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Recent advances in Chinese patent medicines entering the international market

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SUMMARY As an indispensable part of Traditional Chinese medicine (TCM), Chinese patent medicines have played an important role in preventing and treating diseases in China. Since they are easy to use, easy to store, and cost-effective, Chinese patent medicines have been generally accepted and widely used in Chinese clinical practice as a vital medical resource. In recent years, as TCM has developed and it has been accepted around the world, many Chinese patent medicine companies have gained international market access and successfully registered several Chinese patent medicines as over-the-counter (OTC) or prescription drugs in regions and countries that primarily use Western medicine such as the EU, Russia, Canada, Singapore, and Vietnam. Moreover, several Chinese patent medicines have been obtained the US Food and Drug Administration (FDA) approval conducting phase II or III clinical trials in the US. The current work has focused on several Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad. Summarized here are recent advances in the efficacy and molecular mechanisms of these Chinese patent medicines to treat respiratory infectious diseases (Lianhua Qingwen capsules, Jinhua Qinggan granules, and Shufeng Jiedu Capsules), cardiovascular and cerebrovascular diseases (Compound Danshen Dripping Pills, Huatuo Zaizao pills, and Tongxinluo Capsules), cancers (a Kanglaite injection and a Shenqi Fuzheng Injection), and gynecological diseases (Guizhi Fuling Capsules). The hope is that this review will contribute to a better understanding of Chinese patent medicines by people around the world.

Keywords Chinese patent medicines, global acceptance, respiratory infectious diseases, cardiovascular and cerebrovascular diseases, cancers, gynecological diseases

1. Introduction

Traditional Chinese medicine (TCM) is an ancient form of healthcare that has evolved over thousands of years in Asian countries, and especially China. During a long process of accumulating knowledge and practices, TCM has created a unique system of theories, diagnostics, and therapies regarding the prevention and treatment of diseases (*1*). In China, TCM is used daily to treat and prevent various diseases as part of the prevailing medical system. The typical TCM therapies mainly include Chinese herbal medicines, acupuncture, moxibustion, and massage. Chinese herbal medicines mainly include single herbs, prescriptions, and Chinese patent medicines. These TCM therapies have played an important role in the treatment of numerous ailments, providing effective treatments for various complex diseases such as cancer, ischemic stroke, and irritable bowel syndrome (*2,3*). A point particularly worth noting is that TCM has made great contributions to the prevention and treatment of coronavirus disease 2019 (COVID-19), and it has been used in over 90% of treatments across China (*4*). Several Chinese patent medicines in particular have displayed efficacy in combating COVID-19, such as Lianhua Qingwen capsules, Jinhua Qinggan granules, and Shufeng Jiedu capsules (*5*).

Despite the widespread use of TCM in clinical settings, proving its efficacy scientifically and assembling quality clinical evidence is still a challenge. Therefore, the quality of TCM-related clinical trials needs to be rationally and strictly controlled. To achieve the goals of better quality, safety, and efficacy, the national government and regulatory agencies of China are making every effort to encourage and promote the development of the TCM. A number of policies on TCM have been introduced, and a system to manage drug registration has essentially been established for Chinese herbal medicines (*6*).

The international recognition of TCM is increasing, especially since the 2015 Nobel Prize in Physiology or Medicine was awarded to Youyou Tu. On May 25, 2019, the World Health Organization (WHO) included TCM into the eleventh revision of the International Statistical Classification of Diseases and Related Health Problems (ICD), which is a landmark for both TCM and the ICD. This initiative not only recognizes TCM for its past contributions to world healthcare, but it also acknowledges, and attempts to meet, the current need for TCM (*7*). At present, TCM has spread to 196 countries and regions around the world, and 29 member countries of the WHO have enacted laws to regulate TCM. More than one-third of the world's total population has received TCM therapies (*8*). Chinese herbal medicine has gradually entered the international system of medicines, and several Chinese patent medicines have been registered overseas (*9*).

An indispensable part of TCM, Chinese patent medicine mainly refers to a special Chinese medicine preparation prepared from Chinese medicinal materials. There are many dosage forms of Chinese patent medicine, including pills, tablets, capsules, liquids, and injections. Compared to Chinese herbal decoctions, Chinese patent medicines have the advantages of convenient management and accurate dosage, which is why they have been generally accepted and widely used in Chinese clinical practice as a vital medical resource (*10*). According to the 2021 Blue Book on National Supervision of Traditional Chinese Medicine, there were 2,225 Chinese medicine companies and 8,670 varieties of Chinese patent medicine in China by the end of 2021, with an annual operating income of 486.2 billion yuan that year (*11*).

In recent years, many Chinese medicine companies have continuously explored and attempted to register medicines overseas and expand the international market. Thus far, several Chinese patent medicines have successfully been registered as drugs in several countries and regions including Russia, Canada, England, the Netherlands, and Vietnam (Table 1) (*12*). Moreover, as shown in Table 2, several Chinