4	5.1
F63FLPS	1	1.3	M36IM	3	3.8
K70EK	1	1.3	M46I	1	1.3
L10I	3	3.8	V77I	64	82.1
L10IL	4	5.1	V77IV	6	7.7
RT mutations:					
G190A	1	1.3	F116Y	1	1.3
K103KN	4	5.1	F77FL	1	1.3
K103N	9	11.5	I93IL	4	5.1
P236S	1	1.3	I93L	46	59
V106A	1	1.3	K103KR	1	1.3
V106IV	1	1.3	K219N	1	1.3
V179D	2	2.6	K65KR	1	1.3
V179DV	1	1.3	L210LM	1	1.3
Y181C	3	3.8	M184ME	1	1.3
Y181CY	1	1.3	Q151M	3	3.8
Y188L	2	2.6	V118I	4	5.1
F116FHLY	1	1.3	V75IV	1	1.3

Analyzing so many resistance mutations would prove difficult, so mutations were classified by Hierarchical Cluster analysis, the results of which are shown in Figure 1.

3. *Correlation between mutations and HIV viral loads*

The mutations from 68 patients with detectable HIV RNA were classified into 4 classes by Hierarchical Cluster analysis. Class I was L63P, Class II was V77I, Class III was I93L, and Class IV was other mutations. The viral loads of the four groups were analyzed by univariate GLM. The results are shown in Table 3.

Using stepwise regression analysis with LgVL as the dependent variable and the gene mutations as an independent variable yielded the regression equation: LgVL = 4.720 + 0.638 × I93IL. There was statistical significance ($P = 0.023$).

Discussion

Anti-retroviral drug resistance is a major cause of HAART failure. Investigation of the prevalence of resistance is crucial to choosing an appropriate ARV regimen to achieve complete viral suppression, which could in turn reduce mutation accumulation and prevent the spread of resistance strains. Doing so would prove helpful in controlling the disease and reap the full advantages of HAART.

This study revealed that the most common primary mutations were L63P and V77I in treatment-naïve persons with HIV-1 subtype B infection in China's central plains, followed by I93L and other rare mutations. Even though the results were similar to those reported by Si-Xuefeng *et al*. (*1*), there were some differences. The ratios of V77I and I93L were 82.1% and 59.0% respectively, but Si-Xuefeng *et al*. reported respective ratios of 34.15% and 95.73%.

A major resistance mutation present at K103N of the HIV-1 reverse transcriptase gene was found at a higher frequency (11.5%) than as reported abroad (6%) (*2*). While the possibility of a few patients having taken missing doses of HIV medicine could not be excluded, this finding nevertheless warrants attention. The presence of K103N would result in a high level of resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs), so the high prevalence of the mutation could reduce the effects of NNRTIs and even accumulate with other mutations to increase the spread of resistance strains and diminish the effects of antiretroviral regimens containing NNRTIs. The high prevalence of K103N also suggested the necessity and urgency of developing new NNRTIs.

Other major mutations are shown in Table 1. The nucleoside reverse transcriptase inhibitor (NRTI) mutation, Q151M, appeared in 3 patients, one of which also had F116Y while another had F116FHLY, V75IV and F77FL mutations, and there was a potential for the development of Q151M complex. Of the major mutations of NNRTIs, Y181C appeared in 3 patients, Y188L in 2 patients, and V179D in 2 patients, respectively. Of the major mutations of protease

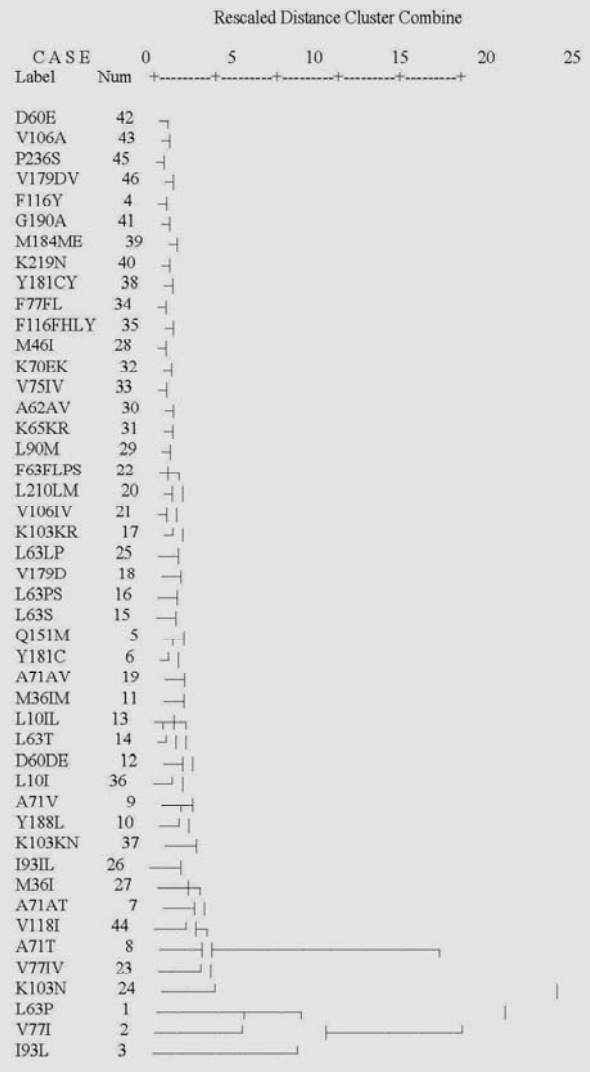

Figure 1. Hierarchical cluster analysis of gene mutations in 78 HIV-infected individuals (Dendrogram using Average Linkage (betwenen groups)).

Table 3. The correlation between gene mutations and logarithm of HIV viral loads (LgVL)

Mutation type	Number	LgVL ($\overline{X} \pm s$)	*P* value
1	54	4.746 ± 0.553	0.324
2	55	4.688 ± 0.541	0.256
3	42	4.737 ± 0.527	0.122
4	39	4.857 ± 0.494	0.003

inhibitors (PIs), M46I and L90M appeared in 1 patient. The low level prevalence of other major mutations besides K103N had less impact on the choice of HAART regimen.

The prevalence of major HIV mutations was still lower in China than that in other countries (*3*). The differences were greater particularly for prevalences of thymine-associated mutations (TAMs) and major protease-associated mutations, which maybe related to the short history of HAART use in China. In addition, frequency of the Q151M complex is higher than that of TAMs in NRTIs mutations in China. When choosing appropriate ARV regimens, the resistance prevalence domestically and abroad should serve as a reference, and especially for resource-limited regions.

The detected mutations were classified into 4 classes by Hierarchical Cluster analysis. Classes I and II had a high prevalence in HIV-1 subtype B-positive patients. Class III, I93L, had a moderate prevalence, and Class IV, which included other mutations, had a low prevalence. Another clustered method combined classes I and II into just one class. L63P, V77I, and I93L were all minor resistance mutations of PIs. Whether the high prevalence of mutations could affect the choice of PIs is still being studied in China. The correlation between the four classes of mutations and the logarithm of HIV viral loads (LgVL) was analyzed using univariate GLM analysis. The results showed that only class IV had a distinguished statistical significance in correlation to LgVL independently ($P = 0.003$). However, there was no statistical significance to the correlation between LgVL and the three mutations of L63P, V77I, and I93L. This fact suggests that the three mutations instead of other rare mutations were unable to decrease the HIV viral loads, so they may not affect the replication and adaptability of HIV. The results of stepwise liner regression analysis showed that LgVL was 0.638 higher in patients with the I93IL mutation. Further research on the clinical significance of this mutation should be done since the current study included few patients with I93IL.

To conclude, the mutations of HIV could not only result in resistance to ARV drugs but also affect the replication capacity of HIV. There was a direct correlation between HIV viral loads and the prognosis of the disease. If the replication capacity of the mutants was less than the wild type virus, the viral loads would remain at a lower level. Thus, ARV drugs would be a better choice when resistance is inevitable. If the replication capacity was greater, however, such drugs would accelerate the progress of the disease. The current study found that these common mutations did not increase viral load but they did have the potential to develop into cumulative mutations. In contrast, some rare mutations such as I93IL could increase HIV viral loads, which would then accelerate the progression of AIDS.

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A silkworm model of pathogenic bacterial infection

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ABSTRACT: Silkworms are invertebrate animals that are killed by bacteria pathogenic against humans, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*. Injection into the hemolymph of antibiotics that are clinically used for human patients abolishes the killing effects. There are several advantages to using silkworms as an infection model, such as low cost, the absence of ethical problems that are associated with the use of mammals, and a body size large enough to handle while injecting sample solution into the hemolymph. We screened *S. aureus* mutants with attenuated virulence against silkworms and found three novel virulence regulatory genes, *cvfA*, *cvfB*, and *cvfC*. These genes contribute to virulence against mice and are required for exotoxin production. The *cvfA* gene is required for expression of the *agr* locus, which regulates most exotoxin genes, and a novel DNA binding protein SarZ. Silkworms are susceptible to *S. aureus* beta toxin, *P. aeruginosa* exotoxin A, and diphtheria toxin. Therefore, silkworms are a promising infection model animal for the identification and evaluation of virulence-associated genes.

Key Words: Silkworms, virulence, *Staphylococcus aureus*, exotoxin

Introduction

Animal models are essential for identifying and evaluating virulence factors of pathogenic microorganisms. The purpose of this review is to provide evidence that silkworms are very useful for evaluating virulence factors of pathogenic bacteria. The review is divided into two parts. First, we introduce the advantages of the silkworm infection model for studying bacterial pathogenicity and evaluating the therapeutic effects of antibiotics. Then, we report the results of our recent studies in which we identified bacterial virulence genes using the silkworm infection model.

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Results and Discussion

1. *Advantages of the silkworm infection model*

To better understand bacterial pathogenicity, the use of animal models is essential. There are problems inherent in the use of animal models, however, such as the high cost housing animals, as well as ethical issues surrounding the use of mammals, which has become more serious in recent years. Especially in European countries, laws strictly regulate the use of mammals for the development of medicine. To overcome these problems, the use of invertebrates, such as the nematode *C. elegans*, the insect *D. melanogaster*, and the amoeba *Dictyostelium discoideum*, has been suggested (*1-6*). In particular, *C. elegans* and *D. melanogaster* are powerful tools for identifying host proteins involved in immune systems because they are genetically tractable and many mutant lines have been constructed (*7-9*). Because these animals are too small to handle, however, they are not suitable for injecting precise volumes of samples into the body fluid, a technique that is essential for quantitative evaluation of bacterial pathogenicity and the therapeutic effects of antimicrobial compounds.

Silkworms have several advantages as model animals for studying bacterial pathogenicity and the therapeutic effects of antibiotics (Figure 1). Silkworm, Lepidoptera *Bombyx mori*, was domesticated over

Advantages of silkworms as animal models

(1) Low cost
(2) No ethical issues
(3) Available to accurate injection into hemolymph and gut
(4) Available to pharmacological test with isolated organs

Figure 1. Silkworm as a model animal. Silkworms have favorable features as a non-mammalian hosts. In particular, No. 3 and 4 are difficult to perform in small-sized non-mammalian hosts, such as flies and nematodes.

the past 5,000 years from the wild species *Bombyx mandarina* for obtaining silk fibers from its cocoons. The long history of the silk industry has provided methods to produce thousands of silkworms at any time of the year. The fertilized eggs can be stored in a refrigerator for a year and can be obtained commercially. Silkworms can be raised from fertilized eggs to 5th instar larvae for three weeks by feeding artificial diet. There are no ethical problems associated with the use of a large number of silkworms. In addition, silkworms have great advantages over other invertebrate animals. Because the body size of silkworm 5th instar larvae (5 cm) is large enough to handle, sample solutions of pathogens and drug samples can be injected into the hemolymph or gut of the larvae using syringes equipped with needles. In *Drosophila* or *C. elegans*, manipulations must be performed under a microscope. When red ink is injected into the hemolymph of silkworms, the whole body color immediately changes to red, because the insect has an open circulatory system. If the needle is introduced deep enough to reach into the midgut, the red ink disperses throughout the midgut without changing the body color (*10*). These characteristics allow for the evaluation of bacterial pathogenisity and the therapeutic effects of antibiotics.

Furthermore, various tissues responsible for multiplying bacteria and drug metabolism can be isolated from silkworm larva, thus allowing for tracing of bacterial infectious process and pharmacologic experiments to study the pharmacodynamics of compounds. The hemolymph of the larvae that was injected with pathogenic bacteria can be easily collected and thus the bacterial cell number in the hemolymph counted. The proliferation process of the pathogenic bacteria in silkworms can be closely monitored (*11*). Drug transport assays can be performed using the isolated midgut. For this, the test sample is injected into the midgut and incubated in an appropriate buffer. If the compound passes through the midgut membrane, it will be detected in the buffer (*12,13*).

Recently, the silkworm genome project was completed by Japanese and Chinese groups (*14,15*). The reverse genetic method, RNA interference, was established in silkworms (*16-18*). The information obtained based on the genome and RNA interference method will facilitate the study of host factors involved in infectious processes.

When *S. aureus* was injected into the hemolymph of silkworm larvae, all of the larvae were killed by *S. aureus* within 2 d (Figure 2). All of the larvae in the control group, which were injected with saline, lived. Chloramphenicol has therapeutic effects. All of the *S. aureus*-injected silkworms injected with 100 μg chloramphenicol lived. Thus, this is a basic system for the study of "infection and therapy". Many pathogenic bacterium, such as *S. pyogenes*, *P. aeruginosa*, *Listeria*

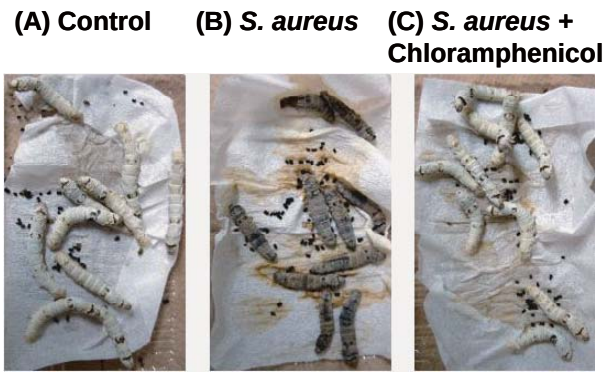

Figure 2. Infection and therapy using the silkworm model. Ten silkworms were injected with saline (A), 1×10^7 cells of *S. aureus* (B), or 1×10^7 cells of *S. aureus* with 100 μg chloramphenicol (C). Photograph was taken 2 d after the injection.

monocytogenes, *Serratia marcescens*, *Salmonella typhimurim*, and *Vibrio cholerae* kill silkworms (*11*). Antibiotics used clinically in humans also have therapeutic effects against silkworms injected with *S. aureus* and *P. aeruginosa* (*11,12*). Bacterial exotoxins kill silkworms (*19*). The 50% lethal dose (LD_{50}) of staphylococcal alpha-toxin is 12 μg/g; and that of staphylococcal beta-toxin is 9 μg/g; that of Pseudomonas exotoxin A is 0.14 μg/g; that of diphtheria toxin is 1.1 μg/g. Most of the LD_{50} values obtained were similar to the reported values in mice, suggesting that silkworms can be used as a model to study the general effects of bacterial exotoxins on multicellular organisms, including humans.

2. *Identification of bacterial virulence genes*

In this chapter, we describe our recent studies for the identification of bacterial virulence genes.

S. aureus causes opportunistic diseases in patients whose ability to protect themselves from bacterial infection is compromised. An understanding of the molecular mechanisms underlying the expression of *S. aureus* pathogenicity in animal bodies will help in establishing therapeutic methods against this pathogen. Based on the genome of this bacterium, there are 589 genes that are conserved among pathogenic bacteria, although their functions remain to be elucidated (*20*).

We constructed 100 gene mutants by disrupting these genes by targeting using a homologous recombination technique (Figure 3). Targeting vectors with the erythromycin-resistant gene and the internal region of the open reading frame were constructed, and introduced into the RN4220 strain. Gene disruption was confirmed by Southern blotting analysis. We injected 100 strains of disruption mutants into the hemolymph of silkworms, and found three mutants with decreased pathogenicity. The parent strain killed half the population of silkworms within 36 h, whereas these three strains required more than 80 h (Table 1). We named these genes *cvfA*, *cvfB*, and *cvfC* (conserved

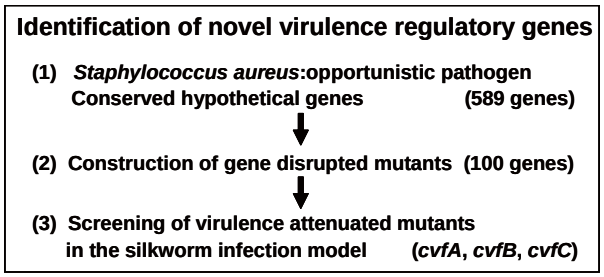

Figure 3. Strategy for identifying of novel virulence genes. The strategy for identifying novel virulence regulatory genes is illustrated. Gene deletions were performed by integrating the targeting vector into the desired hypothetical genes. Silkworms were injected with 50 μL of 20-fold diluted overnight cultures (1×10^7 cells) and larval survival was monitored. When the LT_{50} value of the mutant was 2 times greater than that of the parent strain, the mutant was selected as a virulence-attenuated mutant.

Table 1. Decreased killing ability in silkworms of the *cvfA*, *cvfB*, *cvfC* mutants of *S. aureus* and the *cvfA* mutant of *S. pyogenes*

Strain	Genotype	LT_{50} (h)	Fold change
S. aureus			
RN4220	Parent	36	1
M1129	Δ*cvfA*	84	2.3
M1223	Δ*cvfB*	94	2.6
M1262	Δ*cvfC*	98	2.7
S. pyogenes			
SSI-9	Parent	21	1
M1633	Δ*cvfA*	102	4.9

Ten silkworms were injected with *S. aureus* (1.3×10^7 CFU) and *S. pyogenes* (9×10^8 CFU) and larval survival was monitored. The time point when half of the larvae were killed is represented as LT_{50}. Fold change indicates the ratio of LT_{50} for the mutant to that of the parent.

Table 2. Conservation of the *cvfA* gene among bacteria

Phylum	Section	Genus	Identities
Proteobacteria	*Epsilonproteobacteria*	*Campylobacter*	47%
		Helicobacter	36%
Firmicutes	*Bacillales*	*Bacillus*	69%
		Oceanobacillus	58%
		Listeria	63%
		Staphylococcus	**100%**
	Clostridia	*Clostridium*	55%
		Thermoanaerobacter	60%
	Lactobacillales	*Enterococcus*	65%
		Lactobacillus	53%
		Lactococcus	59%
		Streptococcus	53%
	Mollicutes	*Mycoplasma*	36%
		Ureaplasma	27%
Fusobacteria	*Fusobacterales*	*Fusobacterium*	49%
Thermotogae	*Thermotogales*	*Thermotoga*	64%
Thermus/Deinococcus	*Deinococci*	*Deinococcus*	46%
Spirochaetes	*Spirochaetales*	*Borrelia*	42%
		Treponema	50%
Bacteroidetes	*Bacteroides*	*Bacteroides*	44%
Aquificae	*Aquificales*	*Aquifex*	60%
Chlorobi	*Chlorobia*	*Chlorobium*	39%

Bacterial species that have proteins homologous to *S. aureus* CvfA with more than 20% amino acid identity and E-values lower than e-10.

virulence factor A, B, and C) (*21*).

The *cvfA*, *cvfB*, and *cvfC* genes are conserved among various bacteria. The *cvfA* gene is ubiquitously present in the most bacterial genomes, although its function is unknown (Table 2). To test the ubiquitous function of the *cvfA* gene in other pathogenic bacteria, we constructed a deletion mutant of the *cvfA* homologue in *S. pyogenes* and tested its virulence in the silkworm infection model. The result demonstrated that the deletion mutant also had a delayed killing effect in silkworms (Table 1). When the mutant was complemented with the wild-type *cvfA* gene, the transformant had pathogenicity indistinguishable from the wild-type strain, confirming that deletion of the *cvfA* gene was responsible for the phenotype.

We then tested whether the *cvfA*, *cvfB*, and *cvfC* gene deletion mutants were less pathogenic in mice. The result showed that the number of *S. aureus* and *S. pyogenes* mutant bacterial cells required to kill 50% of the mice was greater than that of the corresponding wild-type bacteria, suggesting that these genes are necessary for pathogenicity in mammals.

The *S. aureus cvfA*, *cvfB*, and *cvfC* gene mutants produce less exotoxin than wild-type strains. In an agar plate assay containing sheep blood erythrocytes, a substrate for beta-toxin, the mutants produced a

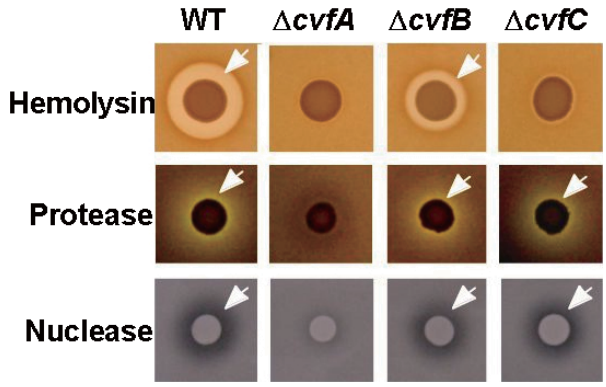

Figure 4. Decreased exotoxin production in the *cvfA*, *cvfB*, and *cvfC* mutants. Overnight cultures of the *cvfA*, *cvfB*, and *cvfC* mutants were spotted onto sheep blood agar plates, skim milk agar plates, and DNA agar plates, and incubated at 37˚C. The cleared zone indicated by white arrows reflects the activity of each exotoxin.

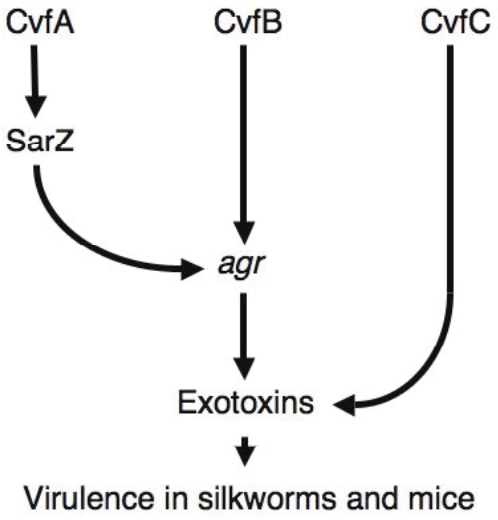

Figure 5. A model of virulence gene regulation. CvfA, CvfB, CvfC, and SarZ contribute to the expression of exotoxins and virulence in both silkworms and mice. CvfA, CvfB, and SarZ are required for maximum expression of the *agr* locus (*30*). CvfA and CvfB also exert their functions via an *agr*-independent pathway, because their deletion in the *agr* null background also attenuated virulence (*21*,*30*).

smaller lysis zone than did the wild-type strain (Figure 4). The phenotype was complemented by introduction of the wild-type genes. For the *cvfA* mutant, the amounts of protease and nuclease secreted from the cells were lower than those secreted by wild-type cells, and the phenotype was complemented by the wild-type *cvfA* gene. We hypothesized that proteins encoded by the *cvfA*, *cvfB*, and *cvfC* genes act to regulate exotoxin genes.

The *agr* locus is responsible for the expression of exotoxins in *S. aureus* (*22*,*23*). We examined whether deletion of the *cvfA* gene affects the expression of the *agr* locus. The results of Northern blot analysis demonstrated that the expression levels of RNAII, a product of the *agr* locus, and RNAIII, whose expression is stimulated by RNAII, were very low in the *cvfA* gene deletion mutants. The results strongly suggest that the *cvfA* gene regulates expression of the *agr* locus, which is required for the expression of exotoxin genes encoding hemolysins, proteases, and nucleases.

The primary amino acid sequence of the CvfA protein indicates that the CvfA protein contains a transmembrane domain, an RNA binding domain named KH (*24*) and a metal-dependent phosphohydrolase domain named HD (*25*,*26*). The KH and HD domains are needed for the pathogenicity of *S. aureus*, because the point mutations of these domains attenuated virulence in the silkworm infection model (*21*). CvfA protein is the first example of a protein harboring both the KH and HD domains having a physiologic role. A database search analysis (*27*) indicates that these proteins might function as ribonucleases, although this has not been verified experimentally.

The isolation and characterization of suppressor mutations is a basic technique in genetics. To understand the molecular network between pathogenic genes of *S. aureus*, including the *cvfA* gene, we screened multicopy suppressors of the *cvfA* gene deletion mutant from an *S. aureus* genomic library. We identified SA2174, which was previously designated as *sarZ*, which suppresses the decreased hemolysin production of the *cvfA* mutant (*28*). SarZ has sequence similarity with the MarR family (*29*), which are known as transcription factors of various genes. Because the *sarZ* gene was initially characterized as a multicopy suppressor of the *cvfA* gene deletion mutant, we considered the possibility that the *cvfA* gene activates the *sarZ* gene. This would explain how overproduction of the SarZ protein restores hemolysin production in the absence of the CvfA protein. Northern blot analysis demonstrated that the amount of *sarZ* transcript was much lower in the *cvfA* deletion mutant than in the parent. Introduction of the wild type *cvfA* gene into the *cvfA* deletion mutant restored the level of the *sarZ* transcript. Therefore, the *cvfA* gene is required for expression of the *sarZ* gene. Because the deletion of the *sarZ* gene induces a loss of pathogenicity in silkworms and mice, the *sarZ* gene itself is a virulence gene that is activated by the *cvfA* gene.

Concluding remarks

We discovered three pathogenic genes in *Staphylococcus aureus* using the silkworm infection model. We suggest that silkworms are useful for identifying and evaluating virulence regulatory genes. The *cvfA*, *cvfB*, and *sarZ* genes locate upstream of the *agr* locus, a well-studied virulence regulatory gene of this bacterium (Figure 5). Together with the CvfB and CvfC proteins, CvfA protein activates the expression of genes encoding hemolysin and other pathogenic genes. We propose that these genes act as regulators of pathogenic gene expression. Their conservation among pathogenic bacteria might indicate their contribution to the central

pathway of the virulence gene expression. Although uncovering the molecular function of novel genes is quite difficult, the effort will benefit our understanding of how pathogenic bacteria exert virulence against humans.

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Secondary metabolites from higher fungi in China and their biological activity

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ABSTRACT: As a part of our search for naturally occurring bioactive metabolites from higher fungi, we investigated the chemical constituents of basidiomycetes and ascomycetes fungi (*Albatrellus confluens, Albatrellus dispansus, Boletus edulis, Boletopsis grisea, Bondarzewia berkeleyi, Cortinarius tenuipes, Cortinarius vibratilis, Daldinia concentrica, Engleromyces goetzii, Hydnum repandum, Hebeloma versipelle, Hygrophorus eburnesus, Lactarius deliciosus, Lactarius hatsudake, Lactarius hirtipes, Lactarius mitissimus, Lactarius rufus, Paxillus panuoides, Pulveroboletus ravenelii, Russula cyanoxantha, Russula foetens, Russula lepida, Russula nigricans, Sarcodon laevigatum, Sarcodon scabrosus, Shiraia bambusicola, Thelephora aurantiotincta, Thelephora ganbajun, Tricholomopsis rutilans, Tylopilus virens, Tuber indicum, Xylaria euglossa, etc.*), and isolated a number of novel terpenoids, phenolics, and nitrogen-containing compounds. The isolation, structural elucidation, and biologically activity of the new compounds are discussed.

Key Words: Higher fungi, secondary metabolites, biological activities, natural products

Introduction

China is extraordinary rich in higher fungi. To date, about 10,000 species of fungi have been reported from China's vast territories. Of them, nearly 6,000 species, belonging to about 1,200 genera, are higher fungi (excluding lichens). In bio-resources, higher fungi belong to very productive biological sources that produce a large and diverse variety of secondary metabolites. Biologically active substances present in untapped and diverse sources of higher fungi from China are interesting. The isolation, structural elucidation, and biologically activity of new compounds prior to 2002 were previously reviewed (*1*,*2*).

Recently several dozen new natural substances and bioactive compounds were found in selected mushrooms on the basis of using our knowledge on the collection of fruiting bodies, strain preservation, fermentation, biologically screening and chemical investigation of higher fungi. The isolation, structural elucidation and biologically activity of the novel terpenoids, phenolics and nitrogen-containing compound from basidiomycetes and ascomycetes fungi (*Albatrellus confluens*, *Albatrellus dispansus*, *Boletus edulis*, *Boletopsis grisea*, *Bondarzewia berkeleyi*, *Cortinarius tenuipes*, *Cortinarius vibratilis*, *Daldinia concentrica*, *Engleromyces goetzii*, *Hebeloma versipelle*, *Hydnum repandum*, *Hygrophorus eburnesus*, *Lactarius deliciosus*, *Lactarius hatsudake*, *Lactarius hirtipes*, *Lactarius mitissimus*, *Lactarius rufus*, *Paxillus panuoides*, *Pulveroboletus ravenelii*, *Russula cyanoxantha*, *Russula foetens*, *Russula lepida*, *Russula nigricans*, *Sarcodon laevigatum*, *Sarcodon scabrosus*, *Shiraia bambusicola*, *Thelephora aurantiotincta*, *Thelephora ganbajun*, *Tricholomopsis rutilans*, *Tylopilus virens*, *Tuber indicum*, *Xylaria euglossa*, *etc*.) are reviewed.

Concentricolide, an anti-HIV agent, and other compounds from the ascomycete *Daldinia concentrica*

Although anti-HIV-1 drugs now available have improved the quality of the lives of HIV/AIDS patients, the rapid evolution of new HIV clades and drug resistant variants in AIDS patients urged the search for new anti-HIV-1 agents and targets. A large variety of natural substances including alkaloids, flavonoids, coumarines, lignans, phenolics, triterpenoids, saponins, sulfated polysaccharides, phospholipids, quinones and peptides with anti-HIV-1 effect have been described, and for a portion thereof the target of interaction has been identified (*3*). Natural substances provide a large reservoir for screening of anti-HIV-1 agents with novel structure and anti-viral mechanisms.

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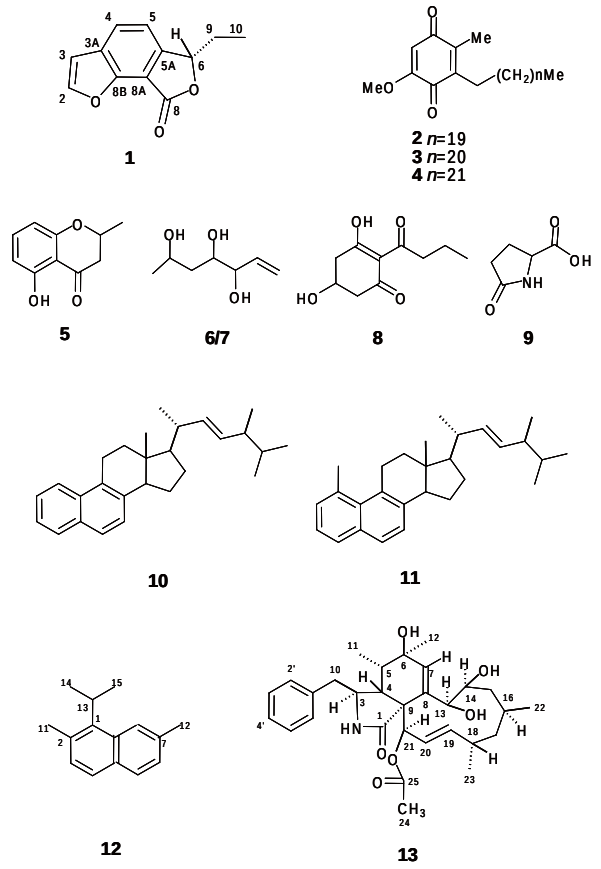


Figure 1. Different compounds of the ascomycete *Daldinia concentrica.*

A novel benzofuran lactone, named concentricolide (**1**), was isolated along with the four known compounds (friedelin, cytochalasin *L*-696,474, armillaramide, russulamide) from the fruiting bodies of the xylariaceous ascomycete *Daldinia concentrica*. The structure of concentricolide was established by spectroscopic methods and X-ray crystallographic analysis. Its anti-HIV-1 activity was tested. Results showed that concentricolide inhibited HIV-1 induced cytopathic effects. The EC_{50} value was 0.31 μg/mL. The therapeutic index (TI) was 247. Concentricolide exhibited the blockage (EC_{50} 0.83 μg/mL) on syncytium formation between HIV-1 infected cells and normal cells (*4*).

Except concentricolide (**1**), a new homologous series of 3-alkyl-5-methoxy-2-methyl-1,4-benzoquinones (**2**-**4**) with chain length C_{21} to C_{23} were isolated from the fruiting bodies of *Daldinia concentrica* (*5*). Two novel heptenetriol stereoisomers, hept-6-ene-2,4,5-triols **6** and **7**, were, along with three known compounds, *i.e.*, 2,3-dihydro-5-hydroxy-2-methyl-4*H*-1-benzo-pyran-4-one (**5**), 3,5-dihydroxy-2-(1-oxobutyl)-cyclohex-2-en-1one (**8**), and pyroglutamic acid (= 5-oxo-*L*-proline) (**9**), isolated from the culture broth of *D. concentrica* (*6*). Compound **5** is reported to be a metabolite from the rice culture solution of the fungus *Phialophora gregata* and has been shown to have biological activity on soybean cells (*7*). Compound **8** has also previously been isolated from the culture broth of the fungus *Nodulisporium* sp. and has been found to have chlorotic activity (with greater activity on monocotyledons than on dicotyledons) (*8*).

The identification of aromatic steroid hydrocarbons bearing a methyl group at positions 1, 2, 3, 4, or 6 in sediments and petroleum is enigmatic since possible steroidal precursors have not yet been reported in living organisms. Two new aromatic steroids (**10** and **11**) were isolated from the fruiting bodies of *D. concentrica*, and one, compound **11,** bears an unusual methyl group at position 1. These compounds presumably originate with the transformation undergone by their precursors due to microbial action. Compounds **10** and **11** could be long-sought, biological precursor steroids for organic matter in the Earth's subsurface (*9*). Two other new compounds, 1-isopropyl-2,7-dimethylnaphthalene (**12**) and 21-acetyloxyl-16,18-dimethyl-10-phenyl-6,13,14-trihydroxyl-[11]-cyto-chalasa-7,19-diene-1-one (**13**), were also isolated from the fruiting bodies of *D. concentrica* (*10*).

Grifolin, a potential natural antitumor substance produced by inducing apoptosis *in vitro* in *Albatrellus confluens*, and other related compounds from the same genus

Grifolin (**14**) is a natural biologically active substance isolated from the fruiting bodies of *Albatrellus confluens*. Here, novel activity of grifolin is described for the first time, namely its ability to inhibit the growth of tumor cells by the induction of apoptosis. Grifolin strongly inhibited tumor cells lines CNE1, HeLa, MCF7, SW480, K562, Raji, and B95-8. Analysis of acridine orange (AO)/ethidium bromide (EB) staining and flow cytometry showed that grifolin possessed apoptosis induction activity with respect to CNE1, HeLa, MCF7, and SW480. Furthermore, cytochrome *c* release from mitochondria was detected by confocal microscopy in CNE1 cells after 12 h of treatment with grifolin. The increase in caspase-8, 9, 3 activity revealed that caspase was a key mediator of the apoptotic pathway induced by grifolin, and the under-expression of Bcl-2 and up-regulation of Bax resulted in the increase in Bax: Bcl-2 ratio, suggesting that the Bcl-2 family is involved in the control of apoptosis. Owing to the combination of its significant antitumor activity by inducing apoptosis and the compound's natural abundance, grifolin represents an interesting antitumor agent that deserves further laboratory and *in vivo* examination (*11*).

In the course of screening for novel naturally occurring fungicides from mushrooms in Yunnan province of China, the ethanol extract of the fruiting bodies of *Albatrellus dispansus* was found to show antifungal activity against plant pathogenic fungi. The active compound was isolated from the fruiting bodies of *A. dispansus* by bioassay-guided fractionation of the

extract and identified as grifolin (**14**) by IR, ^{1}H and ^{13}C NMR, and mass spectral analysis. Its antifungal activity was evaluated *in vitro* against 9 plant pathogenic fungi and *in vivo* against the plant disease *Erysiphe graminis*. *In vitro*, *Sclerotinia sclerotiorum* and *Fusarium graminearum* were the most sensitive to grifolin, and at 0.1 μg/mL their levels of mycelial growth inhibition were 86.43 and 80.90%, respectively. Spore germination of *F. graminearum*, *Gloeosporium fructigenum* and *Pyricularia oryzae* were almost completely inhibited by 12.5 μg/mL of grifolin. The curative effect of grifolin (**14**) on *Erysiphe graminis in vivo* was 65.52% at 100 μg/mL (*12*).

In a previous report, the effects of albaconol (**15**) from *Albatrellus confluens* on vanilloid receptors were studied electrophysiologically in rat ganglion neutrons as well as in recombinant cell lines expressing rat VR1 receptor (*13*). Recently, the effects of albaconol (**15**) on the inhibition of human tumor cell growth, DNA topoisomerase (topo)-mediated DNA cleavage, and direct DNA breakage were investigated. Albaconol (**15**) significantly inhibited the growth of human chronic myelogenous leukemia K562, lung adenocarcinoma A 549, gastric adenocarcinoma BGC-823 and breast carcinoma Bcap-37 cell lines with IC_{50} values of 2.77 ± 0.14, 2.58 ± 0.88, 1.45 ± 0.05, and 1.10 ± 0.31 μg/mL, respectively. Albaconol (**15**) stabilized and increased the topo II-mediated DNA cleavable complex and inhibited the religation activity of topo II in a dose-dependent manner, but it failed to affect the activity of topo I. Albaconol (**15**) acts to break strands of pBR322 DNA at relatively high concentrations but no effect on the macromolecule DNA of K562 cells. These results strongly suggest that albaconol (**15**) specifically targets DNA topo II and that this is one of the mechanisms of albaconol's antitumor action; the direct action of albaconol (**15**) on DNA may contribute to its antitumor activity at high concentrations (*14*).

Contraction and desensitization induced by albaconol (**15**) and the influence of capsazepine, capsaicin, and extracellular Ca^{2+} were investigated to see whether action was mediated *via* a specific VR receptor in guinea pig trachea spiral strips *in vitro*. Both albaconol (**15**) and capsaicin were contractors of tracheal smooth muscle, but albaconol (**15**) was not as potent as capsaicin, with -log (M) EC_{50} values of 4.23 ± 0.18 (n = 10) and 7.33 ± 0.21 (n = 10), respectively. Two-point five and 5.0 μM capsazepine competitively antagonized the contractile response to albaconol (**15**), with -log (M) pK_B values of 6.60 ± 0.39 (n = 10) and 7.36 ± 0.45 (n = 10), respectively. Albaconol (**15**) increased the contraction induced by a low dose of capsaicin (10^{-10}-10^{-9} M) but non-competitively antagonized the contraction induced by a high dose of capsaicin (10^{-8}-10^{-3} M). Either albaconol (1,100 mM) or capsaicin (3.0, 10 μM) was able to desensitize the isolated guinea pig bronchi to subsequent addition of albaconol.

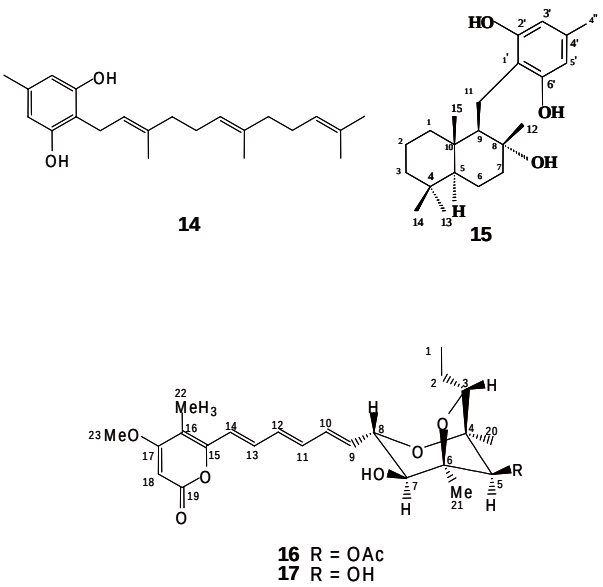


Figure 2. Potential antitumor natural products.

Capsazepine (5.0 μM) significantly prevented the desensitization induced by either albaconol (1, 100 mM) or capsaicin (3, 10 μM). Extracellular Ca^{2+} was essential for albaconol to induce excitation, but it did not affect albaconol- or capsaicin-induced desensitization. These results suggest that albaconol (**15**) induces contraction and desensitization of guinea pig trachea *in vitro* as a partial agonist for VR (*15*).

Albaconol (**15**) inhibited lipid peroxidation in rat liver homogenate with an IC_{50} value of 104.2 μg/mL in comparison to butylated hydroxyanisole (BHA, IC_{50} 40.4 μg/mL) and vitamin E (IC_{50} 127.2 μg/mL). Albaconol increased the activity of SOD (EC_{50} value of 106.3 μg/mL) and BHA (EC_{50} 19.9 μg/mL) (*16*).

When grown in culture, the basidiomycete *Albatrellus confluens* produces a polyene pyrone mycotoxin, aurovertin E (**17**), along with aurovertin B (**16**). This was the first example of the occurrence of aurovertins in macromycetes (*17*). The aurovertins, metabolites from the fungus (anamorphic ascomycetes) *Calcarisporium arbuscula*, are a group of acute neurotoxic substances that act as potent inhibitors of ATP synthesis and ATP hydrolysis catalyzed by mitochondrial enzyme systems (*18-21*).

Radical scavenging activity of natural *p*-terphenyls obtained from three edible mushrooms indigenous to China and other natural *p*-terphenyls

Ten natural *p*-terphenyl derivatives (**18-27**) obtained from the fruiting bodies of three edible mushrooms (*Thelephora ganbajun*, *Thelephora aurantiotincta*, and *Boletopsis grisea*) indigenous to China were assessed in terms of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity. The compounds **18-20** showed potent DPPH radical scavenging activity in comparison

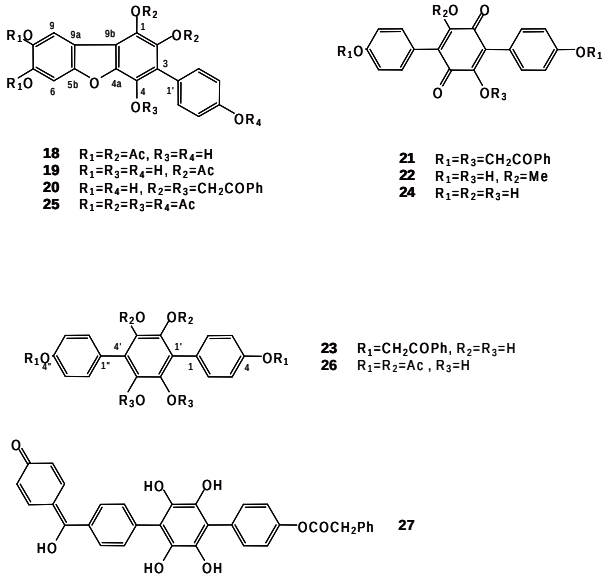


Figure 3. Ten natural *p*-terphenyl derivatives.

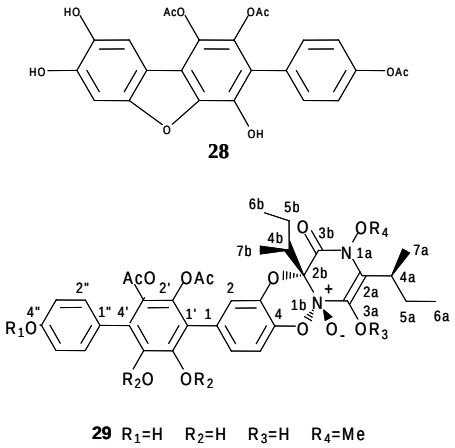


Figure 4. Sarcodan (**28**) and sarcodonin δ (**29**).

to BHA (butylated hydroxyanisol) and α-tocopherol, which are known to be strong activators. The free radical scavenging activity of **19** (EC_{50} = 0.07) was found to be stronger than that of BHA (EC_{50} = 0.09) and α-tocopherol (EC_{50} = 0.25), while that of **18** (EC_{50} = 0.12) and **20** (EC_{50} = 0.13) was similar to that of BHA and stronger than that of α-tocopherol. The formation of furan rings and the numbers and position of hydroxy groups in the molecular structure of *p*-terphenyls have been found to be crucial for modulation of free radical scavenging activity (*22*).

A metabolite with a *p*-terphenyl core, named sarcodon (**28**), was isolated from the fruiting bodies of the basidiomycete *Sarcodon laevigatum* (*23*). Another nitrogenous metabolite with a *p*-terphenyl core, sarcodonin δ (**29**), was isolated from the fruiting bodies of the basidiomycete *Sarcodon scabrosus* (*24*).

Terphenyls are aromatic hydrocarbons consisting of a chain of three benzene rings. There are three isomers, in which the terminal rings are *ortho*-, *meta*-, or *para*-substituents of the central ring. Most of the natural terphenyls are *p*-terphenyl derivatives. The chemical investigation of *p*-terphenyls as one class of the pigments of mushrooms began in 1877 (*25*). In recent years, some terphenyls have been reported to exhibit significant biological activity, *e.g.*, potent immunosuppressant, neuroprotective, antithrombotic, anticoagulant, specific 5-lipoxygenase inhibitory, and cytotoxic activity (see section 5). In addition, terphenyls are, in comparison to other types of complex natural substances, easily synthesized since they contain fewer (or no) chiral centers. Another interesting point is that some popular edible mushrooms are rich in *p*-terphenyls; this is a sign that at least some *p*-terphenyls have a low level of toxicity. Because of their promising biological activity and important properties, terphenyls have generated increasing research interest (*25*).

Antifungal sesquiterpenoid and other compounds from the genera *Lactarius* and *Russula*

The mushrooms belonging to the genus *Lactarius* (family Russulaceae, Basidiomycotina) form a milky juice when the fruiting bodies are injured. In the great majority of *Lactarius* species, different kinds of sesquiterpenes play an important biological role, being responsible for the pungency and bitterness of the milky juice, the change in the color of the latex in air, and constituting a chemical defense system against various predators such as bacteria, fungi, animals, and insects (*26*). Most *Lactarius* sesquiterpenes belonging to the classes of lactaranes, secolactaranes, marasmanes, isolactaranes, norlactaranes, and caryophyllanes are believed to be biosynthesized from humulene (*27-30*).

Rufuslactone (**30**) is an isomer of a previously described lactarane 3,8-oxa-13-hydroxylactar-6-en-5-oic acid γ-lactone (**31**) from *Lactarius rufus*. Its structure was elucidated by spectroscopic means. Rufuslactone (**30**) displayed antifungal properties against plant pathogenic fungi (*31*). *Alternaria brassicae* was the most sensitive to Rufuslactone (**30**), and its mycelial growth inhibition was 68.3 at 100 μg/mL.

A humulene sesquiterpene, named 2β,3α-epoxy-6*Z*,9*Z*-humuladien-8α-ol (**32**), was, together with the known compound lactarinic acid, isolated from the fruiting bodies of *Lactarius hirtipes*. For the subdivision Basidiomycotina, fungal sesquiterpenes formed *via* the humulane-protoilludane biosynthetic pathway are also characteristic. However, no representative of humulene sesquiterpenes has been isolated from higher fungi thus far. Compound **32** was the first humulene-type sesquiterpene found in higher fungi (*32*). Five new humulane-type sesquiterpenes, mitissimols A (**33**), B (**34**), and C (**35**), and a mixture of mitissimyl A oleate (**36**) and mitissimyl B oleate (**37**), were isolated from the fruiting bodies of *Lactarius mitissimus* (*33*).

Their structures were elucidated by comprehensive spectroscopic techniques and necessary chemical methods. The relative stereochemistry of **33** was determined by single crystal X-ray diffraction analysis.

Two new red azulene pigments (**38**, **39**) were isolated from the fruiting bodies of the basidiomycete *Lactarius deliciosus* together with one known pigment (**40**) (*34*). Two other new azulene pigments, 1-formyl-4-methyl-7-(11-hydroxyl) isopropylazulene (**41)** and 4-methyl-7-isopropylazulene-1-carboxylic acid (**42**), were isolated from the fruiting bodies of the basidiomycete *Lactarius hatsudake* (*35*).

A new marasmane sesquiterpene, named lactapiperanol E (**43**), was isolated from the fruiting bodies of *Russula foetens* together with a known sesquiterpene, lactapiperanol A (**44**) (*36*). Sesquiterpenes possessing the marasmane skeleton have been known for more than 50 years (*37*). Marasmic acid was found to be an antibacterial substance in *Marasmius conigenus* (*38*), and its 9-hydroxy derivative, detected in another basidiomycete, displayed antifungal, cytotoxic, and phytotoxic activity (*39*). Velutinal and its fatty acid esters represent interesting examples of prodrugs (*40,41*). In most fungi, only the esters, which are cleaved to velutinal in the event of injury to the fruiting bodies, are present (*42*). Pilatin is an antibiotically active marasmane derivative from the culture of *Flagelloscypha pilatii*. It is a higher oxidized derivative of marasmic acid, causes frameshift mutations in *Salmonella typhimurium*, inhibits the growth of bacteria and fungi, and is highly cytotoxic (*43*). The Russulaceae family is one of the largest in the subdivision Basidiomycotina in Whittaker's kingdom of Fungi and consists of hundreds of species (*44*). While secondary metabolites occurring in the fruiting bodies of European *Lactarius* species have been extensively investigated, the *Russula* mushrooms have received less attention, notwithstanding the larger number of existing species (*45*). Recent investigation of the chemical constituents of *Russula lepida* by the current authors led to the identification of some new terpenoids (*46-48*). The minor constituents of *Russula lepida* were further investigated. A novel nitrogen-containing aristolane sesquiterpenoid compound, lepidamine **45**, was isolated from the fruiting bodies of Basidiomycete *Russula lepida*. It is the first aristolane-type sesquiterpene alkaloid isolated from nature (*49*). It is also interesting that nigricanin (**46**), the first ellagic acid related derivative from higher fungi, has been isolated from the fruiting bodies of the basidiomycete *Russula nigricans* (*50*). Ellagic acid and its derivatives are widely distributed in plants but are rare in fungi. Ellagic acid and its derivatives are known to display multiple forms of biological activity such as DNA damage (*51*) or act as antioxidants (*52*). In the case of actinomycete, *e.g.*, *Streptomyces chartreuses*, only the antibiotics D 329C, chartreusin, and elsamicin have been isolated; and these

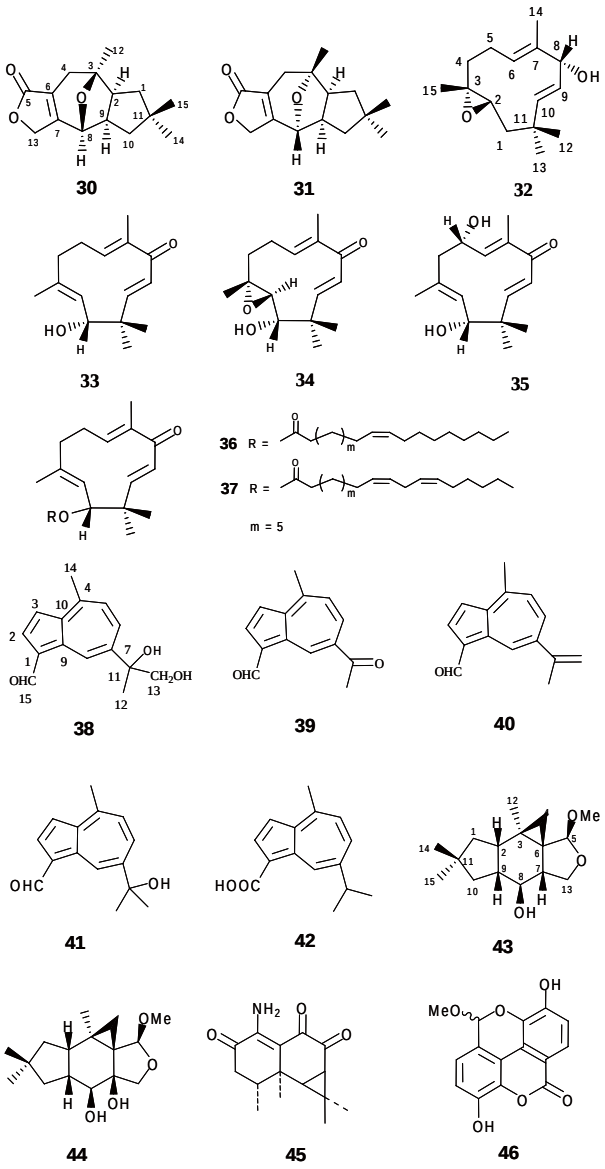


Figure 5. Described chemical compounds.

compounds have been reported to display antibacterial, antineoplastic, and antileukemia activity (*53-55*).

Pigments from *Pulveroboletus ravenelii* and *Xylaria euglossa*

A new butenolide-type fungal pigment, pulverolide (**47**), was isolated from the fresh fruiting bodies of *Pulveroboletus ravenelii* (*56*). *Xylaria euglossa* is a rot-wood-inhibiting ascomycete, mainly occurring on stumps and fallen branches of forested areas in the southwest of China. Many unique secondary metabolites have been found in the fungi of this genus. During the study of *Xylaria sp.*, various new metabolites were discovered, including cytochalasins, globoscin, lactones, maldoxin, sesquiterpenoids, xylaramide, xylarin, and xyloketals (*57*). Detailed chemical investigation of the fungus *Xylaria euglossa* has been performed and a new nitrogen-containing

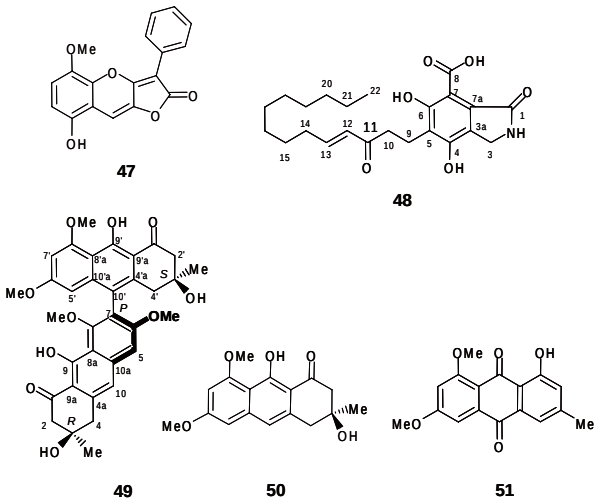


Figure 6. Described chemical compounds.

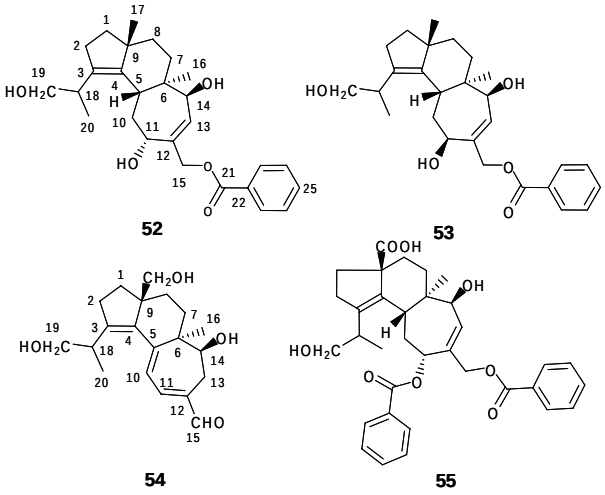


Figure 7. Described chemical compounds.

compound, xylactam (**48**), was isolated along with two known alkaloids, penochalasin B 2 and neoechinulin A, from extracts of the fruiting bodies (*57*).

A new pigment, 8,8'-*O*,*O*-dimethylphlegmacin A (**49**), was isolated from the fruiting bodies of ascomycete *Xylaria euglossa* along with two known fungi pigments, (**50**) and **(51)**. The structure of compound **49** was established as (3*R*,3'*S*,*P*)-2,2',3,3'-tetrahydro-3,3',9,9' -tetrahydroxy-6,6',8,8'-tetramethoxy-3,3'-dimethyl-[7,10'-bianthracene]-4,4' (1H,1'H)-dione by spectroscopic means. Its absolute configuration was deduced from CD and ^{1}H NMR spectra. It represents the first isolation of a phlegmacin-type pigment from an ascomycete (*58*).

Diterpenoids from *Sarcodon sp.* and *Hydnum sp.*

Novel cyathane-type diterpenoids, scabronines G and H and sarcodonin I **(52-54),** were isolated from the fruiting bodies of the basidiomycete *Sarcodon scabrosus* together with four known diterpenoids, allocyathin B_2, sarcodonin A, sarcodonin G, and scabronine F (*59*,*60*). *Sarcodon scabrosus* is a mushroom belonging to the family *Thelephoraceae* and has a bitter taste. Diterpenoids, including sarcodonins A-H, scabronines A-F, and scabronines L and M, have previously been isolated from this mushroom as bitter principles (*61-63*). All of these diterpenoids posses a cyathane skeleton consisting of angularly condensed five-, six- and seven-membered rings and display activity to stimulate nerve growth factor (NGF)-synthesis *in vitro*.

Eleven compounds have been isolated from the fruiting bodies of the basidiomycete *Hydnum repandum*. Their structures were established as sarcodonin A, scabronine B (**55**), 3β-hydroxy-5α,8α-epidioxyergosta-6,22-dien, (22*E*,24*R*)-ergosta-7,22-diene-3β,5α,6β-triol, (22*E*,24*R*)-ergosta-7,22-diene-3β-ol, benzoic acid, 4-hydroxylbenzaldehyde, 4-monopropanoylbenzenediol, ethyl-β-*D*-glucopyranoside, thioacetic anhydrid, and (2*S*,2'*R*,3*S*,4*R*)-2-(2-hydroxytricosanoylamino) hexadecane-1,3,4-triol by spectral methods. Among them, sarcodonin A and scabronine B were first reported from *Hydnum* genus, and the other compounds were isolated from thisfungus for the first time (*64*).

Miscellaneous

A novel *N*-containing compound, vibratilicin (**56**), was isolated from the fruiting bodies of the basidiomycete *Cortinarius vibratilis* (*65*). Compound **56** is a representative of a rare natural substance containing hydroxamic acid moieties and can be viewed as a derivative of neoengleromycin from the fungus *Engleromyces goetzii* (*66*).

Fruiting bodies of the basidiomycete *Thelephora aurantiotincta* contain a *p*-terphenyl, named aurantiotinin A (**57**), together with ganbajunin C and atromentin (*67*). Fruiting bodies of the basidiomycete *Cortinarius umidicola* contain a natural pyridine derivative (3-aldehyde-2-amino-6-methoxypyridine, (**59**), together with (*R*)-glycidyl octadecanoate (**58**) (*68*).

An unique fungal pigment, hypocrellin D (**60**), together with three known perylenequinone derivatives, hypocrellin A (**61**), B, C, was isolated from the fruiting bodies of *Shiraia bambusicola* (*69*). The ROESY experiment and CD of hypocrellin D required that the absolute configuration of the asymmetric carbons of the alicyclic ring of **60** be the same as those of hypocrellin A; *i.e.* 14*S* and 15*R*. *Shiraia bambusicola* (Hypocreaceae), an ascomycete parasitic on bamboo twigs, is recorded only in China and Japan. It has been commonly used as medicinal fungi under the name of "Zhu Huang" in China for treatment of rheumatism and pneumonia in traditional Chinese medicine (TCM). The new perylenequinone pigments hypocrellin A-C and shiraiachrome A-C were previously isolated from *S. bambusicola* as fungal metabolites that exhibit photodynamic activity on bacteria and fungi (*70*,*71*). Recently, the methanolic extract of the mycelium of the fungus *S. bambusicola* was found to show significant cytotoxicity in A-549 and HCT-8 solid tumor cells.

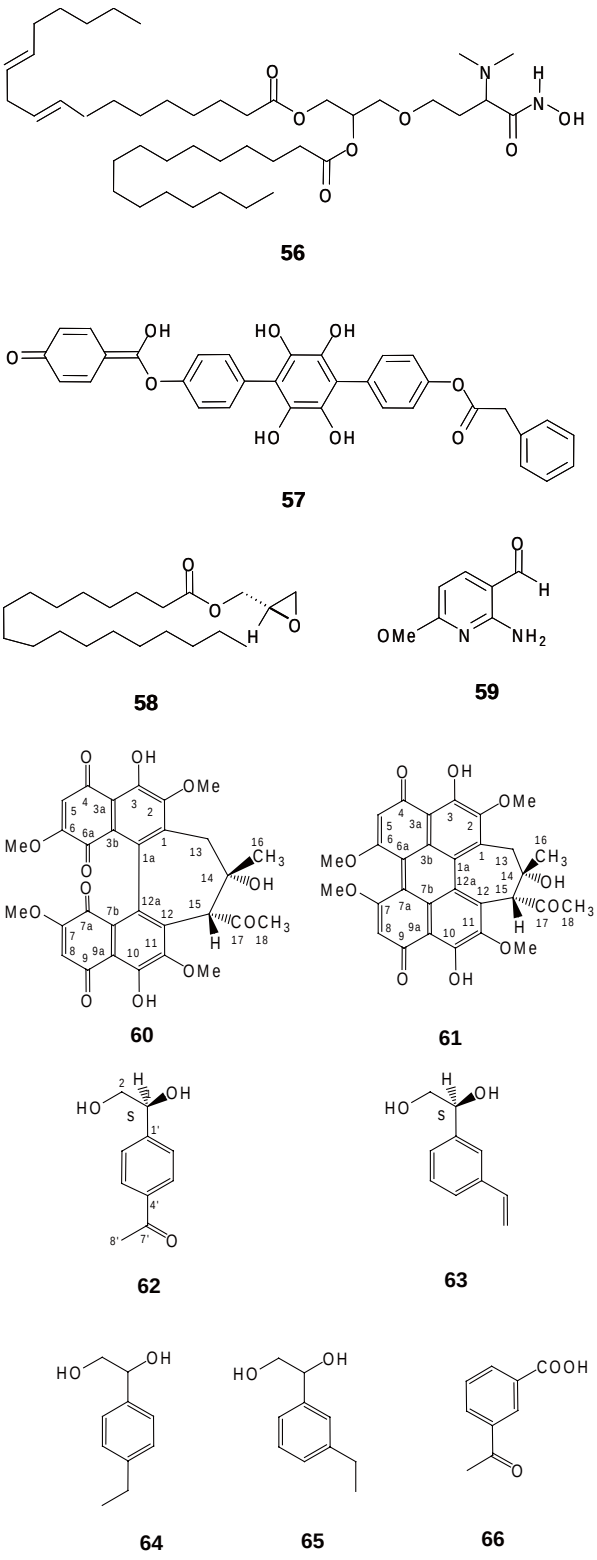


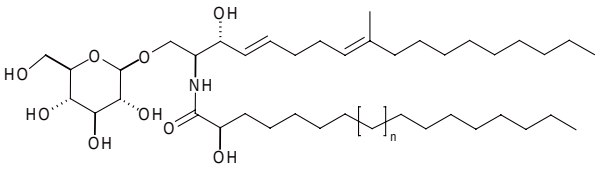
Figure 8. Described chemical compounds.

Subsequent bioassay-guided fractionation in HCT-8 *in vitro* led to the isolation and characterization of shiraiachromes A and B as two major cytotoxic principles (*72*). A series of new perylene derivatives related to shiraiachrome-A and -B as well as calphostin-C has been synthesized and evaluated for its cytotoxicity, antiviral activity, and inhibitory activity against protein kinase C (*72*).

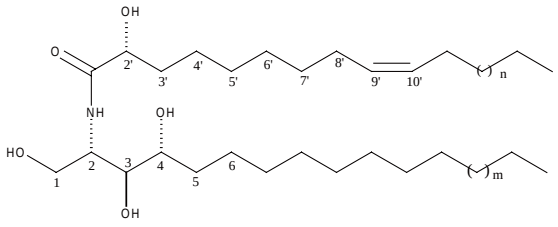
When grown in culture, the basidiomycete *Boletus edulis* produces two phenyl-ethanediols, 1-(3-ethenylphenyl)-1,2-ethanediol **62** and 1-(4-acetylphenyl)-1,2-ethanediol **63**, together with three known compounds, 1-(3-formylphenyl)-ethanone **64,** 1-(3-ethylphenyl)-1,2-ethanediol **65,** and 1-(4-ethylphenyl)-1,2-ethanediol **66** (*73*). Compound **62** has often been used as a type of rubber composition and was isolated for the first time as a new natural substance.

Five cerebrosides, including three new ones named cortenuamide A (**67**), cortenuamide B (**68**), and cortenuamide C (**69**), were isolated from the fruiting bodies of the Basidiomycetes *Cortinarius tenuipes*. The structures of those compounds were elucidated as (4*E*,8*E*)-*N*-*D*-2'-hydroxytetracosanoyl-1-*O*-β-*D*-glucopyranosyl-9-methyl-4,8-sphinga-dienine (**67**), (4*E*,8*E*)-*N*-*D*-2'-hydroxytricosanoyl-1-*O*-β-*D*-gluco-pyranosyl-9-methyl-4,8-sphingadienine (**68**), (4*E*,8*E*)-*N*-*D*-2'-hydroxyl-docosanoyl-1-*O*-β-*D*-glucopyranosyl-9-methyl-4,8-sphingadienine (**69**), (4*E*,8*E*)-*N*-*D*-2'-hydroxyoctasanoyl-1-*O*-β-*D*-glucopyranosyl-9-methyl-4,8-sphingadienine, and (4*E*,8*E*)-*N*-*D*-2'-hydroxypalmitoyl-1-*O*-β-*D*-gluco-pyranosyl-9-methyl-4,8-sphingadienine by spectral and chemical methods (*74*). A new ceramide, named hygrophamide (**70**), was isolated from the fruiting bodies of the Basidiomycetes *Hygrophorus eburnesus*. The structure of the compound was elucidated as (2*S*,3*R*,4*R*,2'*R*)-2-(2'-hydroxy-9'*Z*-ene-tetracosanoylamino)-octadecane-1,3,4-triol (**70**) by spectral and chemical methods (*75*).

Ceramide fractions were isolated from the fruiting bodies of *Tuber indicum* and separated into three kinds of molecular species, **71**, **72**, and **73,** by normal and reverse phase silica gel-column chromatography. According to NMR spectroscopy, FAB-MS, and chemical degradation experiments, the component sphingoid base for **71** and **72** was uniformly



67 n=9, **68** n=8, **69** n=7



70 n=11, m=4

Figure 9. Described chemical compounds.

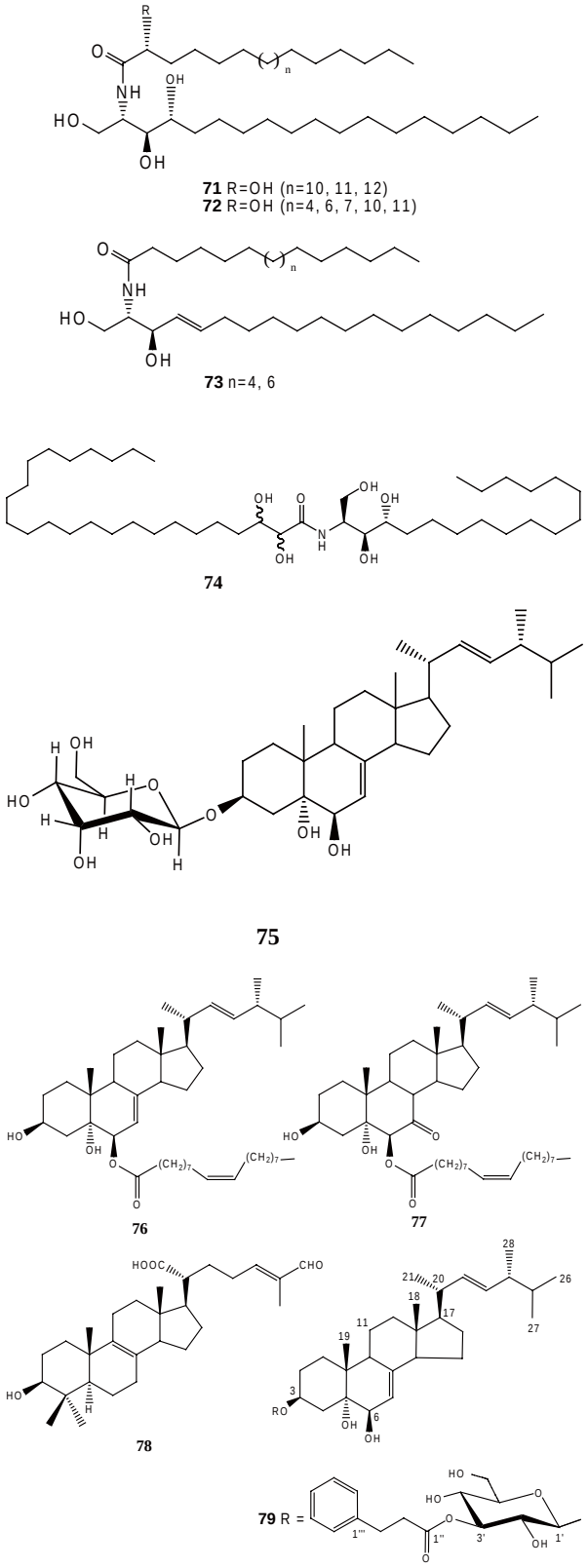


Figure 10. Described chemical compounds.

(2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecantriol, while the sphingoid of **73** was *D-erythro*-sphingosine, and their structures have been determined unequivocally to be (2*S*,2'*R*,3*S*,4*R*)-2-(2'-*D*-hydroxyalkanoylamino) octadecane-1,3,4-triol, the fatty acid composition of which consists of 2-hydroxydocosanoic, 2-hydroxytetracosanoic, and 2-hydroxytricosanoic acids; (2*S*,3*S*,4*R*)-2-(alkanoyl-amino) octadecane-1,3,4-triol, the fatty acid composition of which is unusual and consists of docosanoic, hexadecanoic, tricosanoic, octadecanoic and nonadecanoic acids; and (2*S*,3*R*,4*E*)-2-(alkanoylamino)-4-octadecene-1,3-diol, the component fatty acids of which were hexadecanoic (predominant) and octadecanoic acids, respectively (*76*). A new phytosphingosine-type ceramide **74**, named paxillamide, was isolated from the fruiting bodies of the basidiomycete *Paxillus panuoides* (*77*).

A new steroidal glucoside with a polyhydroxy ergosterol nucleus, tuberoside (**75**), has been isolated from the fruiting bodies of ascomycete *Tuber indicum*. This is the first example of isolation of a polyhydroxylated ergosterol glucoside from higher fungi in nature (*78*). Two new oleate esters of polyhydroxylated ergostane-type nucleus, 3β,5α-dihydroxy-(22*E*,24*R*)-ergosta-7,22-dien-6β-oleate (**76**) and 3β,5α-dihydroxy-(22*E*,24*R*)-ergosta-22-en-7-one-6β-oleate (**77**), were isolated from the fruiting bodies of the basidiomycete *Tricholomopsis rutilans* along with three known sterols (*79*). A new cytotoxic lanostane triterpenoid (**78**) was isolated from the basidiomycete *Hebeloma versipelle* (*80*). **78** exhibited cytotoxic activity against the tumor cell lines HL60, A549, SGC-7900 and Bel-7402, with IC_{50} values of 11.2, 20.9, 22.6, and 25.0 μg/mL, respectively. A new ergostane-type glycoside, named tylopiloside (**79**), was isolated from the fruiting bodies of the basidiomycete *Tylopilus virens*. Its structure was elucidated as (22*E*,24*R*)-ergosta-7,22-dien-5α,6β-diol-3β-*O*-[3-(3-phenylpropanoyloxy)]-β-*D*-glucopyranoside (*81*).

The fungus *Bondarzewia berkeleyi* (Fr.) Bond. et Singer of the family Bondarzewiaceae (Basidiomycota) grows at the base or roots of *Abies* and other conifers of the family Fagaceae. There are no reports of its chemical constituents in the literature. Steglich and Anke reported a cytotoxic metabolite, montadial A, isolated from the polypore *B. Montana* (*82*). They noted that treatment of these mycelial roots with aqueous KOH causes an intense yellow color. Taxonomically, the genus *Bondarzewia* has been placed in the order Russulales, which is supported by the occurrence of stearoyl-velutinal, the chemotaxonomic marker compound for this order (*82*).

Summary

Among many diverse organisms, higher fungi are a major source of biologically active natural substances. They have often been found to contain biologically active compounds, and they provide a rich variety of active secondary metabolites. There are potentially many compounds still to be discovered in higher fungi since until now only a relatively small number of higher fungi have been chemically investigated, and many

of the remaining species are involved in interesting biological phenomena. These as yet unstudied species hold the promise of providing new natural substances. That these fungi are often involved in interesting biological processes indicates not only that the new metabolites involved will be chemically interesting but also that the new metabolites may be biologically interesting and significant. The large biodiversity of higher fungi provides a huge resource for extending the chemodiversity of natural substances and for finding new lead structures for medicinal chemistry.

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Progress in cell membrane chromatography

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ABSTRACT: Cell membrane chromatography (CMC) was first established by He *et al.* in 1996. A bioaffinity chromatography technique, CMC has since proven to be an important method for studying drug-receptor interactions and screening active compounds from medicinal herbs. This paper briefly reviews the characteristics of the cell membrane stationary phase (CMSP), the CMC analytical system, and its applications.

Key Words: CMC, preparation, characteristics, applications

Introduction

The receptor concept proposed by Ehrlich and Langley in the early 1900s had little immediate impact upon pharmacology until Clark posited the interaction between the drug and the receptor in 1937 (*1*,*2*). Since that time, drug-receptor interaction has been a major part of receptor pharmacology. Applications of various new techniques in this field, such as the widespread radioligand-binding assay (RBA), have brought about the formation and development of receptor pharmacology (*3*,*4*). However, RBA results have difficulty reflecting the type of force and stereo-selectivity of drug-receptor interactions. There is also limited ability to incorporate radioactive atoms into the structures of most drugs. Thus, direct information on drug-receptor interactions cannot be obtained using the RBA method for drugs that are not radioactively labeled. In 1996 (*5*), the authors developed a new technique for bioaffinity chromatography called cell membrane chromatography (CMC). Since then, it has been used to study drug-receptor interactions and screen for active components from medicinal herbs. In the CMC system, the cell membrane stationary phase (CMSP) was prepared by immobilizing a cell membrane containing special receptors on a silica carrier. Interactions between drugs and receptors have been investigated directly using the CMC system. This system can readily identify active components acting on receptors in the CMSP. The CMC method is considered an important type of bio-membrane chromatography. The following briefly discusses several studies using the CMC method.

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Characteristics of the CMSP

In contrast to normal liquid chromatography, an enzyme-activity-like cell membrane preparation must be maintained in RBA for the CMSP used in CMC. The procedures usually used for preparation of the CMSP and measurement of the surface features of the CMSP are described below (*6*-*8*).

Preparation of the cell membrane

Cells from tissues or cultured cells are dissociated by a hypoosmotic solution and centrifuged to remove nuclei and then centrifuged again to yield the cell membrane. The purity of the cell membrane is verified using a scanning electron microscope. The fact that the flaps of the cytoplasm membrane and the vesicles of the membrane structure can be clearly observed indicates that the procedure for preparing the cell membrane is suitable for use. The total ATPase activity and the protein level of the membrane should be determined when the cell membrane is stored.

Preparation of the CMSP

The key step in CMC is the preparation of the CMSP. Activated silica is placed in a reaction tube, which is followed by suspension of the cell membrane. Adsorption of the cell membrane on the activated silica surface takes place until equilibrium is reached. The whole adsorption process is carried out under vacuum and ultrasonication so that the cell membrane is distributed uniformly on the silica surface. Afterwards, the reaction mixture is diluted with an equal volume of deionized water. The phospholipids of the living cell membrane are able to fuse spontaneously with

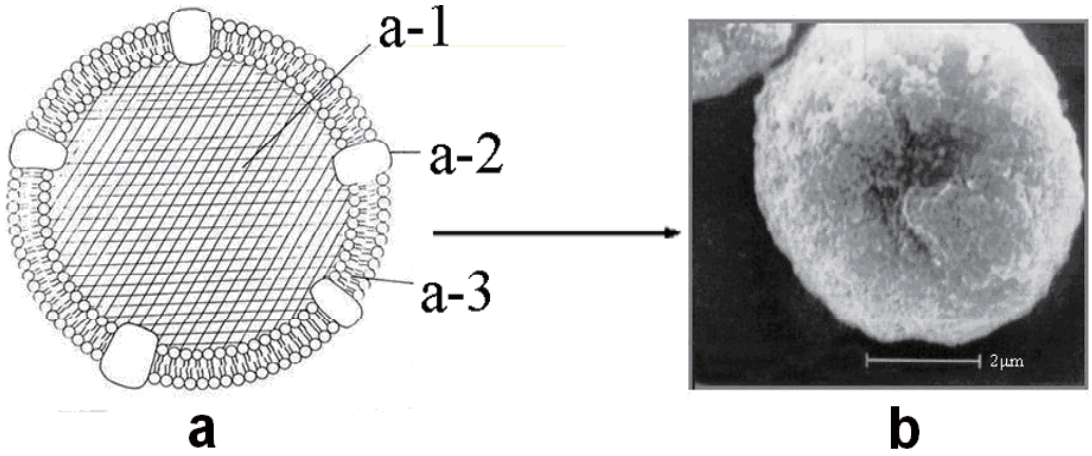

Figure 1. An ideal image and actual micrograph of the CMSP. a: ideal image of the CMSP. a-1, silica carrier; a-2, membrane receptor; a-3, phospholipid layer. b: actual micrograph of the CMSP.

each other (self-fusion) on the silica surface in the aqueous solution until a resealed cell membrane layer is obtained. The supernatant in the reaction mixture is removed by centrifugation and the CMSP is then washed with Tris-HC1 buffer until no residual free cell membrane is detected on its surface. The purity of the cell membrane is verified using a scanning electron microscope.

Surface characteristics of the CMSP

In an aqueous solution, silanol groups (Si-OH) on a silica surface are very strongly polar and usually display strong, irreversible adsorption of biopolymers. Thus, the cell membrane is immobilized on the silica surface. However, one of the characteristics of cell membranes is to have a phospholipid bilayer with two kinds of strong interactions: ionic interaction among membrane polar heads and hydrophobic interaction among carbon chains in the interior of the cell membrane. An ideal image and actual micrograph of the CMSP are shown in Figure 1.

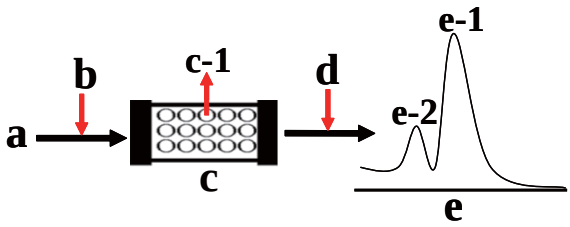

Figure 2. A diagram of the CMC/UV system. a, transfer pump for mobile phase; b, sample; c, CMC column; c-1, CMSP; d, UV-D; e, retention curve; e-1, solvent peak; e-2, retention peak.

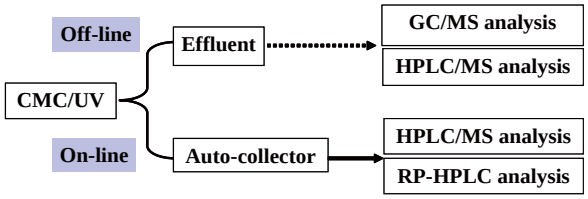

Figure 3. Combined system depicting CMC.

CMC Model System

Chromatographic system

The cell membrane is an important type of biomembrane that has enzymatic activity. In the CMC model system, the chromatographic conditions must imitate the physiological state as much as possible in order to maintain the activity of the CMSP while this system is running. Thus, typical CMC conditions usually include a sodium phosphate buffer or double-distilled water as a mobile phase and a column temperature of 37°C. Additional conditions include a lower flow rate and a detection wavelength suited to detection with a UV detector.

Analytical instruments in CMC

(1) *CMC/UV system*

The CMC/UV system is a general one (Figure 2). It can be used to study drug-receptor interactions. The results obtained from this system correspond well with those of RBA. The system can also be used to screen for active components.

(2) *Combined system*

A combined system can improve CMC qualitatively and quantitatively. This system is well-suited to screening for or identifying active components or compounds from traditional Chinese medicines, natural plants, and reaction mixtures in chemical synthesis through use of either an off-line or an on-line system as shown in Figure 3.

Typical Applications

Drug-receptor interactions

The CMC system has been applied extensively to the study of drug-receptor interactions and measurement of the affinity between drugs and receptors. Yuan and colleagues (*9,10*) used nine ligands of the α_1-adrenergic receptor (AR) to investigate their chromatographic affinity for the α_{1D}-AR subtype. Human embryonic kidney (HEK) 293 cells expressed by cDNA of α_{1D}-AR subtypes were cultured and the CMSP was prepared. Then, the interactions between ligands and α_{1D}-AR in the CMSP were investigated using CMC. Their results showed that the prepared CMSP and CMC method were useful in evaluating affinities of drug-receptor and drug-receptor subtypes and screening for drugs selective to α_{1D}-AR. Yuan and He (*11-13*) prepared a CMSP and used it for rapid on-line chromatographic evaluation of ligand binding affinity to muscarinic acetylcholine receptor (mAChR) by immobilizing the rat cerebral cell membrane and guinea pig jejunum membrane on the surface of a silica carrier. Their data reflected the selectivity and specificity of interactions between drugs and mAChR and proved that CMC can be used to evaluate drug-receptor affinity for drug candidates. At the same time, Yuan and He (*14*) also prepared a CMSP of an expressed cell line and rabbit hepatocytes to study drug-receptor interactions.

In light of these findings, the CMC method can be used to investigate drug-receptor interactions. The results obtained from CMC correspond well with those of RBA.

Screening for active components from medicinal herbs

Medicinal herbs are very important natural resources for finding active compounds as part of new drug development. An effective screening technique is needed for such studies. The CMC method can be used for this purpose because it has both characteristics of chromatographic separation and active recognition from a mixture sample. In actual usage, different CMC models established for several target cells can be used for different medicinal herbs. Several CMC models were used to screen for the effective components from the following natural resources:

(1) *Traditional Chinese medicines*

Traditional Chinese medicines (TCMs) are clinically more effective at treating some diseases. The pharmacological effects of a TCM are usually produced by its active components. Thus, identifying components by means of modern screening techniques is crucial to elucidating the mechanisms and controlling the quality of TCMs. In this area, the CMC model has proven to be a useful screening tool (*15*).

In previous studies, Zhang *et al*. (*16*) and Liang *et al*. (*17*) used a cyno-blood vessel CMC model and rabbit arteriae aorta CMC model in pharmacological trials *in vitro* to screen for effective fractions and effective components of *Angelica sinensis*. They found that the effective fraction was the eluate of hexane-ethyl acetate from the separated extract, and the effective components in the fraction were ligustilide, dimethyl phthalate, and diethyl phthalate, respectively. Li *et al*. (*18*) identified the effective components of *Radix Notoginseng*, *Radix Salviae Miltiorrhizae*, and *Radix Angelicae* with cardiac muscle, cerebrum, and blood vessel CMC models. In accordance with these screening results, a method of controlling the quality of Xinkangping as a TCM prescription for the treatment of coronary heart disease was studied. Zhao *et al*. (*19*) studied the effective components YYH-214 and YYH-216 in the roots and leaves of *herba epimedii* (Yin Yang Huo in Chinese, YYH) screened for using a blood vessel CMC model. They found that YYH-214 and YYH-216 exhibited potent vasodilatation *in vitro*. Screening results provided by the CMC model correlated well with pharmacological effects. Zhang *et al*. (*20*) screened for the effective components of *Cladonia alpestris* (Tai Bai Hua in Chinese, TBH) using a CMC model and studied their correlation with pharmacological effects. They found that TBHG8 was an effective component of TBH1 as an active fraction in TBH for cardiac muscle contractions *in vitro*. Liang *et al*. (*21*) identified the effective components ligustilide and butylidenephthalide from Ligusticum Chuanxiong, used as a traditional Chinese medicine, using a rat artery CMC model. Their results showed that the components effectively inhibited vasoconstriction of rat abdominal aorta segments *in vitro*. These effective components in Ligusticum Chuanxiong are mainly used to treat blood vessel diseases.

(2) *Natural medicinal plants*

In the research and development of new drugs, natural medicinal plants are another important resource in which to search for effective or leading compounds. Using a special target receptor in a CMC model allows the ready identification of bioactive components that react with receptors from natural medicinal plants. Zhang *et al*. (*22*) screened for the active components inhibiting HeLa cell proliferation in *Libanotis buethorimensis* using CMC and found that osthol in *Libanotis buethorimensis* may inhibit HeLa cell proliferation. He *et al*. screened the anti-angiogenesis activity of taspine from *Leontice robustum* using a human umbilical vein endothelial cell (HUVEC) CMC model (*23,24*). Further studies found that taspine may inhibit proliferation and migration of HUVEC and inhibit CAM neovascularisation. These results indicate

that there is a correlation between CMC screening results and a drug's pharmacological effects. In addition, the anti-inflammatory activity of atractylenolide I and atractylenolide III from the rhizomes of *Atractylodes macrocephala Koidz* was screened using a white blood CMC model (*25,26*). Atractylenolide I and atractylenolide III exhibited good anti-inflammatory action in later studies.

In summary, the CMC system provides an analytical method with a high level of performance, selectivity, and efficiency not only for the study of drug-receptor interactions but also for the identification of active compounds from medicinal herbs. The technique behind the system should prove extremely useful in areas like pharmaceutical analysis, receptor pharmacology, and pharmacochemistry.

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Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis

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ABSTRACT: Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) is one of the most prominent analytical techniques owing to its inherent selectivity and sensitivity. In LC/ESI-MS/MS, chemical derivatization is frequently used to enhance the MS/MS detectability. The derivatization improves the separation and ionization efficiency. Moreover, the generated derivatives give particular product ions by CID (collision induced dissociation), which allow for the sensitive detection. In this review, we present an overview of the derivatization reagents which have been applied to LC/ESI-MS/MS, focusing on the applications involving small molecules in biomatrices.

Key Words: LC/ESI-MS/MS (liquid chromatography/electrospray ionization tandem mass spectrometry), derivatization, reagent

Introduction

The development of sensitive and selective determination methods of trace level compounds is essential to elucidate their biological roles and functions in living systems. Recently, liquid chromatography/mass spectrometry (LC/MS) is frequently utilized for this purpose. In particular, high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipped with electrospray ionization (ESI) ion source is the most prominent method, as it requires lower temperature for ionization compared with other ionization methods, and thus it can be used for thermally unstable compounds. In addition the low background noise level in MS/MS technology enables sensitive detection of the targeted compounds.

However, all the compounds can not be favorably analyzed by LC/ESI-MS/MS. For example, the ionization efficiencies are often extremely low and such compounds cannot be sensitively detected. An analyte should have the following properties to be sensitively analyzed by LC/ESI-MS/MS. Firstly, it must be in its ionic form in the solution phase or be chargeable through adduct formation in gas-phase reaction. Secondly, the analyte must have a non-polar region, since hydrophobic compounds can be well separated from salts and interfering compounds possessing suppression effects (*1*). And the non-polar ions prefer the droplet-air interface and reside at the droplet surface. Consequently these ions enter the gas phase more readily than those in the droplet interior and show a higher response (*2-5*). Thirdly, it is desirable that the target analyte fragments efficiently upon collision induced dissociation (CID) and generates an intense product ion for the sensitive MS/MS detection.

Chemical derivatization of the analyte is often used to enhance the detection sensitivity in ESI-MS. In the past decade, it was reported that the chemical derivatization of the analyte by the chargeable compounds improved ESI-MS responses of the target analytes (*6-9*). Since then, several reagents originally utilized in ultraviolet or fluorescence detection were borrowed for this purpose followed by the synthesis of others to be specifically used in LC/ESI-MS. These reagents were summarized in several review papers (*10-13*). These regents are aimed to enhance the ESI response but they are not designed to generate a particular product ion by CID. Therefore, the fragment patterns of the derivatives and the generation of a particular product ion depended on the structures of the generated derivatives, not on the reagents. On the contrary, the reagents developed specifically for LC/ESI-MS/MS were designed to carry a structure suitable for MS/MS detection. They are efficiently fragmented by CID to generate particular product ions. In this review, we present an overview of the derivatization reagents which have been applied to LC/ESI-MS/MS,

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Table 1. Derivatization reagents and analytes in LC/ESI-MS/MS

Reagent	Analyte	Detection limit	Ref
for ketones and aldehydes			
hydroxylamine	testosterone (human serum)	0.035 nmol/L	*14*
hydroxylamine	altrenogest (horse urine)	13 pg/mL	*15*
hydroxylamine	succinylacetone (human urine)	0.063 μM (LOQ)	*16*
DNPH	aliphatic aldehydes (environmental water)	μg/L range	*17*
cyclohexanedione	aliphatic aldehydes (plasma)	20-100 pg	*19*
5,5'-dimethyl-1,3-cyclohexanedione	aliphatic aldehydes (human brain tissue)	5 pg (LOQ)	*20*
Gir P	17-hydroxyprogesterone (dried filter paper blood)	10 ng/mL	*21*
Gir T	5-formyl-2'-deoxyuridine (Hela-S3 cell)	3-4 fmol	*22*
Dns-Hz	succinylacetone (dried blood spot, urine)	0.005 μM	*23*
HMP	testosterone, 5α-dihydrotestosterone (prostatic tissue)	1.0 ng/g tissue	*24*
HMP	testosterone, 5α-dihydrotestosterone (human prostate)	1.0 fmol for testosterone	*25*
HMP	testosterone (rat brain, serum)	0.06 ng/g tissue	*26*
HMP	dehydroepiandrosterone (human silva)	25 pg/mL	*27*
HMP	5α-reduced pregnane type neurosteroids (rat brain, serum)	0.25 ng/g tissue	*28*
HP	5 kinds of steroids such as testosterone	2 fmol	*29*
DAABD-MHz	aliphatic aldehydes	30-60 fmol	*30*
for alcohols and phenols			
Dns-Cl	17β-testosterone (mouse plasma, brain)	50 pg/mL	*31*
Dns-Cl	estrone, 17β-estradiol; estriol, 17α-ethinylestradiol (water sample)		*32*
Dns-Cl	17α-ethinylestradiol (aqueous environmental sample)	1.0 ng/mL	*33*
Dns-Cl	15 kinds of endogenous estrogen (human urine)	2 pg	*34*
Dns-Cl	ethinylestradiol (monkey plasma)	0.2 fg/mL	*35*
Dns-Cl	17β-estradiol (human serum)	0.6 pg	*36*
Dns-Cl	norethindrone, ethinylestradiol (human plasma)	2.5 pg/mL for ethinylestradiol	*37*
Dns-Cl	ethinyl estradiol (human plasma)	2.5 pg/mL	*38*
Dns-Cl	propfol (rat plasma)	20 ng/mL (analytical range)	*39*
Dns-Cl	1-hydroxypyrene (human urine)	20 pg/mL	*40*
pocolinic acid	7α-hydroxy-4-cholesten-3-one (human plasma)	100 fg	*41*
propionyl anhydride; benzoyl anhydride	ribosides, nucleotides (plant)	attomole-low femtomole range	*1*
NA	estrone, estradiol, androsterone	2 fmol	*29*
for carboxylic acids			
HCl-butanol	methylmalonic acid (human serum, plasma urine)	0.05 μmol/L	*56*
HCl-butanol	methylmalonic acid (human plasma, urine)	0.12 μmol/L (S/N = 40)	*57*
HCl-butanol	methylmalonic acid (human serum, plasma)		*58*
HCl-butanol	nitrotyrosine (rat plasma)	0.07 pmol	*59*
HCl-butanol	homocarnosine (cerebrospinal fluid)	20 nmol/L	*60*
trimethylamino-ethylalcohol (TAME)	very long chain fatty acids (human plasma)		*61*
4-dimethylamino-benzylamine	valproic acid (VA) and its metabolites (human plasma)	200 ng/mL for VA	*67*
DAABD-AE	C5-C6 di-carboxylic acids	0.025 μM for glutaric acid	*70*
DAABD-AE	pristanic, phytanic, C22:0, C24:0 and C26:0		*71*
for amines			
NBD-F	biogenic amines (tryptamine, histamine, agmatine, *N*-methylsalsolinol) (rat tissues)	0.6 ng/mL	*72*
NBD-F	*D,L*-amino acids (central nervous system)	95-150 ng/mL	*73*
acetic anhydride	shingosine-1-phosphate dihydrosingosine-1-phosphate (bovine serum, human endotherial cell)	less than 50 fmol	*74*
acetic anhydride pentafluorobenzyl cloride	aminonitropyrene diaminopyrene (rat urine, blood)	0.1 pg for diaminopyrene	*75*
NIT	18 kinds of volatile primary and secondary amines (air sample)	0.12-0.25 ng/μL	*76*
pentafluoropropionic acid anhydride	diamines (human urine, plasma)	0.2-0.3 fmol	*77*
nitrobenzyl chloroform	GABA agonist (rat plasma)	10 ng/mL	*78*
Dns-Cl	muscimol, iboteic acid (mushroom)	25 ppm	*79*
THAS	amino acids	atto-mole level	*80*
for miscellaneous compounds			
NBA	3,5-dinitrosalicylic acid anhydride (poultry muscle and liver)	0.04 mg/kg	*81*
NBA	nitrofuran metabolites (food samples)	0.11-0.21 μg/kg	*82*
ammonia	phenethyl isothiocyanate (human plasma, urine)	2 nmol/L	*83*
DBA	isocyanates (airsample)	10 atto mole	*84*
diazomethane	biphosphates (serum, urine)	pg/mL level	*85*
hexamethyleneimine	deoxyguanine adduct	low femto mole range	*86*

Abbreviations, DAABD-AE: 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; DAABD-MHz: 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole; DBA: di-*n*-butyl amine; DNPH: 2,4-dinitrophenyl hydrazine; Dns-Cl: dansyl chloride; Dns-Hz: Dansyl hydrazine; Gir P: 1-(carboxymethyl)pyridium chloride hydrazide; Gir T: (carboxym ethyl)trimethylammonium chloride hydrazide; HMP: 2-hydrazino-1-methyl-pyridine; HP: 2-hydrazinopyridine; NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NA: isonicotinyl azide; NBA: 2-nitrobenzaldehyde; NIT: naphtyl isothiocyanate; LOQ: limit of quantification; THAS: 4-(trimethylammonium)anilyl-*N*-hydroxysuccidimidyl carbamate iodide.

focusing on the applications to low molecular weight compounds (Table 1).

Derivatization of analytes

Ketones and aldehydes (carbonyl compounds)

Ketones and aldehydes are neutral functional groups. The ionization efficiencies in ESI of these compounds are usually low. To overcome this, a chargeable moiety was introduced to these compounds to enhance the ionization efficiency. Hydroxylamine reacts with ketones to form the corresponding oximes. The derivative contains nitrogen atom and is expected to improve the ionization efficiencies of the analytes. Hydroxylamine was applied to LC/ESI-MS/MS analysis of ketones such as testosterone (*14*), altrenogest (a steroid used for the control of estrus in horse) (*15*), and succinylacetone (the hallmark of hepatorenal tyrosinemia) (*16*). A significant increase of detection sensitivity for altrenogest was observed (*15*). The product ions obtained by CID were dependant on the analyte structures.

DNPH (2,4-dinitrophenylhydrazine) was used for the determination of aldehydes and ketones in disinfected water (*17*). The derivatives were detected in the negative-ion mode. Several common product ions such as *m/z* 163, *m/z* 152, *m/z* 122, derived from the reagent skeleton, were observed. The product ion spectra were complicated by the several fragment ions generated by CID. The transitions of the $[M-H]^-$ ions to these product ions were used for MRM (multiple reaction monitoring). DNPH derivatives were also analyzed by LC/APCI (atmospheric pressure chemical ionization)-MS/MS (*18*). Cyclohexanedione was used for the determination of aliphatic aldehydes (C3-C10) including 4-hydroxynonenal and nonanal, the peroxidation products of fatty acids. Aldehydes were condensed with two molecules of cyclohexanedione in the presence of ammonia to form the tri-cyclic compounds (Hantzsch reaction). These compounds gave the common product ion at *m/z* 216 by CID, derived from the tri-cyclic structure. The transitions of $[M+H]^+$ ions of the derivatives to *m/z* 216 were used for MRM (*19*). Similarly, 5,5'-dimethyl-1,3-cyclohexanedione was used for the derivatization of biogenic aldehydes. The derivatives gave the common product ions at *m/z* 273 or 274 by CID (*20*). The transitions of $[M+H]^+$ ions of the derivatives to *m/z* 273 or 274 were used for MRM.

Girard's reagent P (1-(carboxymethyl)pyridium chloride hydrazide; Gir P) and Girard's reagent T ((carboxymethyl)trimethylammonium chloride hydrazide; Gir T) are reagents that possess a permanent cationic charge. Gir P was used for the determination of 17-hydroxyprogesterone (17-OHP), the marker for congenital adrenal hyperplasia. Several fragment ions (*m/z* 80, 93, 121) were generated by CID. The transition of *m/z* 299 ($[M]^{2+}$) to *m/z* 80 was used for MRM (*21*). Gir T was used for the determination of 5-formyl-2'-deoxyuridine, a major thymidine lesion generated by reactive oxygen species. The generated derivative gave the product ion at *m/z* 195, by the loss of a trimethylamino moiety (59 Da). The detection limit (3-9 fmol) was about 20 fold better than that for the direct analysis of the underivatized compound (*22*). Dansyl hydrazine (5-dimethylaminonaphthalene-1-sulfonyl hydrazine; Dns-Hz) was used for the analysis of succinylacetone in dried blood spot specimens. The generated dansyl hydrazone selectively gave the product ion at *m/z* 170 by CID, assigned to the cleavage of dimethyaminonaphtyl moiety originated from the reagent (Figure 1a). The product ion spectra of the derivatives were rather simple and clear. The transition of *m/z* 462 ($[M+H]^+$) to *m/z* 170 was used for MRM (*23*).

One of the most promising reagents for aldehydes and ketones is HMP (2-hydrazino-1-methyl-pyridine) (*24*). HMP has a 1-methylpyrizino group as a permanently charged moiety and a hydrazine group as a reactive site. HMP reacted with carbonyl compounds at 60ºC within 1 h. The ionization efficiencies of HMP derivatives were rather high and the generated derivatives gave a product ion at *m/z* 108 by CID, derived from 1-methylpyrizinoamino moiety (Figure 1b). HMP was applied to the LC/ESI-MS/MS analysis of keto-steroids such as testosterone, 5α-dihydrotestosterone in prostate and prostatic tissue (*24,25*), testosterone in rat serum and brain (*26*),

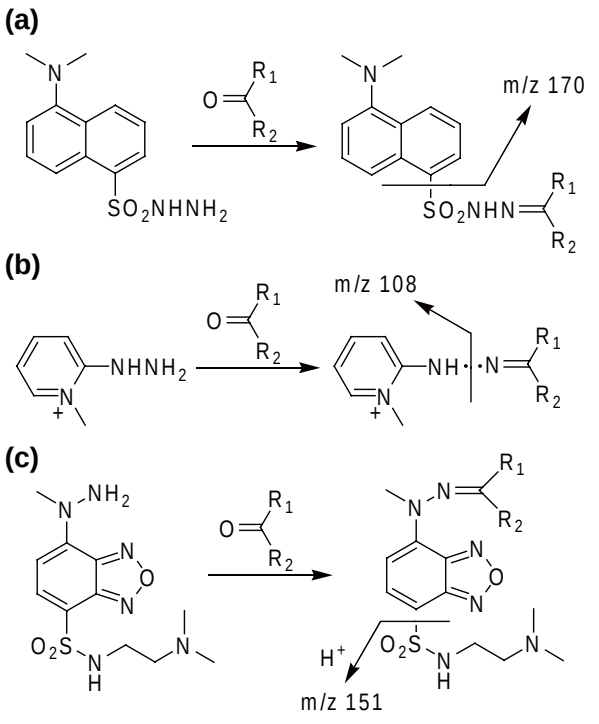

Figure 1. Derivatization reaction for ketones and aldehydes, and the product ion of the derivative obtained by CID, (a) Dns-Hz; (b) HMP; (c) DAABD-MHz.

dehydroepiandrosterone in saliva (*27*), 5α-reduced pregnane-type neurosteroids in rat brain and serum (*28*). HMP is not effective for increasing the detection responses of di-oxo-steroids. This phenomenon was due to the fact that small molecules with a multi-charge are unstable in the gas phase and provided multiple ions. To overcome these problems, HP (2-hydrazinopyridine) was used for di-oxo-steroids such as androsterone and progesterone. The generated derivatives gave the intense product ions at *m/z* 322 and 348 for the derivatives of androsterone and progesterone, respectively (*29*). Recently DAABD-MHz (4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole) for aldehydes was developed. It has a dimethylamino group as a chargeable moiety and a hydrazino group as a reaction site. The generated derivative showed a predominant product ion at *m/z* 151 by CID, derived from the protonated (*N*,*N*-dimethylamino)ethylaminosulfonyl moiety of the reagent (Figure 1c). The transition of $[M+H]^+$ ions to *m/z* 151 was used for MRM (*30*).

Alcohols and phenols

Alcohols and phenols are neutral compounds. Therefore, derivatization is required to enhance the ionization efficiency. One of the most often used reagents is dansyl chloride (5-Dimethylamino-1-naphthalenesulfonyl chloride; Dns-Cl). Dns-Cl has a dimethylamino group as an ionization moiety and a reactive sulfonylchloride group. It reacts with phenols at 60ºC within several minutes. The generated derivatives provided an almost single product ion at *m/z* 171 by CID, which was assigned to protonated dimethylaminonaphtyl moiety (Figure 2a). SRM (selected reaction monitoring) using the transition of quasi-molecular ion of the derivative to *m/z* 171 or 170 resulted in a sensitive detection of the derivative. So far, it was used for determination of steroids having hydroxyl group such as 17β-estradiol, estrone, 17α-ethinyl estradiol (*31-38*), propofol (veterinary medicine) (*39*), and 1-hydroxypyrene (biomarker to monitor the exposure to polycyclic aromatic hydrocarbons) (*40*).

The derivatization to picolynoyl ester was used for 7α-hydroxy-4-cholestene-3-one, a biomarker for bile acid biosynthesis. The derivative gave the product ion at *m/z* 383 by CID, due to the loss of picolinic acid moiety. The transition of *m/z* 506 ($[M+H]^+$) to *m/z* 383 was used for SRM (*41*) (Figure 2b). Propionyl and benzoyl anhydride were used for bases, ribosides, and intact nucleotides such as AMP, ADP and AMP (*1*). The ESI response was enhanced by the formation of hydrophobic derivatives. In addition, the retention on a reversed-phase column was greatly increased, and the derivatives were separated without the need for an ion paring reagent, known for its unwanted suppression effects on ionization. Propionated cytokinins were analyzed by

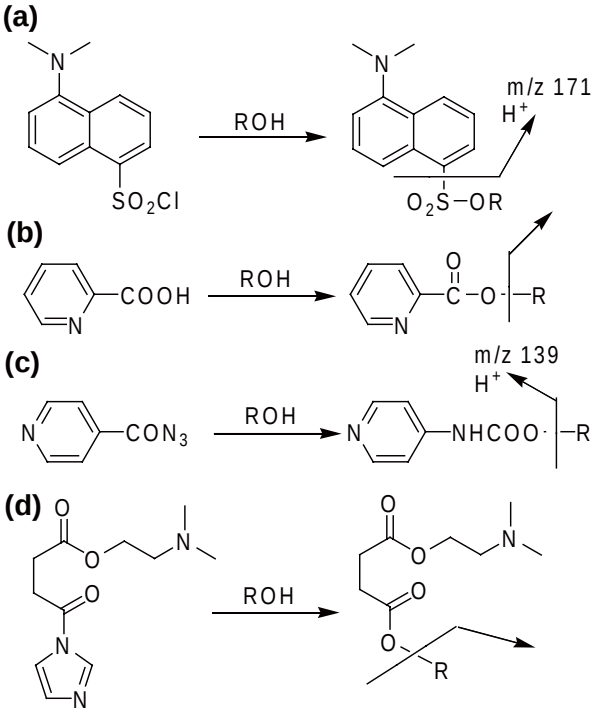

Figure 2. Derivatization reaction for alcohols, and the product ion of the derivative obtained by CID, (a) Dns-Cl; (b) picolynic acid; (c) NA; (d) MDMAES imidazole.

LC/ESI-MS/MS with detection limits in the sub femto-mole range. NA (isonicotinoyl azide) was used for the derivatization of di-hydroxysteroids such as estradiol and 5α-androstane-3α,17β-diol. NA reacted with two hydroxyl groups of estradiol at 80ºC for 30 min and the generated derivative gave the product ion at *m/z* 139 by CID, which was assigned to the protonated pyridyl carbamic acid (Figure 2c). The transition of $[M+H]^+$ ion to *m/z* 139 was usable for SRM analysis (*29*). MDMAES (mono-(dimethylaminoethyl) succinyl) imidazole was used for cholesterol and dehydrocholesterol. MDMAES imidazole reacted with hydroxyl group at 70ºC for 10 min and generated MDMAES ester. The ester gave the product ion at *m/z* 369 by CID, due to the neutral loss of MDMAES moiety (189 Da) (Figure 2d). Cholesterol and dehydrocholesterol in dried spot of plasma were analyzed by ESI-MS/MS without chromatographic separation (*42*). This reagent is usable for the derivatization of alcohol and SRM analysis.

LC/APCI-MS/MS is suitable for neutral derivatives. Acetic anhydride was used for budesonide in human plasma (*43*), and benzoyl chloride for propylene glycol in rat plasma and lung tissue (*44*). Estrone and related compounds were derivatized with pentafluorobenzyl bromide (*45*).

Carboxylic acids (including amino acids)

Carboxylic acids are detectable in the negative ESI-MS. However their sensitivity is known to be poor, because of the high background noise. In addition the mobile phases for the carboxylic acids separation are

not always compatible with ESI-MS. In one approach, carboxylic acids such as fatty acids are derivatized to their esters, and analyzed by LC/APCI-MS. Fatty acid esters are neutral or rather hydrophobic having an atom with proton affinity such as oxygen and are therefore suitable for APCI-MS detection.

One of the successful examples for ESI-MS/MS detection of carboxylic acids with derivatization is the simultaneous analysis of amino acids and acylcarnitines in dried blood spots for the diagnosis of organic acidemias and amino acidpathies developed by Rashed and coworkers, which paved the way for the reliable automated newborn screening (*46-48*). Amino acids were derivatized with butanolic HCl and the generated butyl esters were introduced to ESI-MS/MS without chromatographic separation. Most of α-amino acids butyl esters gave the intense product ions correspond to the loss of $HCOOC_4H_8$ (102 Da) by CID. Therefore, amino acids profile in biological samples can be obtained by the neutral loss scan of 102 Da. Acylcarnitines serve as marker metabolites for inherited disorders related to organic acid and fatty acid metabolism (*49*). Acylcarnitines have a quaternary ammonium group and a carboxylic group in their structure. Their butyl esters were introduced to ESI-MS/MS and gave the common product ion at *m/z* 85 by CID (*46*). Therefore, acylcarnitine profile can be obtained by precursor ion scan of *m/z* 85. These methods are currently widely used for the analysis of amino acids and acylcarnitines in urine, plasma, serum, or blood (dried blood spot). This topic was the subject of several excellent papers (*50-55*).

Butanolic HCl derivatization was also used for LC/ESI-MS/MS of carboxylic acids. Methylmalonic acid is the marker for a group of metabolic disorders caused by deficiency in methylmalonyl-CoA mutase or a defect in vitamin B_{12} metabolism. The di-butyl ester gave the product ion at *m/z* 119, due to the loss of 2 C_4H_8. The transition of *m/z* 231 ($[M+H]^+$) to *m/z* 119 was used for MRM (*56-58*). Nitrotyrosine is the tyrosine nitration product. Its butyl ester gave the product ion at *m/z* 181 or *m/z* 227, due to the loss of C_4H_8 (56 Da) or the loss of C_4H_8 and formic acid (102 Da) by CID. The transitions of *m/z* 283 ($[M+H]^+$) to these product ions were used for MRM detection (*59*). Homocarnosine is a brain specific di-peptide and the marker of heritable defect in GABA pathway. The transition of protonated homocarnosine butyl ester, *m/z* 297 ($[M+H]^+$) to *m/z* 212, derived from the loss of 85 Da, due to the loss of the aminobutyryl moiety, was used for SRM experiments (*60*).

Recently, several reagents having an ionization moiety, a reaction site, and a suitable structure for MS/MS detection were used for derivatization of carboxylic acids. TMAE (trimethylaminoethyl) ester derivatives were prepared for LC/ESI-MS/MS of very long chain fatty acids, the diagnostic markers for peroxisomal disorders (*61*). Fatty acids were treated with oxalyl chloride, dimethylaminoethanol, followed by the methylation with methyl iodide. These derivatives gave the product ion by the loss of 59 Da, derived from $(CH_3)_3N$ moiety of the derivatization reagent, and each fatty acid derivative was detected by MRM (Figure 3a). The generated derivatives were suitable for MS/MS detection. However, the three step derivatization reaction was tedious for routine assay. TMAE or DMAE (dimethylaminoethyl) ester derivatization of fatty acids and ESI-MS/MS analysis without chromatographic separation were also reported (*62-66*). Valproic acid and its metabolite were derivatized with 4-dimethyla minobenzylamine and analyzed by LC/ESI-MS/MS. The generated amide derivative gave the product ion at *m/z* 120 by CID, derived from dimethylamino phenyl moiety of the reagent (Figure 3b). The transition of *m/z* 277 ($[M+H]^+$) to *m/z* 120 was used for MRM for the determination of valproic acid (*67*). Recently, DAABD-AE, (4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole) was developed as a derivatization reagent for carboxylic acids with excellent mass spectrometric charcteristics (*68,69*). DAABD-AE was used for the derivatization of dicarboxylic acids such as glutaric acid and 3-hydroxyglutarate, the marker metabolites for glutaric acidemia type 1 in human urine (*70*). The generated amide derivative gave the product ion at *m/z* 151 by CID, derived from the protonated (*N*,*N*-dimethylamino)ethylaminosulfonyl moiety of the reagent (Figure 3c). The transition of $[M+H]^+$ ions to *m/z* 151 was used for MRM. An attractive diagnostic method for peroxisomal disorders was also developed based on DAABD-AE derivatization and LC-MS/MS analysis. Compared to standard gas chromatography-mass spectrometric methods routinely used for this

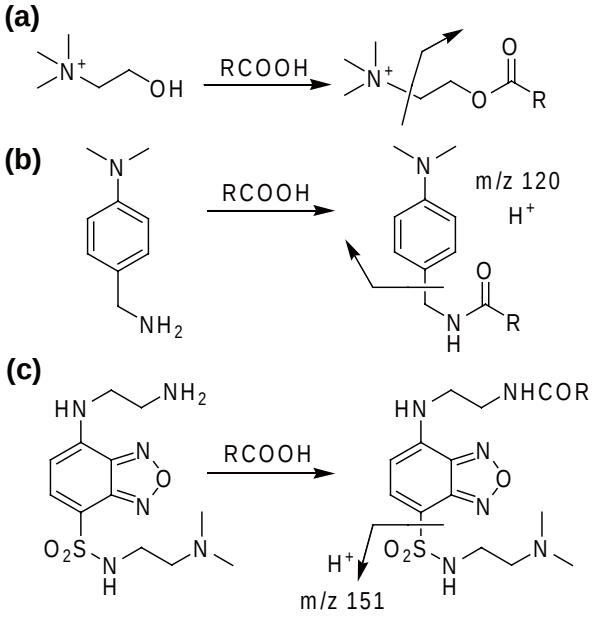

Figure 3. Derivatization reaction for carboxylic acids, and the product ion of the derivative obtained by CID, (a) trimethylaminoethyl (TMAE) alcohol; (b) 4-dimethylaminobenzylamine; (c) DAABD-AE.

purpose, this LC-MS/MS method is more simple, saves 75% of instrument time and requires one tenth of biological sample volume (*71*).

Amines

The compounds having amino group are easily protonated under acidic conditions and suitable for ESI-MS. However, the analysis of amines is often troublesome because of their high polarity, basicity, and high water solubility. Chemical derivatization makes amines more hydrophobic and the generated derivatives can be more easily separated from the interfering compounds on the reversed-phase column, and can be more sensitively detected in ESI-MS. In addition, the increase in the molecular weight decreases the background noise from the matrix, since the background is generally lower in the higher mass range.

NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) was used for biogenic amines such as tryptamine, histamine, *N*-methylsalsolinol, and agmatine. The characteristic product ions were produced for each derivative by CID. In case of agmatine, the transition of *m/z* 294 ($[M+H]^+$) to *m/z* 277 was used for MRM (*72*). NBD-F was applied to the determination of *D*-amino acids. The characteristic precursor to product ion transitions, *m/z* 297 to 279 (NBD-Asp), *m/z* 269 to 223 (NBD-Ser), *m/z* 311 to 293 (NBD-Glu) were monitored for quantification (*73*). Acetyl anhydride was used for Sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate, an endogenous sphingolipid and the analog. Acetyl chloride reacted with an amino group and a hydroxyl group, and generated bi-acetylated derivatives. The transition of *m/z* 462 ($[M-H]^-$) to *m/z* 402 for S1P by CID, due to the neutral loss of acetic acid, was used for MRM (*74*). Acetyl chloride was used for polyaromatic amines such as diaminopyrenes and aminonitropyrenes, human carcinogens. The acetylated derivatives gave two sensitive MS/MS transitions, which were used for MRM, one was for quantification and the other for confirmation (*75*). Acetylation improved peak shapes and reproducibility in LC of diaminopyrene resulting in high signal to noise ratios. NIT (naphtylisothiocyanate) was used for the determination of eighteen kinds of primary and secondary amines in air samples. The derivatives of the primary amines gave the common base peak at *m/z* 144 and the fragment ion at *m/z* 127 by CID, whereas those of secondary amines gave the common base peak at *m/z* 186 and the fragment ion at *m/z* 128 by CID. This method provided the structural information of the analytes, and was suited for the analysis of complex environmental samples (*76*). Pentafluoropropionic acid anhydride was used for diamines, markers for the exposure to isocyanates. The reaction proceeded within 10 sec and the generated derivatives were analyzed by LC/ESI-MS in the negative ion mode. The $[M-H]^-$ ion was selected as precursor ions. And the product ions of $[M-H-120]^-$ or $[M-H-240]^-$, due to the neutral loss of one or two CF_3CHF_2 group, or *m/z* 119 corresponding to $CF_3CF_2^-$ ion, were monitored (*77*). Nitrobenzyl chloroformate was used for 3-amino-2(*S*)-hydroxypropylmethylphospheric acid, $GABA_B$ receptor agonist. The derivative gave the product ion at *m/z* 152, assigned to 4-nitrobenzyl alcohol anion, which was generated by the cleavage of ester bond of the reagent (Figure 4a). The transition of *m/z* 371 ($[M-H]^-$) to *m/z* 152 was used for SRM (*78*). Dansyl chloride was used for musimol and ibotenic acid, bioactive compounds in mushroom. The generated derivatives gave the product ion at *m/z* 171, derived from protonated dimethylaminonaphtyl moiety of the reagent (Figure 4b). The transitions of (M^+) ions to *m/z* 171 were used for MRM (*79*).

THAS (4-(trimethylammonium)anilyl-*N*-hydroxysuccidimidyl carbamate iodide) was the reagent designed for LC/ESI-MS/MS. It reacted with amino acids to form urea compounds. The derivatives gave the characteristic cleavage at the urea bond that is the binding position between the reagent and amino group, and produced characteristic fragment ions derived from the reagent skeleton (Figure 4c). Amino acids were analyzed with the detection limits of atto-mole level (*80*).

Amines are sometimes derivatized with reagents for ultraviolet or fluorescence detection, converted to neutral compounds, and analyzed by LC/APCI-MS. The readers should refer to the previous review (*10*).

Miscellaneous

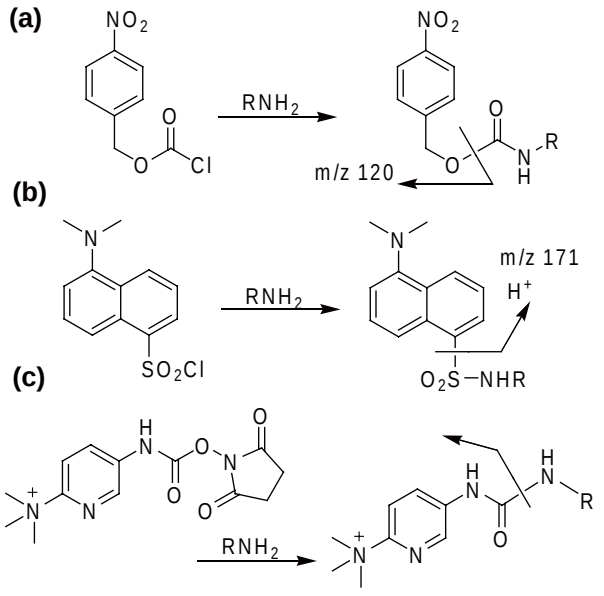

Figure 4. Derivatization reaction for amines, and the product ion of the derivative obtained by CID, (a) 4-nitrobenzyl chloroformate; (b) Dns-Cl; (c) THAS.

NBA (2-nitrobenzaldehyde) was used for the determination of 3,5-dinitrosalicylic acid hydrazide (DSH), nifursol (histomoniasis prevention compound) metabolites. DSH, generated by the acid-catalysed hydrosis of tissue-bound metabolites, was derivatized in situ with NBA and isolated by liquid-liquid extraction, and analyzed by LC/ESI-MS/MS in the negative-ion mode. The derivative gave the fragment ions at *m/z* 182, 183, and 226 by CID, derived from the analyte skeleton. The transitions of *m/z* 374 (quasi-molecular ion) to *m/z* 182 and 226 were used for MRM (*81*). NBA (2-nitrobenzaldehyde) was also used for nitrofuran metabolites, effective compounds against pathogenic bacteria. The transitions of ($[M+H]^+$) ions to the characteristic product ions were used for MRM (*82*).

Ammonia was used as a derivatization reagent for phenethyl isothiocyanate, a dietary compound present in cruciferous vegetables that has cancer-preventive properties. The derivative gave the fragment ion at *m/z* 105 due to the loss of the thiourea moiety. The transition of *m/z* 181 ($[M+H]^+$) to *m/z* 105 was used for SRM (*83*). DBA (di-*n*-butylamine) was used for the derivatization of isocyanates. The generated thiourea derivatives gave the fragment ions at *m/z* 130 and 156, assigned to $[DBA+H]^+$ and $[DBACO]^+$, respectively. The transitions of ($[M+H]^+$) ions to these fragments were used for MRM (*84*).

Diazomethane was used for the derivatization of biphosphonates such as risedronate and alendronate, the compound effective to osteoporosis and bone-related diseases. Risedronate is extremely hydrophilic and structurally similar to many endogenous phosphorylated compounds. Four hydroxyl groups of risedronate were methylated by diazomethane on silica-based anion-exchange sorbents. The derivatization made the analyte more hydrophobic, and improved chromatographic separation and ESI response. The transition of *m/z* 340 to *m/z* 214 for risedronone, and *m/z* 344 to 214 for alendronate were use for MRM (*85*). Hexamethyleneimine was used for the derivatization of deoxyguanosine monophosphate adducts, the indicator for the onset of tissue carcinogenicity. The hydrophobic derivatives showed increases in ionization efficiency and improved peak shape. The derivatization showed 3-4 fold signal enhancement compared to underivatized deoxyguanosine nucleotide (*86*).

Conclusion

The derivatization reagents applied to LC/ESI-MS/MS in biomedical analysis were reviewed. These reagents were used for the derivatization of ketones, aldehydes, alcohols, carboxylic acids, amines, and other compounds. The derivatization improved the separation efficiency, ionization efficiency and MS/MS detectability of the analytes. Some regents are aimed to enhance the ESI response but they are not designed to generate a particular product ion by CID. The fragment patterns of the derivatives and the efficiencies depended solely on the structures of the analytes. These reagents are not always useful for LC/ESI-MS/MS. In some cases, the derivatives gave particular product ions efficiently and were sensitively and selectively detectable in MRM. On the contrary, the reagents designed for LC/ESI-MS/MS have the suitable structure for MS/MS detection. They fragment easily by CID and efficiently generate particular intense product ions. Although derivatization is very useful to enhance the detectability in LC/ESI-MS/MS, it should be noticed that excess reagents can cause ionization suppression of the derivatives. Thus, separation of the derivatives from excess reagent by LC is often required. Prominent derivatization regents for LC/ESI-MS/MS are still desired in the fields of biomedical analysis.

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Synthesis and cytotoxic activity of 3-phenyl-2-thio-quinoxaline 1,4-dioxide derivatives in hypoxia and in normoxia

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ABSTRACT: A series of novel 3-phenyl-2-ethylthio/ethylsulfinyl/ethylsulfonyl/phenylthio/ phenylsulfonyl-quinoxaline 1,4-dioxide derivatives were synthesized and screened for their cytotoxicity *in vitro* on human leukaemia cell line HL-60, human esophagus cancer cell line ECA-109, human prostate cancer cell line PC-3, human gastric carcinoma cell line SGC-7901, and human breast cancer cell line MCF-7 in hypoxia and in normoxia. Half of tested compounds showed higher cytotoxic activity both in hypoxia and in normoxia. The mechanism of one potent compound, 67, in hypoxia showed that the mitochondria pathway is involved in the antitumor activity of this class of compounds.

Key Words: Quinoxaline 1,4-dioxides, antitumor, hypoxia and normoxia

Introduction

Hypoxic tumor cells in a solid tumor cause resistance to radiotherapy and chemotherapy (*1-6*). Traditional chemotherapeutic agents have no or little effect on hypoxic tumor cells. Bioreductive prodrugs can effectively kill this kind of cell. One of the most promising bioreductive prodrugs is quinoxaline 1,4-dioxide (*7-10*), and the known compound 3-amino-2-carbonitrile quinoxaline 1,4-dioxide (TPZCN) is an important lead compound with beneficial biological activity *in vitro* (*11*). The 3-methyl-2-phenylthio-quinoxaline 1,4-dioxides were reported to have several forms of beneficial biological activity such as antimycobacterial and anticandidal activity (*12-14*). There are, however, no reports on the antitumor activity of this kind of compound. 3-phenyl-quinoxaline 1,4-dioxide derivatives should be effective antitumor agents in hypoxia since they contain the quinoxaline 1,4-dioxide pharmacore. Therefore, a series of novel 3-phenyl-2-thio-quinoxaline 1,4-dioxides were synthesized and screened for their cytotoxic activity in hypoxia and in normoxia.

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Materials and Methods

Chemistry

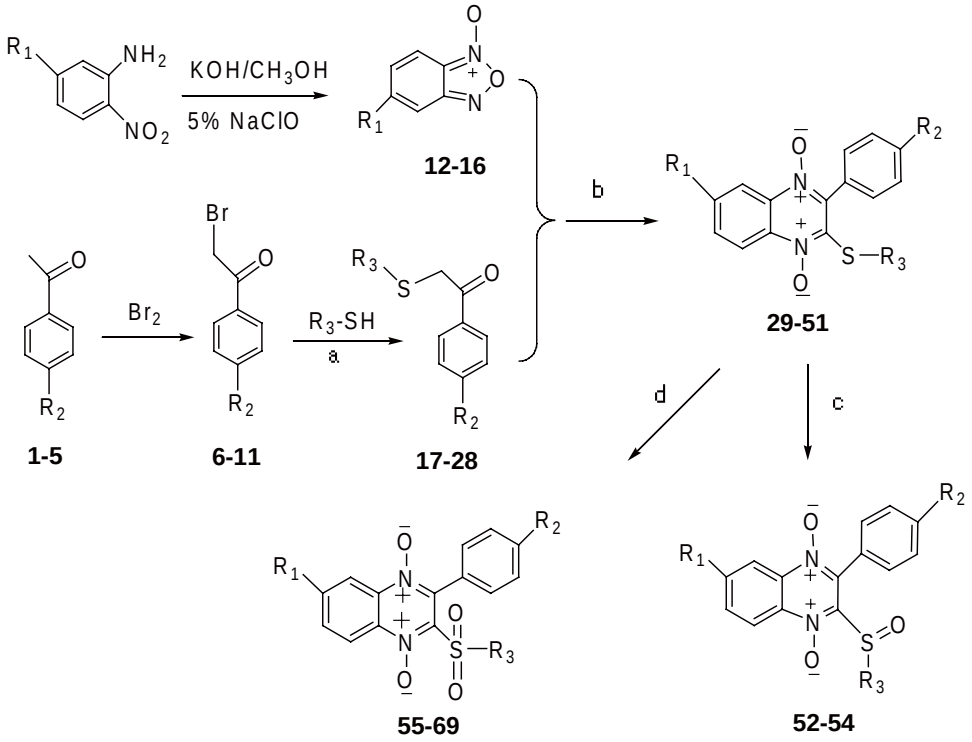
The synthetic pathway of the target compounds **20-59** is shown in Scheme 1. Compounds **12-16** were prepared by reaction of 2-nitroanilines with 5% sodium hypochlorite solution in the presence of KOH. Cyclocondensation of compounds **12-16** with appropriate 2-ethylthio (or phenylthio)-1-phenyl ethanone by the well-known Biuret reaction afforded 3-phenyl-2-ethylthio/phenylthio-quinoxaline 1,4-dioxides **29-51** (*15*). Compounds **29-51** were oxidized by different amounts of m-chloroperoxybenzoic acid (MCPBA) to produce target compounds 3-phenyl-2-ethylsulfinyl-quinoxaline 1,4-dioxides **52-54** or 3-phenyl-2-ethylsulfonyl/ phenyl sulfonyl-quinoxaline 1,4-dioxides **55-69**, respectively. All of the prepared compounds were confirmed by spectral data including IR, ^{1}H NMR, and MS (*16*).

X-ray analysis

In order to identify the structures of the class of compounds, the single-crystal structure of **55** was determined by X-ray crystallography as illustrated in Figure 1. In **55**, all H atoms were placed in geometrically idealized positions. The quinoxaline 1,4-dioxide system is almost planar. The quinoxaline 1,4-dioxide and phenyl planes are approximately perpendicular, with a dihedral angle of 85.8.

Biological evaluation

All of the prepared compounds were evaluated for their cytotoxic activity *in vitro* on human leukaemia cell line



29 R_1=R_2=H, R_3=CH_2CH_3;
30 R_1=H, R_2=F, R_3=CH_2CH_3;
31 R_1=H, R_2=Cl, R_3=CH_2CH_3;
32 R_1=H, R_2=Br, R_3=CH_2CH_3;
33 R_1=H, R_2=CH_3, R_3=CH_2CH_3;
34 R_1=H, R_2=OCH_3, R_3=CH_2CH_3;
35 R_1=Cl, R_2=H, R_3=CH_2CH_3;
36 R_1=Cl, R_2=F, R_3=CH_2CH_3;
37 R_1=Cl, R_2=Cl, R_3=CH_2CH_3;
38 R_1=Cl, R_2=Br, R_3=CH_2CH_3;
39 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
40 R_1=CH_3, R_2=F, R_3=CH_2CH_3;
41 R_1=CH_3, R_2=Cl, R_3=CH_2CH_3;
42 R_1=CH_3, R_2=Br, R_3=CH_2CH_3;
43 R_1=R_2=H, R_3=Ph;
44 R_1=Cl, R_2=H, R_3=Ph;
45 R_1=CH_3, R_2=H, R_3=Ph;
46 R_1=H, R_2=Cl, R_3=Ph;
47 R_1=Cl, R_2=Cl, R_3=Ph;
48 R_1=CH_3, R_2=Cl, R_3=Ph;
49 R_1=H, R_2=Br, R_3=Ph;
50 R_1=Cl, R_2=Br, R_3=Ph;
51 R_1=CH_3, R_2=Br, R_3=Ph;
52 R_1=R_2=H, R_3=CH_2CH_3;
53 R_1=Cl, R_2=H, R_3=CH_2CH_3;
54 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
55 R_1=R_2=H, R_3=CH_2CH_3;
56 R_1=Cl, R_2=H, R_3=CH_2CH_3;
57 R_1=CH_3, R_2=H, R_3=CH_2CH_3;
58 R_1=OCH_3, R_2=H, R_3=CH_2CH_3;
59 R_1=H, R_2=F, R_3=CH_2CH_3;
60 R_1=Cl, R_2=F, R_3=CH_2CH_3;
61 R_1=CH_3, R_2=F, R_3=CH_2CH_3;
62 R_1=H, R_2=Cl, R_3=CH_2CH_3;
63 R_1=Cl, R_2=Cl, R_3=CH_2CH_3;
64 R_1=CH_3, R_2=Cl, R_3=CH_2CH_3;
65 R_1=H, R_2=Br, R_3=CH_2CH_3;
66 R_1=Cl, R_2=Br, R_3=CH_2CH_3;
67 R_1=CH_3, R_2=Br, R_3=CH_2CH_3;
68 R_1=H, R_2=CH_3, R_3=CH_2CH_3;
69 R_1=R_2=H, R_3=Ph

Scheme 1. The synthetic route of the compounds **29–69**. Reagents and conditions: (a) K_2CO_3, THF, reflux, 12 h; (b) NH_3; (c) 2.0 equiv of MCPBA, Chloroform; (d) 4.0 equiv of MCPBA, Chloroform.

HL-60, human esophagus cancer cell line ECA-109, human prostate cancer cell PC-3, human gastric-carcinoma cell line SGC-7901, and human breast cancer cell line MCF-7 in hypoxia and in normoxia according to reported methods (*17*). The IC_{50} values of the tested compounds in normoxia and in hypoxia are summarized in Table 1.

Results and Discussion

SAR studies

As shown in Table 1, half of the tested compounds displayed higher cytotoxic activity on all tested cancer cell lines than the reference drug both in hypoxia and in normoxia. Obviously, the cytotoxic potency of tested compounds on these five cancer cell lines was highly dependent on structures of the 2-position side chains. When 2-position was occupied by an ethylthio group (*e.g.*, **29-42**) or phenylthio group (*e.g.*, **43-51**),

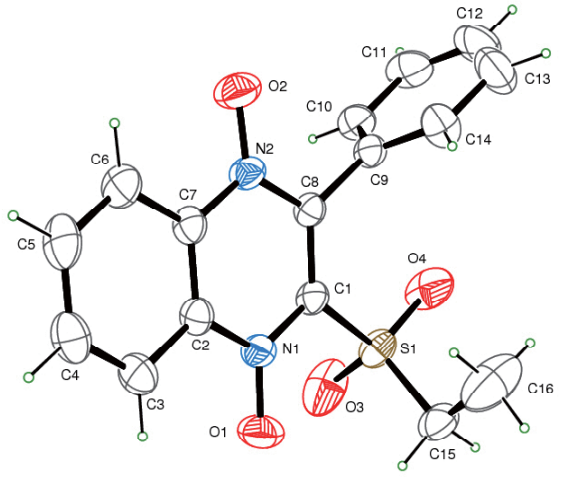


Figure 1. Single-crystal structure of **55**.

the compound showed almost no or weak activity. For the 2-phenylthio series, the substituents both at the 5-position of quinoxaline and on the 3-benzene ring can slightly affect cytotoxic activity. Compounds with electron-withdrawing substituents (**44, 46, 47, 50**) such as fluorine, chlorine, and bromine showed higher activity than those with electron-donating substituents (**45**). On the other hand, 3-phenyl-2-ethylsulfinyl/ethylsulfonyl/phenyl-sulfonyl-quinoxaline 1,4-dioxides (**52-69**) exhibited impressive cytotoxic activity on most tested cancer cell lines. Most traditional antitumor agents are useless on hypoxic cells, while the current class of compounds showed higher activity both in hypoxia and in normoxia may thus be effective agents in tumor therapy.

Mechanism studies

Further study of the mechanisms of cytotoxic activity

Table 1. Cytotoxicity of quinoxaline 1,4-di-N-oxides derivatives (**29-69**) on five human cancer cell lines in hypoxia and in normoxia *in vitro*

Comd.	Cytotoxicity (IC_{50}, μM)[a]									
	K562		Eca109		SGC7901		PC3		SMMC7721	
	H[b]	N[c]	H	N	H	N	H	N	H	N
29	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
30	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
31	>50	>50	>50	>50	>50	>50	>50	44.9	>50	>50
32	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
33	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
34	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
35	18.6	>50	>50	>50	>50	>50	>50	>50	>50	>50
36	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
37	6.5	27.8	11.9	8.8	43.1	29.1	39.6	34.8	>50	>50
38	10.0	>50	>50	30.6	38.3	19.6	>50	>50	34.9	>50
39	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
40	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
41	>50	>50	>50	>50	>50	>50	>50	>50	>50	40.4
42	>50	38.5	>50	>50	>50	>50	46.7	>50	>50	>50
43	47.6	23.2	>50	>50	>50	>50	46.1	4.6	>50	>50
44	12.9	3.5	18.8	38.2	35.5	33.2	18.0	>50	>50	28.2
45	>50	45.8	>50	>50	>50	>50	>50	>50	>50	>50
46	15.0	14.9	26.2	19.9	>50	35.9	40.0	43.6	33.1	38.9
47	4.7	3.9	13.5	2.6	14.2	12.5	4.9	4.3	21.4	9.9
48	12.7	0.5	>50	43.4	>50	32.4	>50	16.1	35.9	>50
49	5.0	10.0	>50	>50	>50	34.2	29.3	23.0	31.4	38.7
50	3.0	3.2	23.9	31.8	27.9	18.3	39.0	42.3	>50	>50
51	6.2	6.5	26.0	49.4	41.8	11.3	31.1	15.5	>50	19.0
52	1.6	0.6	8.6	23.3	5.8	5.3	7.0	6.2	31.3	19.3
53	1.2	0.3	6.0	7.6	3.1	1.7	3.4	2.4	8.8	3.6
54	1.2	1.3	10.9	14.1	13.0	22.7	7.0	11.4	5.3	4.2
55	1.3	1.0	4.7	9.7	6.5	11.8	2.5	5.1	>50	29.2
56	1.5	0.6	3.4	6.4	2.9	1.3	8.4	4.1	4.7	5.9
57	1.8	1.2	5.2	6.8	6.9	8.2	4.3	2.3	13.5	6.3
58	1.6	1.3	9.6	3.3	5.2	5.5	6.8	3.8	18.5	6.0
59	1.1	1.3	7.0	9.5	8.8	2.9	0.9	1.0	1.6	0.7
60	3.2	0.1	5.2	5.0	2.2	4.9	0.7	0.8	2.6	3.7
61	1.0	1.4	10.6	4.4	5.1	6.2	4.4	1.8	12.0	7.5
62	1.8	0.5	9.7	7.9	7.9	9.7	0.7	5.3	11.9	5.0
63	3.7	4.7	4.9	3.1	1.8	1.4	1.6	>50	2.6	3.1
64	3.8	4.2	4.0	1.4	4.5	2.9	0.5	3.2	10.2	12.2
65	0.8	2.5	1.8	1.2	3.1	0.6	13.3	3.1	2.2	4.6
66	0.8	0.6	6.2	2.2	1.5	1.5	13.8	1.0	0.1	16.1
67	0.2	0.3	6.4	0.5	8.9	6.8	5.0	8.7	2.2	3.5
68	1.9	0.7	7.1	3.1	3.6	20.8	13.9	1.1	8.1	8.3
69	1.2	0.5	4.7	4.4	3.4	9.4	7.6	1.9	14.4	16.3

[a]Each experiment was independently performed three times; [b]H=Hypoxia: 3% oxygen; [c]N=Normoxia: 20% oxygen

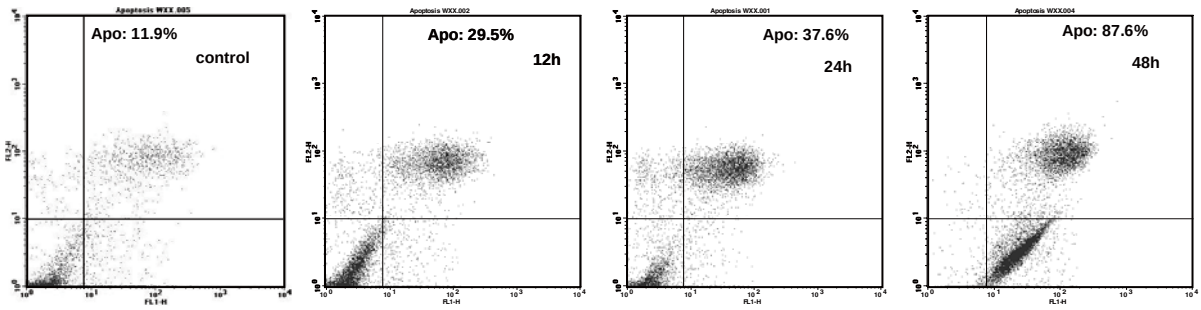


Figure 2. Compound **67** induced apoptosis in K562 cells. K562 cells were treated with 6.0 μM **67** in hypoxia for 0, 12, 24 and 48 h. Apoptosis was assessed by Annexin V-FITC/Propidium iodide (PI) staining.

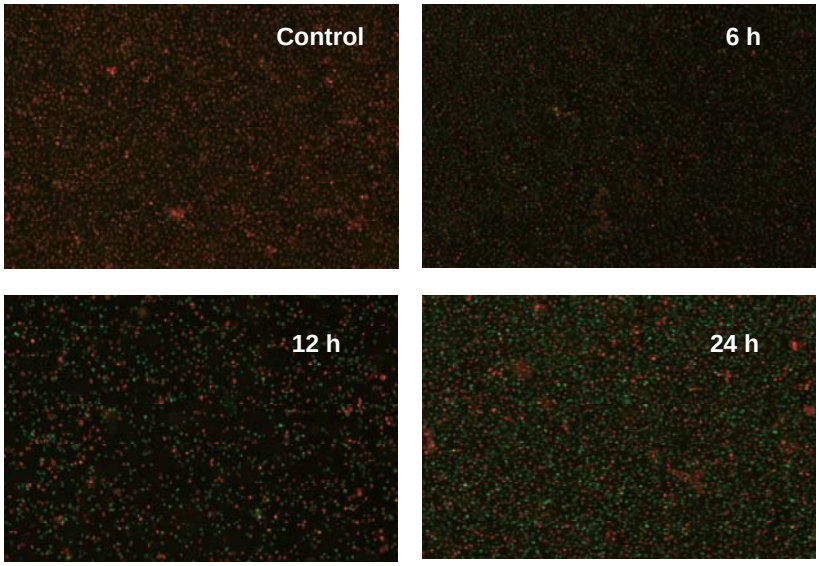


Figure 3. Compound **67** induced ΔΨm loss in K562 cells. K562 cells were treated with 6.0 μM **67** in hypoxia for 0, 6, 12 and 24 h. ΔΨm loss was assessed by JC-1 staining in which mitochondria depolarization is indicated by an increase in the green-to-red fluorescence intensity ratio.

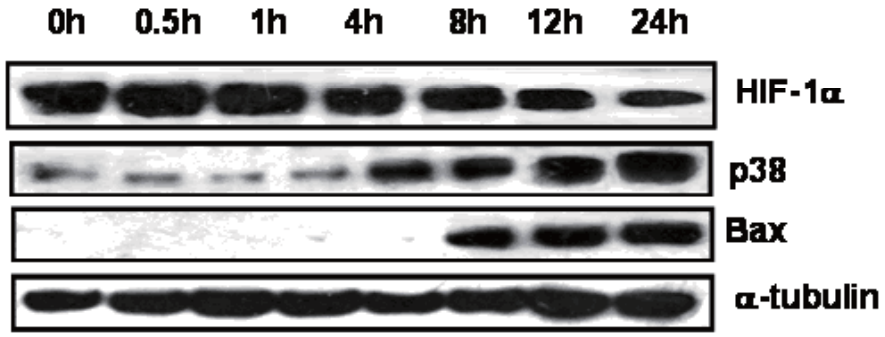


Figure 4. Protein expressions of HIF-1α, P38, and Bax in K562 cells treated with 6.0 μM compound **67** in hypoxia for 0.5, 1, 4, 8, 12 and 24 h. Each lane was loaded with 40 μg of protein.

in hypoxia was performed with one potent compound (**67**) in K562 cells. K562 cells were treated with tested compound for 0 h, 6 h, 12 h or 24 h respectively, and then apoptosis, the mitochondrial membrane potential (ΔΨm), and protein expression were determined according to reported methods (*17*). The results are shown in Figures 2-4. All experiments were repeated three times.

K562 cells were cultured in complete medium with 6.0 μM **67** for 0-48 h in 3% O_2. Every six hours the cells were collected and the apoptotic percentage was analyzed by flow cytometry (Figure 2). As shown in Figure 2, an apoptotic phenomenon was observed at 12

h after cells were exposed to **67**. After K562 cells were incubated with **67** for 0, 12, 24 and 48 h, the percentage of apoptotic cells was 11.9%, 29.5%, 37.6% and 87.6%, respectively. This increase occurred in a time-dependent manner, indicating that the apoptotic pathway was involved in the mechanisms of compound **67**-mediated cytotoxic activity.

To investigate the pathway of apoptosis induced by tested compounds, ΔΨm loss and protein expression of HIF-1α, Bax, and P38 in K562 cells treated with 6.0 μM 67 for 0-24 h in hypoxia were determined. With JC-1 staining, mitochondria depolarization is specifically indicated by a fluorescence emission shift from red to green. ΔΨm loss in K562 cells was reduced by **67** after 0-24 h treatment; corresponding data are shown in Figure 2. Compared to the control, K562 cells treated with **67** exhibited a mass of green fluorescence, suggesting that **67** might possess highly potent cytotoxic activity *via* a mitochondrial pathway.

P38, Bax, and HIF-1α were the key proteins involved in cell apoptosis and DNA damage. The expression of P38, Bax, and HIF-1α in K562 cells treated with **67** was performed by Western blot analysis. As shown in Figure 3, 6.0 μM **67** increased P38 and Bax levels in K562 cells after 24 h of exposure in hypoxia and reduced the HIF-1α protein level. The data obtained confirmed that higher cytotoxicity of **67** was related to the P38 and Bax-mediated apoptosis pathway.

Conclusions

In summary, a new series of novel 2-substituted-phenyl-3-ethylthio/ethylsulfinyl/ethyl sulfonyl/phenylthio/phenylsulfonyl-quinoxaline 1,4-dioxides were synthesized and screened for their antitumor activity *in vitro* on five cancer cell lines in hypoxia and in normoxia. Half of the tested compounds showed higher antitumor activity both in hypoxia and in normoxia. When treated with compound **67**, K562 cells exhibited overexpression of P38 and Bax, and K562 cells also induced down-regulation of HIF-1α, suggesting modulation of protein and mitochondria pathways involved in the anti-cancer activity of compound **67**.

Acknowledgments

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16. All compounds gave satisfactory spectroscopic data in accordance with their proposed structures. Selected data are as follows: compound **67** Mp: 209-211°C; MS (ESI): 425 (M+H); ^{1}H NMR ($CDCl_3$) δ: 8.51 (d, 1H, *J* = 8.8 Hz, Ar-H), 8.44 (s, 1H, Ar-H), 7.79 (d, 1H, *J* = 8.8 Hz, Ar-H), 7.68 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.28 (d, 2H, *J* = 8.4 Hz, Ar-H), 3.78~3.83 (m, 2H, CH_2), 2.67 (s, 3H, CH_3), 1.33 (t, 3H, *J* = 7.6 Hz, CH_3); IR (KBr): 3082, 1610, 1492, 1457, 1395, 1312, 1219, 1182, 1128, 1089, 1012, 935, 916, 843, 781, 760, 716.
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A novel method to suppress the dispersal of Japanese cedar pollen by inducing morphologic changes with weak alkaline solutions

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ABSTRACT: Inhalation of airborne pollen causes irritative symptoms in humans, known as pollinosis. The changing global climate and increased pollution contribute to enhance the release of pollen, thereby increasing the number of people suffering from allergies. We examined the effect of spraying weak alkaline solutions onto cedar trees, the main allergenic culprit in Japan, on pollen release. Weak alkaline solutions were sprayed onto Japanese cedar blossoms to disrupt the external walls of the pollen, and to induce swelling of the cytosolic components containing the nucleus. This morphologic change of the pollen grains depended on the pH of the suspending solution, with a threshold pH of near 7.5. As the breakdown of the external walls and swelling of the cytosolic components are inhibited by high osmolarity, the influx of water triggered the morphologic changes. Weak alkaline solutions sprayed onto cedar blossoms decreased the amount of pollen released from the anthers in a pH-dependent manner. The addition of detergent to the sodium bicarbonate solution facilitated this effect on cedar pollen release. We suggest that spraying cedar and cypress forests with a weak alkaline solution might prevent the scattering of pollen that causes allergies in humans.

Key Words: Pollinosis, *Cryptomeria japonica*, alkaline solutions, pollen release

Introduction

Various environmental substances cause allergic diseases (*1*). The inhalation of airborne pollen leads to irritative symptoms such as runny nose, sneezing, and eye hyperemia, an allergic reaction known as pollinosis. The allergens in airborne pollen have been purified, and their molecular structures have been analyzed (*2*). Environmental factors such as microparticles generated from diesel engines (*3*,*4*) and pathogenic bacteria (*5*) worsen the allergic symptoms of pollinosis. Various types of host cells are involved in pollinosis (*6*), such as acquired immune cells like helper T cells and immunoglobulin-producing B cells (*7*), innate immune cells like dendritic cells and macrophages (*8*), mast cells and eosinophils (*9*), mucosal epidermal cells, *etc*. These cells communicate with each other through the cytokine network (*10*,*11*), and inflammatory mediators such as histamine cause the allergic pathology (*12*,*13*). These aspects of the molecular mechanisms of pollinosis are extremely complicated. As our understanding of the system is currently insufficient, it is difficult to develop effective drugs to target pollinosis at the molecular level.

The number of patients suffering from pollinosis is increasing, along with the number of patients suffering from other allergic disorders such as atopy and asthma. One reason for the worldwide increase in pollinosis is that the increased concentration of atmospheric carbon dioxide and the accompanying global warming promotes pollen release from trees (*14*,*15*). When plants are raised in high concentrations of carbon dioxide or high temperatures, metabolic processes such as photosynthesis and water uptake are facilitated, and the production of pollen is promoted (*16-19*). Both the concentration of carbon dioxide in the air and the climate temperature are predicted to increase over time. Therefore, it is anticipated that the number of patients suffering from pollinosis will further increase due to a global overproduction of pollen. To cope with the situation, methods targeting pollen release from trees might be effective. The development of tree races that produce less total or less allergenic pollen has been attempted (*20*). Planting these new races in place of widely distributed anemophilous plants, however, is time-consuming and costly. Thus, simpler and more large-scale methods to suppress pollen dispersal are necessary.

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In Japan, pollinosis caused by Japanese cedar pollen (*Cryptomeria japonica*) is considered to be the most problematic (*21*). As a result of historical large-scale cedar planting, cedar trees are distributed throughout the country. For geographic reasons due to monsoons, pollen grains are likely to scatter in the air and reach the cities. Moreover, once pollen grains fall onto the paved roads in cities, they tend to remain there and are easily swept up into the air, thereby coming into contact with people. Therefore, a method that efficiently inhibits pollen release from cedar trees might be useful for suppressing pollinosis. In this report, we suggest a novel way to reduce the amount of pollen released from trees by focusing on the characteristics of cedar pollen, which undergoes morphologic changes in weak alkaline solutions.

Materials and Methods

Pollen

Pollen from cedar and cypress was purchased from the Japan Forest Tree Breeding Association. Cedar branches and blossoms were collected at the Gunma Prefectural Forestry Experiment Station (Gunma, Japan) in March, 2007.

Observation of morphologic changes of pollen grains

Pollen grains in a plastic tube were suspended in distilled water (pH 6), 25 mM sodium- or potassium-phosphate buffer solution (pH 5-9), or 1 M sodium bicarbonate solution (pH 9). After incubation at 20˚C for 10 min, an aliquot was observed under a microscope.

Measurement of the number of pollen grains released from blossoms

Cedar branches with blossoms (0.4-0.7 g) were sprayed with 10 mL of distilled water, 0.1 M phosphate buffer solution (pH 5-8), or 1 M sodium bicarbonate solution. The branches were dried for 3 h on a paper towel and placed in a 50-mL plastic tube. The tubes were shaken vigorously for 10 sec with a vortex mixer. The blossoms were collected and their weights measured. The collected pollen was suspended in 1 mL of distilled water. An aliquot of the suspension was observed under a microscope and the number of pollen grains was counted using a cytometer. The average number of pollen grains collected from three branches was calculated.

Results

Morphologic changes of Japanese cedar pollen in weak alkaline solutions

In the nasal cavity of humans, the external walls of cedar pollen grains break down, which results in the release of the cytosolic components surrounded by a transparent membrane (*22*). The high pH (> 8) of the mucus is considered to contribute to this reaction. Morphologic changes of cedar pollen were induced in 50 mM Tris/HCl solution (pH 8) (Figure 1). The external walls of the pollen grains were fluorescent under a fluorescence microscope, but the released cytosolic particles surrounded by the transparent membrane were not (Figure 1). We suspended cedar pollen in buffered solutions with various pHs, and counted the number of pollen grains that underwent morphologic changes after 10 min. Weak alkaline solutions induced morphologic changes of the pollen, with a threshold of pH 7.5 (Figure 2). The transparent membranes of the cytosolic particles disappeared when the morphologically changed pollen was incubated with pectinase (data not shown), indicating that the membrane covering the particle is made of pectin. Because the transparent membrane was not degraded by organic solvents or detergents (data not shown), lipids are not responsible for maintaining the rigidity of the transparent membrane.

Japanese cypress (*Chamaecyparis obtusa*) is another gymnosperm that causes pollinosis (*23,24*). The structure and antigenicity of the cypress pollen components are similar to those of cedar pollen (*24,25*). Therefore, we assumed that cypress pollen might also undergo morphologic changes in weak alkaline solutions. We suspended cypress pollen in 1 M sodium bicarbonate solution, and observed the samples under a microscope after incubating them for 10 min at room temperature. The majority, 50% to 90%, of the cypress pollen grains released cytosolic particles from the external walls (Figure 3), whereas in distilled water most pollen did not change morphologically. Thus, as with cedar pollen, weak alkaline solutions induced morphologic changes in cypress pollen.

The external walls of cedar pollen grains are permeable to water, ions, and small hydrophilic molecules (*26,27*). We hypothesized that water absorption through the external walls is the initial step of the morphologic changes in cedar pollen. If so, the process might be influenced by osmotic pressure. Thus, we tested the effect of high osmolality on the morphologic changes in cedar pollen in weak alkaline solutions. The addition of 1 M sucrose to 1 M sodium bicarbonate solution decreased the number of swollen cytosolic particles (Figure 4). This finding indicates that a pH shift increases the permeability of the external surfaces of cedar pollen, leading to a rapid uptake of water, which results in the breakdown of the outer walls and the release of transparent particles. Further swelling of the transparent membrane was inhibited by 5 M sucrose. Therefore, the swelling is also triggered by the osmotic inflow of water.

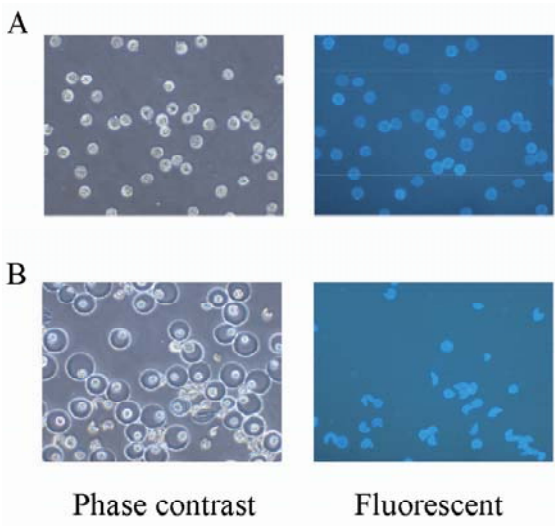

Figure 1. Morphologic changes of Japanese cedar pollen in weak alkaline solutions. Japanese cedar pollen was suspended in distilled water (pH 5-6) (A) or 50 mM Tris/HCl (pH 8) (B), incubated at 25˚C for 10 min, and samples were observed under a microscope equipped with a fluorescent lens.

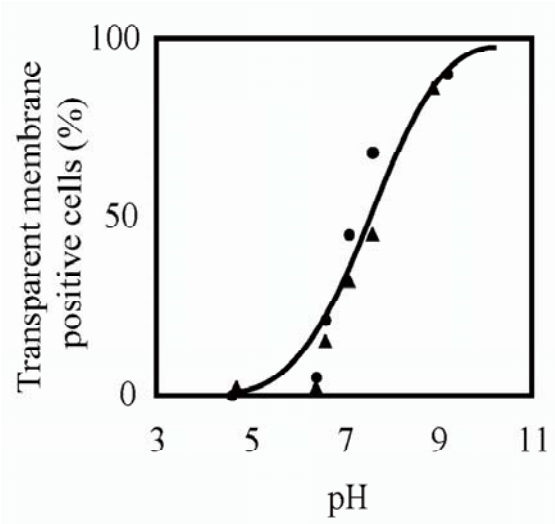

Figure 2. pH-dependence of the morphologic changes of Japanese cedar pollen. Japanese cedar pollen was suspended in 25 mM sodium-phosphate buffer (*triangles*) or 25 mM potassium-phosphate buffer (*circles*) with various pH values at 25˚C for 10 min. Samples were analyzed under a microscope with a phase contrast lens. The number of pollen grains with morphologic changes was counted.

Suppression of cedar pollen release from blossoms by spraying with weak alkaline solutions

In general, mature pollen grains are in a dried state when released from the anthers in response to the oscillation of branches caused by wind. Pollen grains that undergo morphologic changes lose their outer coats, which are necessary for floating in the wind and for protecting the interior contents from the environment. The altered morphology of cedar pollen suspended in weak alkaline solutions might therefore render them unsuitable for scattering, and such pollen might be easily destroyed in the environment. We evaluated whether spraying the branches with weak

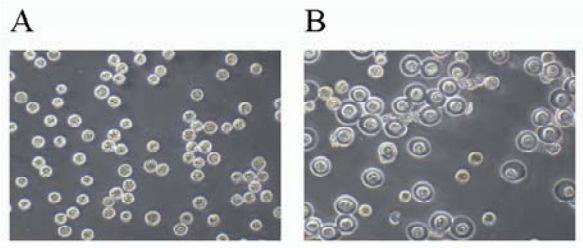

Figure 3. Morphologic changes of Japanese cypress pollen in weak alkaline solutions. Japanese cypress pollen was suspended in distilled water (A) or 1 M $NaHCO_3$ (B), incubated at 25˚C, and samples were observed under a microscope.

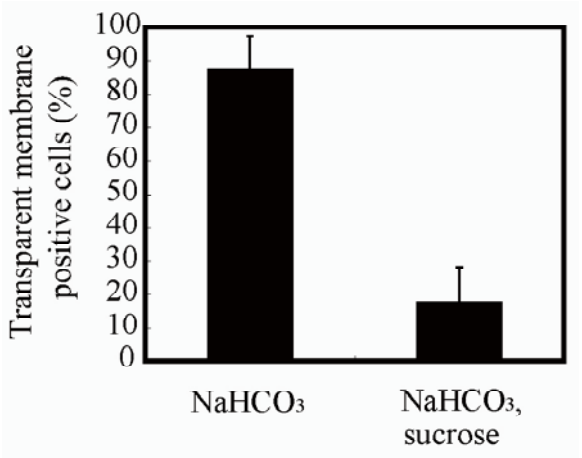

Figure 4. Inhibitory effect of hypertonicity on the morphologic changes of Japanese cedar pollen. Japanese cedar pollen was suspended in 1 M $NaHCO_3$ or 1 M $NaHCO_3$ containing 1 M sucrose and incubated at 25˚C for 10 min. Samples were observed under a microscope. The number of pollen grains with morphologic changes was counted.

alkaline solutions decreased the amount of cedar pollen released from the anthers. First, pollen release was inhibited when sodium bicarbonate solutions were sprayed onto cedar blossoms (Figure 5A). Next, we sprayed phosphate-buffered solutions of various pHs onto cedar blossoms and examined the effect on pollen release. The amount of released pollen decreased in a pH-dependent manner (Figure 5B). Cedar pollen is covered by a waxy material that protects it against the invasion of weak alkaline solutions. We examined the effect of detergents that are generally added to agrochemicals against insects and pathogens. The addition of NP-40 to a 1 M sodium bicarbonate solution suppressed the pollen release from cedar blossoms in a dose-dependent manner (Figure 6). Therefore, the addition of detergents allowed for penetration of the waxy material and suppressed pollen release from the anthers.

Discussion

Suppression of the dispersal of cedar pollen by spraying with weak alkaline solutions

Pollinosis is a clinically important allergic disease caused by airborne pollen from various plants.

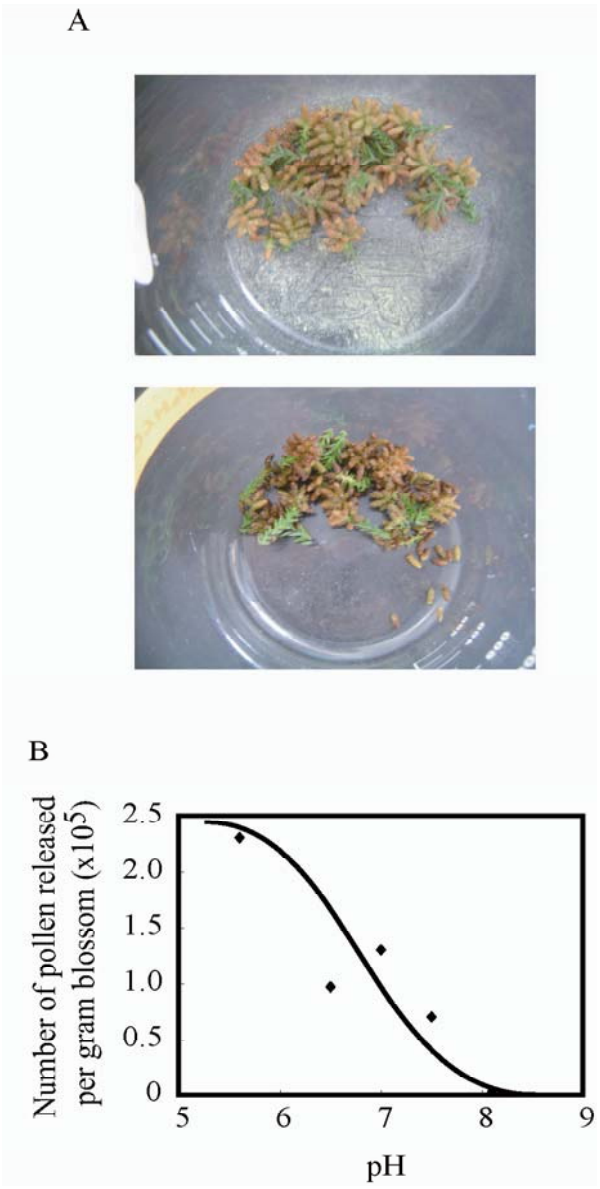

Figure 5. Suppression of pollen release from cedar blossoms by spraying with weak alkaline solutions. (A) Japanese cedar blossoms were sprayed with 10 mL of distilled water (*top*), or 1 M $NaHCO_3$ (*bottom*), and dried at 37˚C for 10 h in 1-L beakers. Release of yellow-colored pollen was observed in (*top*), but not in (*bottom*). (B) Japanese cedar blossoms were sprayed with 10 mL of 0.1 M sodium phosphate buffer with various pHs. After drying on a paper towel for 3 h, cedar blossoms were placed in a 50-mL plastic tube, and agitated vigorously with a vortex mixer. Released pollen grains were suspended in distilled water, and counted under a microscope.

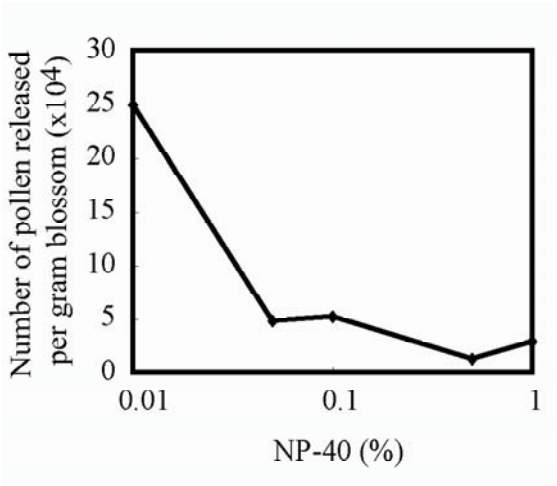

Figure 6. Influence of NP-40 on pollen release from cedar blossoms sprayed with a sodium bicarbonate solution. Japanese cedar blossoms were sprayed with 1 M $NaHCO_3$ containing various concentrations of NP-40. Blossoms were dried on paper towels at 25˚C for 3 h, and agitated vigorously in a 50-mL tube with a vortex mixer. Released pollen grains were suspended in distilled water and counted under a microscope.

The number of patients suffering from pollinosis is increasing, especially in industrialized cities. In Japan, cedar pollinosis was first reported in 1964, and Cryj1, a major allergen of cedar pollen, was discovered in 1983. Since then, there have been a number of attempts to overcome the disease, such as the development of medicines that inhibit inflammatory mediators released from activated mast cells (*28*), vaccination and desensitization against pollen allergens (*29-31*), and the generation of new races of cedar that release less pollen. Unfortunately, because these drugs and remedies often have unwanted side effects (*32*,*33*), and because planting new strains is time-consuming and costly, these attempts have not been successful. Therefore, the establishment of an inexpensive, simple method to suppress pollinosis is highly desirable. In this report, we suggest that 1) cedar pollen undergoes morphologic changes in weak alkaline solutions, and 2) spraying cedar blossoms with alkaline solutions induces morphologic changes of the pollen inside the anthers, resulting in a decreased amount of pollen released from the blossoms.

The transparent membrane covering the cytosolic component of pollen is made of pectin (*34*,*35*). As pectin contains acidic sugars, water molecules are easily trapped on the surface. Thus, cedar pollen that undergoes morphologic changes in weak alkaline solutions has increased adhesiveness. Furthermore, pollen lacking the external wall might be easily destroyed by environmental changes in temperature or humidity, and by physical force. Therefore, treatment with weak alkaline solutions is expected to be useful for suppressing the dispersal of cedar pollen. We propose that by reducing the amount of scattered pollen by spraying cedar forests with weak alkaline solutions, such as sodium bicarbonate solutions, the release of allergens might be decreased. Spraying a harmless alkaline solution, such as sodium bicarbonate, onto paved roads might also be effective to prevent the pollen from scattering back up into the air. Because cedar blossoms are covered with a waxy substance that repels water, detergents in the sprayed solution will facilitate the induction of morphologic changes of the pollen inside the anthers.

Mechanism of disruption of the external walls of cedar pollen in weak alkaline solutions

The external walls of cedar pollen function as barriers

against physical force or environmental changes. The walls are extremely rigid, and only severe conditions such as acetolysis by strong acid can break the structure. In contrast, cedar pollen that enters the nasal cavity of humans seems to readily release allergen molecules. This process in the nasal cavity is considered to be triggered by the high pH of the mucosal surface. Moreover, as the changes in pollen morphology are inhibited by adding sucrose to the alkaline solutions, the process is suggested to involve water absorption into the wall structure due to osmotic pressure. Cellulose is a major constituent of the external walls of cedar pollen. *D*-glucose, a structural unit of the external walls, possesses hydroxyl groups at the C2, C3, and C6 positions, which are involved in hydrogen bonding with other oxygen molecules of cellulose fibrils. These hydrogen bonds might be weakened in alkaline solutions, as the hydroxyl groups of glucose undergo deprotonation. As a result, the loosened fibrils of the external walls might become permeable to water molecules. Further studies are required to understand the mechanism of the sharp pH-threshold for the morphologic changes of cedar pollen.

Morphologic changes of other types of pollen that cause pollinosis

Cypress is another anemophilous gymnosperm that causes pollinosis. The structure of cypress pollen is similar to that of cedar pollen, and it is covered by a cellulose wall. Cypress pollen undergoes the same morphologic alterations as cedar pollen in a weak alkaline solution, which suggests that spraying weak alkaline solutions onto other types of gymnosperms might help to minimize pollinosis in humans.

Acknowledgements

We are grateful for Mr. Yoshikazu Kanazawa (Gunma Prefectural Forestry Experiment Station) for his help harvesting cedar branches with blossoms.

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The effects of antidepressant drug on ethanol-induced cell death

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ABSTRACT: Alcoholism is a serious health problem. Alcohol-dependent subjects have many health-related problems, such as severe cognitive impairments, alcoholic liver disease and coronary heart disease, resulting from ethanol-induced cell injury or cell death. Understanding the mechanisms underlying the cell death may provide clues for novel treatment strategies to prevent alcohol-induced cell damage. Prolonged ethanol consumption causes apoptotic activity in a host of cell types – more obviously affecting the liver, heart and surprisingly affecting the brain. This study uses four cell lines: neuronal cell line (SH-SY5Y), glia cell line (U-118 MG), liver cell line (E47) and heart cell line (the rat H9c2), and addresses that alcohol does, in fact, cause cell death in these four cell types, whether ethanol induced cell death is through apoptotic pathway, and whether an monoamine oxidase (MAO) inhibitor (*e.g.* deprenyl) protects cells from the effects of alcohol. We have found that ethanol exposure lowers cell proliferation in all cell types, but affects brain cell lines (neuron and glioma) the most, while ethanol and deprenyl exposure in unison increases cell viability largely in brain cells, and then in liver cells. Our results suggest that MAO-mediated apoptosis may contribute to ethanol-induced cell death. Individuals suffering from alcoholism or alcohol abuse may be treated with deprenyl to alleviate the apoptotic activity resulting from alcohol consumption and protect the body's cells from alcohol-induced death. In summary, this study demonstrates the effects of deprenyl as an anti-apoptotic agent against the detrimental effects of alcohol.

Key Words: Alcohol, neuroblastoma, glioma, cell culture, cell viability

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Introduction

Alcohol (ethanol) is one of the most commonly used drugs around the world and like all other drugs it has profound effects on the body. In particular, it affects the brain and alters its normal function (*1*). This includes altering the effects of neurotransmitters, suppressing nerve signals and causing cell death (*2*). In rodents, ethanol exposure during development significantly reduces the size of the brain as well as brain/body weight ratios (*3*). There are many adverse physical effects from long-term exposure to alcohol including the increased activity in the liver that cause cell death and hardening of the tissue (cirrhosis of the liver) (*4*) and an increase in the number of apoptotic cells in various brain areas (*5*). This study investigates the effects of ethanol on neuronal cell death, liver cell death and heart cell death. Furthermore, this investigation determines the effects of deprenyl on cell death.

Deprenyl (selegiline), an irreversible inhibitor of monoamine oxidase B (MAO B), was synthesized as an antidepressant and used to treat Parkinson's disease (*6*). Because MAO degrades serotonin and produces reactive oxygen that may cause cell death, an MAO inhibitor prevents cell apoptosis (*7-9*). Deprenyl or related compounds may be neuroprotective in general through the inhibition of "death" signal transduction-mediated by MAO, induced by endogenous and environmental factors (*10*). Deprenyl in low concentrations that induce MAO B inhibition (0.001 nM to 1.0 nM) potently inhibits serum withdrawal induced apoptosis in tissue cultures of neuro-ectodermal origin (*11*). These studies demonstrated that ethanol can induce apoptosis in both neuronal and non-neuronal cells and compared the effects of deprenyl on the protection of cells from the harmful effects of ethanol in cell lines-derived from neuron, glioma, liver and heart.

Materials and Methods

Cell lines

A human neuroblastoma cell line, SH-SY5Y, a human glioma cell line, U-118 MG, and a rat heart cell line,

H9c2, were purchased from The American Type Culture Collection (ATCC). A human liver cell line, E47, is a gift from Dr. Arthur Cederbaum, Mount Sinai School of Medicine, New York. E47 cells are HepG2 cells which were transfected with human P450 enzyme CYP2E1 cDNA in the sense orientation and constitutively express CYP2E1. CYP2E1 produces increased oxidative stress and liver cell toxicity, therefore, E47 cell line responds to ethanol in ways similar to liver parental cells (*12,13*).

SH-SY5Y was cultured in Minimum Essential Medium (MEM) containing Earle salts, fetal bovine serum, 1.0 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. U-118 MG and H9c2 were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, fetal bovine serum and 200 mM glutamine. E47 was cultured in Minimum Essential Medium (MEM) containing Earle salts and fetal bovine serum.

Cell culture and treatments with ethanol and deprenyl

Before treatments, SH-SY5Y, U-118 MG, E47 and H9c2 cells were seeded on 6-well plates. After overnight culture in medium, the medium was replaced with new medium containing different concentrations of ethanol and deprenyl, daily, for three days. The ethanol concentration we used (200 mM for examining the effect of deprenyl) was within the standard range of in vitro study (*14*). This concentration was also decreased to ~1/4 after one day by evaporation (data not shown), thus the medium with ethanol was changed every 24 h. When a heavy drinker's ethanol concentration in blood reaches ~50 mM, he probably shows slurred speech and unsteadiness (*15*). Thus, the ethanol concentration for this study is around the physiological effect of ethanol in alcoholics.

MTT assay for proliferation rate/cell viability evaluation

Cell viability and proliferation was measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (*9*). The medium in excess of 2 mL (6-well plates) was removed and 40 μL of MTT dye (5 mg/mL) in sterile PBS was added to 360 μL of medium or PBS depending on cell confluence. More confluent cell lines used PBS and plates with fewer cells used medium. Plates were incubated for 4 to 5 h, during which time the mitochondria in living cells converted the soluble yellow dye (MTT) into an insoluble purple formazan crystal. Cells and dye were then solubilized by the addition of 800-1000 μL of DMSO to the 6-well plates. Optical density of each well at 572 nm was determined using the NanoDrop Spectrophotometer.

Western blot analysis

Cells were cultured in medium with ethanol (0, 200 and 400 mM) for 2 d, washed by PBS (pH 7.4), and sonicated in 500 μL of RIPA lysis buffer (10 mM Tris·HCl, pH 7.4/160 mM NaCl/1% Triton/1% Na dexycholate/0.1% SDS/1 mM EDTA/1 mM EGTA) supplemented with protease inhibitors (Sigma, Japan). Thirty micrograms (for MAO B assay) of total proteins were separated by 10.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the transfer, membranes were blocked at room temperature for 2 h with 5% nonfat dry milk in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20). The membranes were then incubated with mouse anti-caspase 3 antibody (1:500) or mouse anti-actin antibody (1:1000) overnight at 4°C. After incubation with respective secondary antibody at room temperature for 2 h, the bands were visualized by hoseradish peroxidase (HRP) reaction using SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

Statistical analysis

The statistical significance was evaluated using a Student's *t* test for two-group comparisons when needed. A value of $P < 0.05$ was considered to be significant.

Results

Ethanol induces cell death in a concentration dependent manner

SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with different concentrations of ethanol (0, 100, 200, 300 and 400 mM) for three days. Then the cell viability was determined by MTT assay. The results showed that ethanol induced cell death in a concentration dependent manner as determined by MTT assay (Figure 1). In addition, ethanol affected the brain cell lines (SH-SY5Y and U-118 MG) more than liver (E47) and heart cell (H9c2) lines at the concentration of 200 mM (Figure 1).

Ethanol induced cell death is through apoptotic pathway

To test whether cell death induced by ethanol is through apoptotic cascade, the levels of apoptotic protein, caspase 3, was determined in each cell line treated without or with ethanol, because caspase 3 mediated apoptotic pathway has been found to participate in MAO involved apoptotic signaling pathway (*9*). As determined by western blot (Figure 2A), the relative intensity of each casapase 3 protein band was quantified by a PhosphorImager system (Figure 2B) and expressed as -fold control, in which the cells without ethanol treatment was taken as 1. These quantitative data showed that the levels of caspase 3 was increased significantly by ethanol treatment (200 and 400 mM for

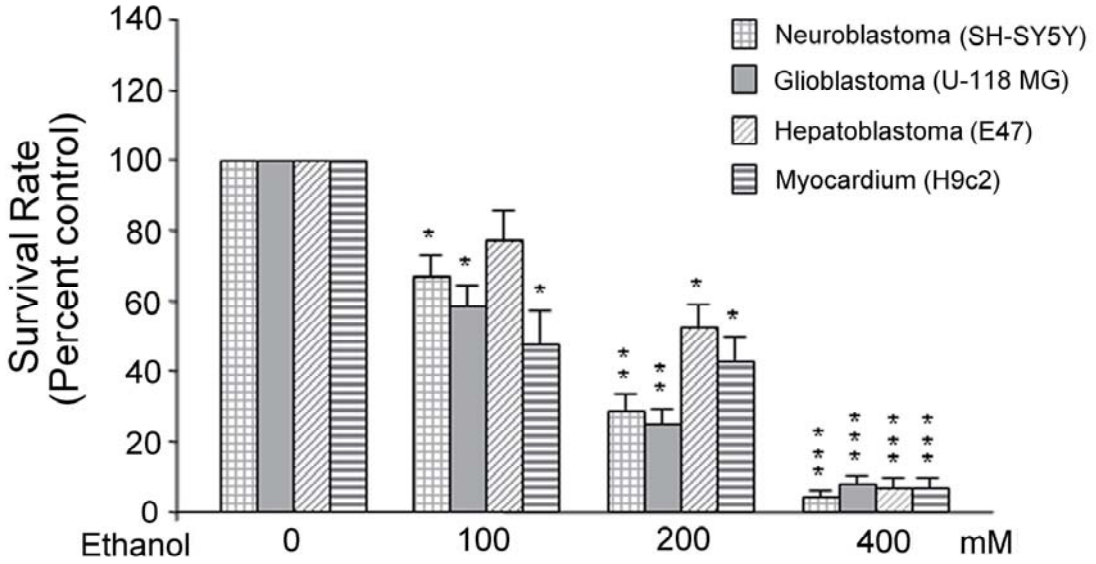

Figure 1. Effects of different concentrations of ethanol on cell survival rate. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with different concentrations of ethanol (0, 100, 200, 300 and 400 mM) for three days. Then the cell viability was determined by MTT assay. Controls were untreated cells (0 mM) which were taken as 100%. Data represent the mean ± S.D. of three independent experiments (**P* < 0.05, ***P* < 0.02, ****P* < 0.005 versus respective control cells without ethanol treatment).

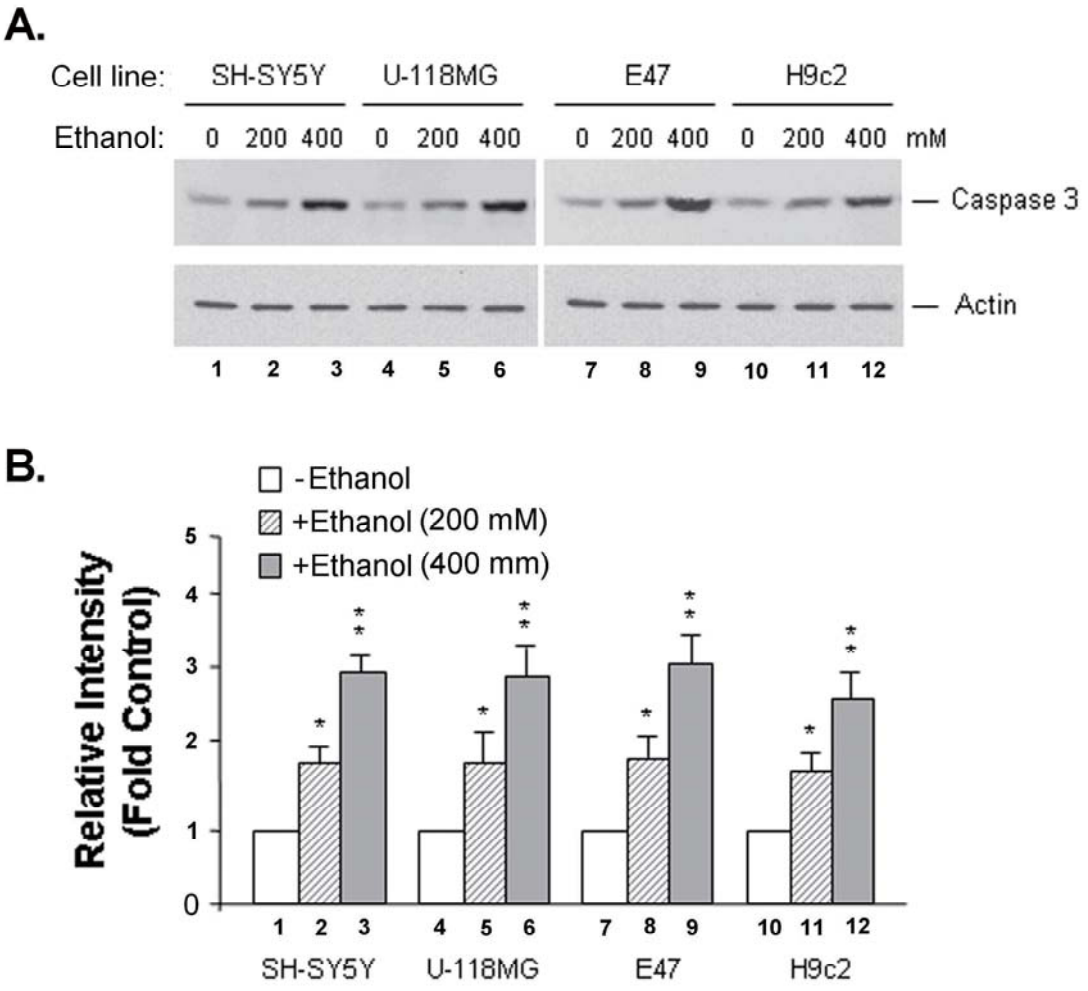

Figure 2. The expression of the apoptotic protein, caspase 3, after ethanol exposure. (A) Cells were exposed to 0, 200 and 400 mM ethanol for 48 h, and the cell lysates were subjected to the western blot analysis using anti-caspase 3 antibody. (B) Quantitative analysis. The relative intensity of each caspase 3 band was quantified by PhosphorImager. Values were expressed as fold of control, in which the cells without ethanol treatment was taken as 1. Data represent the mean ± S.D. of three independent experiments (**P* < 0.05, ***P* < 0.02 versus respective control cells without ethanol treatment).

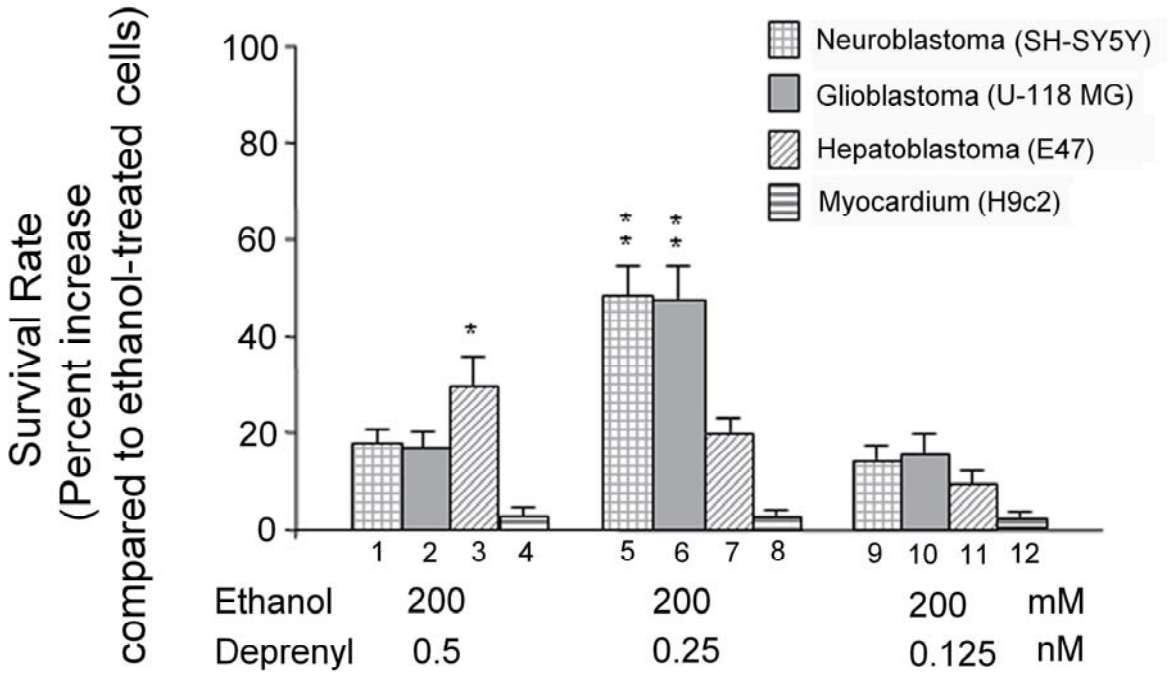

Figure 3. Effects of different dosage of deprenyl (MAO inhibitor) on the protection of ethanol induced cell death. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with or without 200 mM ethanol in the conjunction with 0.5, 0.25 or 0.125 nM of deprenyl as indicated in the figure for three days. Then the cell viability (survival rate) was determined by MTT assay. The values of first group (+ ethanol) were obtained by comparing ethanol-treated group with normal control group which was taken as 100%. The values of second group (+ ethanol and deprenyl) were obtained by comparing ethanol/deprenyl-treated group with deprenyl-treated group which were taken as 100%. Then the percent increase in the survival rate by deprenyl is expressed by comparing the values of second group (+ ethanol and deprenyl) with the values of first group (+ ethanol) which was taken as 100%. All data are presented as the mean ± S.D. of at least three independent experiments (*$P < 0.05$, **$P < 0.02$ versus respective control cells with ethanol treatment but without deprenyl).

48 h) compared to control group (ethanol 0 mM) in a concentration dependent manner.

The inhibitor of MAO, deprenyl, against ethanol induced cell death

The physiologically relevant concentration of ethanol has been found to increase the MAO B gene expression and catalytic activity in the human glioma 1242-MG cells (*16*). Therefore we studied whether the MAO inhibitor (deprenyl) could reduce the ethanol-induced cell death. First, the different concentrations (0.125, 0.25 and 0.5 nM) of deprenyl were used in order to establish an ideal concentration for the cellular protection (Figure 3). The results showed that 200 mM ethanol treatment in conjunction with 0.25 nM deprenyl provided the most protection against apoptotic activity for brain cells SH-SY5Y and U-118 MG (Figure 3, compare column 5 and 6 to column 1, 2, 9 and 10). For the liver cells, the best protection was at 0.5 nM deprenyl (Figure 3, compare column 3 to column 7 and 11). However, for the heart cells, there was no protection for deprenyl at any concentrations (Figure 3, column 4, 8 and 12).

According to above optimal condition, cells were exposed to 200 mM ethanol in conjunction with 0.25 nM (for brain cell lines) or 0.5 nM (for liver cell line) deprenyl for three days, and the cell viability were in survival rate was observed in both SH-SY5Y and U-118 MG cells with 200 mM ethanol and 0.25 nM deprenyl treatment compared to those of cells treated with 200 mM ethanol alone (Figure 4, column 2 and 4 *vs.* column 1 and 3). In addition, ~1.5-fold increase in survival rate was found in liver cells, E47 (Figure 4, column 6 *vs.* column 5). However, heart cells H9c2 were not protected at 200 mM ethanol treatment with 0.5 nM deprenyl (Figure 4, column 8 *vs.* column 7).

These data suggested that lower concentration of deprenyl (0.25 nM) protects brain cells, higher concentration (0.5 nM) protects liver, but deprenyl at either concentration does not protect heart cells.

Discussion

An aberrant increase of MAO B activity has been implicated in several psychiatric and neurodegenerative disorders (*17-19*). The MAO B gene is located on the X chromosome (*20*) and its activity increases progressively in the brain throughout adult life (*21,22*). MAO B is the enzyme that deaminates a number of biogenic amines and produces reactive oxygen (H_2O_2) which causes toxicity to cells. Thus one predicted mechanism for cell death is an abnormal increase in monoamine oxidase (*23*). Previously, the physiologically relevant concentration of ethanol has been found to increase the MAO gene expression and catalytic activity in the human glioma 1242-MG cells (*16*). The increased activity of MAO may thereby increase production of hydrogen peroxide (H_2O_2, a

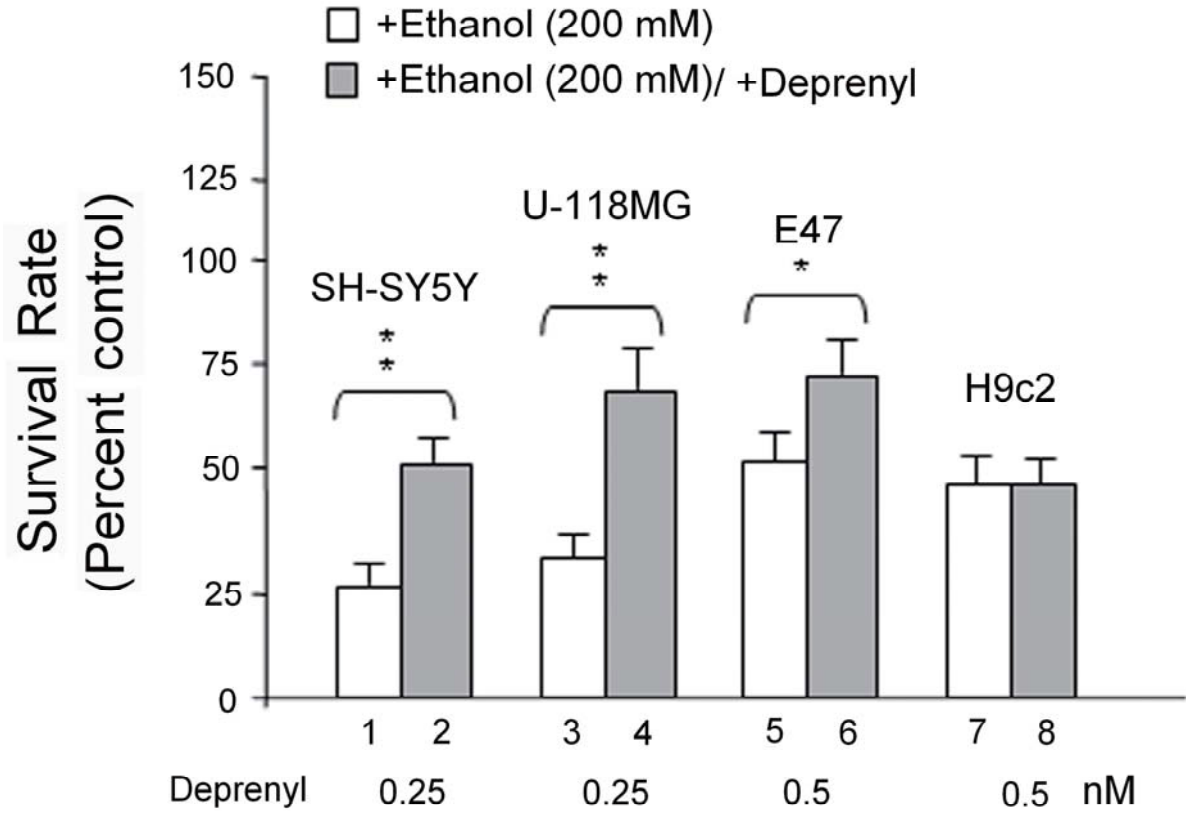

Figure 4. Effects of deprenyl on the protection of ethanol induced cell death. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with or without 200 mM ethanol in the conjunction with 0.25 or 0.5 nM of deprenyl as indicated in the figure for three days. Then the cell viability was determined by MTT assay. The values of first group (+ ethanol) were obtained by comparing ethanol-treated group with normal control group which was taken as 100%. The values of second group (+ ethanol and deprenyl) were obtained by comparing ethanol/deprenyl-treated group with deprenyl-treated group which were taken as 100%. Then the percentages of both groups were shown in the figure. All data are presented as the mean ± S.D. of at least three independent experiments (*$P < 0.05$, **$P < 0.02$).

major source for oxidative stress) and cause apoptosis (*24*). Our present study shows that the levels of caspase 3, an apoptotic mark protein, were increased significantly by ethanol, suggesting that ethanol-induced cell death is mediated by apoptotic pathway.

Based on previous publications, deprenyl provides cell protection against apoptosis even in minute doses (*11,25*). Therefore we studied whether the MAO inhibitor (deprenyl) could protect the ethanol-induced cell death. Our results show that deprenyl specifically protects brain cells from ethanol treatment at a lower dose (0.25 nM) which is consistent with previous discovery. Further, it is interesting that deprenyl at a higher dose (0.5 nM) is able to protect liver cells from the harmful effect of ethanol, although the protection is less than that in the brain cells. One of anti-apoptotic mechanisms of deprenyl has been found to elevate the expression of antiapoptotic Bcl-2 protein for protecting against a toxic metabolite 1-methyl-4-phenylpyridinium ion (MPP+)-induced neurotoxicity (*26*). Therefore, the protective effect of deprenyl on ethanol-induced cell death might be through the disruption of MAO-mediated apoptotic pathway.

Based on our results, deprenyl may not protect heart cells. Our unpublished data show that the MAO catalytic activity has been increased upon ethanol treatment in SH-SY5Y, U-118 MG and E47, but not changed significantly in the rat heat cell line (H9c2). Therefore, the MAO-mediated apoptotic pathway might not be important in this rat heart cell line compared to human brain- and liver-derived cell lines. However, the heart cell line we used is a rat cell line (because a human heart cell line is not available commercially at present). Therefore, whether deprenyl can protect heart cells need to be further studied.

In summary, alcohol abuse causes a decrease in cell proliferation rates as a result of ethanol-induced cell death. Based on the findings, deprenyl, at the correct dosage, consistently provides cellular protection against the apoptotic effects of alcohol consumption, especially for the brain, and may also protect other organs or tissues, such as the liver. MAO inhibitors/anti-depressants such as deprenyl may be used as a means of treatment for individuals suffering from alcoholism to counteract the detrimental effects of alcohol abuse.

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A simple and rapid high performance liquid chromatography method to determine levofloxacin in human plasma and its use in a bioequivalence study

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ABSTRACT: A simple and rapid HPLC-UV method has been developed for determination of levofloxacin in human plasma. Chromatographic separation was performed on a Kromasil C_{18} column with the mobile phase consisting of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v) and flow rate was 1.0 mL/min. The method used ultraviolet detection set at a wavelength of 294 nm. The standard curves were linear over a concentration range of 0.05-5.0 μg/mL (r > 0.99). The method was simple, rapid, precise, accurate, and suitable for routine bioequivalence study. The method was successfully used in a bioequivalence study of two different levofloxacin hydrochloride capsules in healthy Chinese volunteers.

Key Words: Levofloxacin, high performance liquid chromatography, bioequivalence

1. Introduction

Levofloxacin, one of the commonly used fluoroquinolone antimicrobials, is the active S-isomer isolated from the racemic ofloxacin. Its antibacterial action is twice as active as the racemate ofloxacin *in vitro*. Levofloxacin possesses a broad spectrum of activity against various bacteria, including gram-positive and gram-negative microorganisms (*1*). It is also active against causes of atypical respiratory infection such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* (*2*). Because of its excellent antibacterial activity and low frequency of adverse effects on oral administration, levofloxacin has been widely used for the treatment of infectious diseases, such as community-acquired pneumonia and acute exacerbation of chronic bronchitis (*3*).

Numerous HPLC methods for the quantification of levofloxacin in biological samples have been reported. These methods involved the use of protein precipitation (*4*) and liquid-liquid extraction (*5*,*6*) and full automated extraction (*7*) and solid-phase extraction (*8*) coupled with ultraviolet detection and protein precipitation (*9*) and solid-phase extraction (*10*) followed by fluorescence detection. Capillary electrophoresis and nuclear magnetic resonance spectroscopy have also been used to determine drugs in biological fluids (*11*,*12*). The high performance liquid chromatography-tandem mass spectrometry method (HPLC/MS/MS) has been used to determine levofloxacin in human plasma (*13*). Recently, use of the hydrophilic interaction liquid chromatography-tandem mass spectrometry method (HILIC/MS/MS) to quantify levofloxacin has also been described (*14*). However, these methods suffer from a number of disadvantages, including tedious and time-consuming sample preparation and insufficient selectivity and stability.

In bioequivalence studies, the proposed method should be simple and able to process hundreds of samples in a limited time. This paper describes a simple, rapid, precise, and accurate HPLC method for determining levofloxacin in human plasma. Once developed and validated, this method was successfully used for bioequivalence investigation of two different levofloxacin hydrochloride capsules in 20 healthy Chinese volunteers.

2. Materials and Methods

2.1 *Chemicals and reagents*

Levofloxacin (99.7% purity) and ciprofloxacin (99.5% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC-grade) was obtained from Concord Tech (Tianjin, China) and the other chemicals and reagents were of analytical grade. Drug-free human plasma was provided by the central

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blood bank of Shenyang (Liaoning, China).

2.2 *Chromatographic conditions*

The HPLC system (JASCO, Kyoto, Japan) used consisted of a model PU-2080 pump, a fixed injection-loop of 20 μL, and a model UV-2075 UV detector; data acquisition was performed with the Sepu3000's processor (Hang Zhou, China). The analytical column employed was a Kromasil C_{18} column (200 mm × 4.6 mm, i.d., 5 μm) with a guard column (10 mm × 4.6 mm i.d., 5 μm) of the same packing material. The mobile phase consisted of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v) and was filtered through a 0.45 μm cellulose membrane filter (Tianjin Auto Science, China) and degassed before use. The detection wavelength was set at 294 nm. Chromatography separation was performed at room temperature and flow rate was maintained at 1 mL/min.

2.3 *Standard solutions and quality control samples*

Primary stock solutions of levofloxacin (1 mg/mL) and ciprofloxacin (1 mg/mL) were prepared in water. Levofloxacin stock solution was further diluted with water to obtain different working standard solutions ranging from 0.25 to 25.0 μg/mL. A working standard solution of ciprofloxacin was prepared by diluting the stock solution with water to yield a final concentration of 25.0 μg/mL. Human plasma calibration standards of levofloxacin (0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/mL) were prepared by spiking an appropriate amount of the working standard solutions into drug-free human plasma. Quality control (QC) samples were prepared at low (0.1 μg/mL), medium (0.5 μg/mL), and high (4.0 μg/mL) concentrations.

2.4 *Sample preparation*

To a 5-mL test tube were added 0.5 mL plasma, 100 μL of the internal standard working solution (25.0 μg/mL), and 100 μL of water. Then, 100 μL of perchloric acid (0.6 M) were added to precipitate protein in plasma; the result was vortex-mixed for 30 sec and centrifuged at 10,000 × g for 5 min, and a 20-μL aliquot of the supernatant was injected into the HPLC system.

2.5 *Validation of this method*

Six randomly selected blank plasma samples were processed by a similar extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to interference at the retention time of levofloxacin and ciprofloxacin. The calibration curves were constructed each day before the analysis of the samples by plotting the peak-area ratio (levofloxacin/ciprofloxacin) versus the drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a coefficient of variation (CV) of less than 20% and accuracy of 80-120%. The accuracy and precision of the method were assessed by determining QC plasma samples at concentrations of 0.1, 0.5, and 4.0 μg/mL on three consecutive days, accompanying by a standard calibration curve on each analytical run. The recovery of levofloxacin was evaluated by comparing peak areas of pre-treated quality control plasma samples (n = 6) with mean peak areas of those spiked-after-extraction samples at the same nominal concentrations. The recovery of I.S. from plasma was evaluated at a concentration of 5.0 μg/mL using the same process. Stability quality control plasma samples were subjected to short-term (24 h) incubation at room temperature, three freeze/thaw cycles, and storage for 30 days (-20ºC).

2.6 *Bioequivalence study*

The present method has been used in a randomized crossover bioequivalence study in which the concentration of levofloxacin was measured in plasma samples from twenty healthy male Chinese volunteers after single oral doses (2 × 100 mg) of two different levofloxacin hydrochloride capsules from either YangZiJiang (reference, JiangSu , China) or DiSha (test, WeiHai, China) pharmaceutical companies under fasting conditions. After a one-week wash-out period, the subjects were crossed-over. Blood samples (3 mL) were drawn from the forearm at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 11, 15 and 24 h after administration, transferred to heparinized tubes, and gently mixed. After centrifugation (3,000 × g, 10 min), the separated plasma samples were collected and stored at -20ºC prior to analysis. Pharmacokinetic parameters were calculated by DAS Software-Version 2.0 (Chinese Pharmacological Society, China).

3. Results

3.1 *Specificity*

The current method showed excellent chromatographic specificity with no endogenous plasma interference at the retention times of levofloxacin and ciprofloxacin. Chromatograms obtained from human blank plasma and human blank plasma spiked with levofloxacin (2 μg/mL) and ciprofloxacin (5 μg/mL) are shown in Figure 1A and B, respectively. Levofloxacin and ciprofloxacin were well resolved with respective retention times of 8.4 and 9.4 min. Figure 1C shows the chromatogram of a plasma sample obtained at 1.0 h after a single oral dose of 200 mg levofloxacin from a healthy volunteer.

3.2 *Calibration curve and limit of quantification*

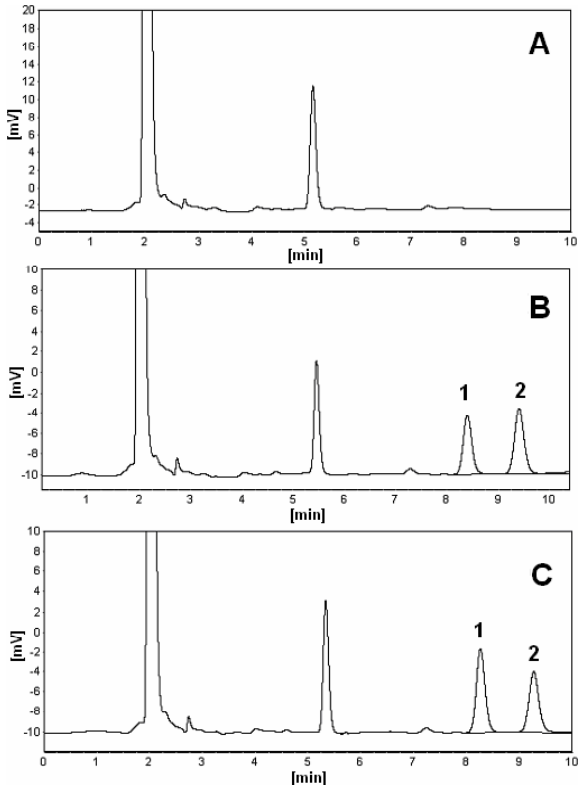

Figure 1. Representative chromatogram of human blank plasma (A), human blank plasma spiked with levofloxacin (1) and (2) (B), and plasma sample obtained at 1.0 h after a single oral dose of 200 mg of levofloxacin from a healthy volunteer (C).

Table 1. Accuracy and precision from the determination of levofloxacin in human plasma (n = 18)

Concentration (μg/mL)	Mean ± SD (μg/mL)	RE (%)	RSD (%)	
			Intra-day	Inter-day
0.1	0.098 ± 0.008	-1.9	12.4	6.9
0.5	0.477 ± 0.023	-4.7	6.0	4.4
4.0	4.27 ± 0.16	6.9	7.3	2.9

Table 2. Stability data of levofloxacin in human plasma

Concentration	Mean ± SD (μg/mL)	RE (%) (μg/mL)	RSD (%)
Short-term stability for 24 h in plasma at room temperature			
0.1	0.105 ± 0.008	4.6	7.5
0.5	0.527 ± 0.009	5.4	1.6
4.0	3.99 ± 0.093	-0.3	2.3
Long-term storage at -20ºC for 30 days			
0.1	0.095 ± 0.005	-4.7	5.0
0.5	0.495 ± 0.029	-0.9	5.8
4.0	4.15 ± 0.262	3.9	6.3
Three freeze/thaw cycles			
0.1	0.092 ± 0.005	-8.2	5.7
0.5	0.490 ± 0.013	-2.0	2.6
4.0	4.15 ± 0.041	3.9	1.0

The calibration curves were linear over the concentration range of 0.05-5.0 μg/mL with a mean correlation coefficient of 0.9972. The mean (± SD) regression equation from replicate calibration curves on different days was: Y = (0.4677 ± 0.0294)X + (0.0045 ± 0.0083), where Y is the peak area ratio of levofloxacin to ciprofloxacin and X is the plasma concentration of levofloxacin. The lower limit of quantification with a coefficient of variation of less than 20% was 50 ng/mL.

3.3 *Precision and accuracy*

The coefficient variation values of both inter- and intra-day analysis were less than 12.4% whereas the relative error was less than 6.9%. The inter- and intra-day precision and accuracy values of the assay method are presented in Table 1.

3.4 *Recovery*

The mean extraction recoveries of levofloxacin at concentrations of 0.1, 0.5, and 4.0 μg/mL were 86.9 ± 6.1, 95.0 ± 4.9 and 93.3 ± 3.7%, respectively. The extraction recovery of the I.S. was 93.5 ± 4.5%.

3.5 *Stability*

Treated plasma samples were found to be stable at least 24 h when the samples were kept at room temperature (RE < 5.4%). The concentrations of levofloxacin in plasma which underwent three freeze-thaw cycles or storage at -20ºC for 30 days were found to be stable with relative error of less than 8.2%. The stability data of levofloxacin stored under various conditions and subjected to freeze-thaw cycles are shown in Table 2.

4. Discussion

4.1 *Preparation of plasma samples*

Protein precipitation has the advantages of simplicity and universality, so PPT was used to prepare the plasma samples.

Protein precipitation with methanol, acetonitrile, and perchloric acid has been investigated, but when a supernatant treated with methanol or acetonitrile was injected into HPLC system a poor peak was obtained. Direct protein precipitation with perchloric acid was simple and rapid and good separation of the drug and I.S. was achieved using the precipitation method. Results indicated that 0.6 M perchloric acid per 100 μL plasma successfully removed all of the protein in the plasma. The use of a lower amount of perchloric acid results in incomplete precipitation, but a higher amount may reduce chromatographic column lifetime. Thus, 0.6 M perchloric acid was selected as the protein precipitation

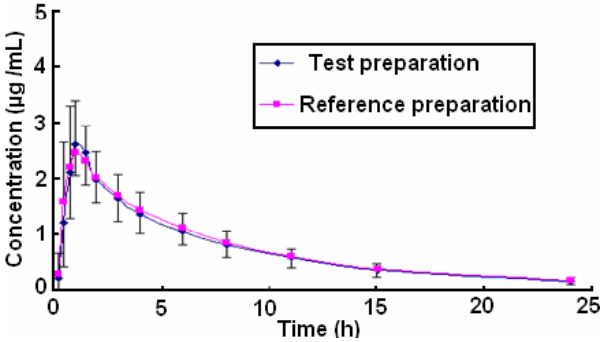

Figure 2. Mean plasma concentration-time curve of levofloxacin in a test with oral administration of a 200 mg levofloxacin hydrochloride or reference preparations to 20 healthy volunteers.

Table 3. Main pharmacokinetics parameters of levofloxacin (Mean ± SD, n = 20)

Pharmacokinetic parameters	Test preparation	Reference preparation
$AUC_{0\text{-}24}$ (μg·h/mL)	17.12 ± 3.83	17.55 ± 3.36
$AUC_{0\text{-}\infty}$ (μg·h/mL)	19.27 ± 4.15	19.26 ± 3.18
C_{max} (μg/mL)	2.95 ± 0.54	3.03 ± 0.87
T_{max} (h)	1.1 ± 0.3	1.1 ± 0.4
$t_{1/2}$ (h)	7.21 ± 3.01	7.69 ± 1.34
K_e (h^{-1})	0.09 ± 0.01	0.10 ± 0.01

agent.

4.2 *Optimization of mobile phase*

The chromatographic conditions were optimized by injecting analytes with mobile phase containing varying percentages of organic phase to achieve good resolution and symmetric peak shapes for levofloxacin and ciprofloxacin, as well as a short retention time. As expected, the retention times and resolutions of levofloxacin and the I.S. increased with decreasing acetonitrile percentage. The high organic solvent content shortened the chromatographic cycle time and an acidic modifier (phosphoric acid) in the mobile phase ensured stable chromatographic retention times for levofloxacin and ciprofloxacin. Triethylamine was added to improve peak shapes. Thus, optimal conditions were a mobile phase consisting of acetonitrile, water, phosphoric acid, and triethylamine (14:86:0.6:0.3, v/v/v/v). Under optimum conditions, the chromatographic run time for each sample was completed within 10 min.

4.3 *Advantages of the method*

In comparison to previously published HPLC methods for separation and quantitation of levofloxacin, the major modifications incorporated into the current method include: simple sample preparation procedures, common and cheap HPLC equipment and mobile phase additives, and a short analysis time as well. Thus the assay is suitable for routine analysis of large batches of biological samples to adjust drug dosage or perform pharmacokinetic studies. A simple, rapid, precise, and accurate HPLC method for determining levofloxacin in human plasma has been presented. Although lower sensitivity was obtained in comparison to previously published LC methods with fluorescence or mass spectrometry detection, the resulting LOQ (50 ng/mL) was sufficient for human pharmacokinetic studies.

4.4 *Bioequivalence evaluation*

The mean plasma concentration-time profiles and mean pharmacokinetic parameters after a test with a single oral dose of 200 mg and reference products administrated to twenty healthy male subjects are presented in Figure 2 and Table 3, respectively. Both the mean values and SD for the two preparations were close together. Pharmacokinetic parameters of the two preparations obtained from the statistical calculation exhibited bioequivalence.

5. Conclusion

An analytical method developed for levofloxacin quantification in plasma samples showed good specificity, sensitivity, linearity, precision, and accuracy over the entire range of clinically significant and therapeutically achievable plasma concentrations, thereby enabling its use in bioequivalence trials. Results of such a trial with the method revealed that test levofloxacin hydrochloride capsules from DiSha (WeiHai, China) and reference levofloxacin hydrochloride capsules from YangZiJiang (JiangSu, China) are bioequivalent.

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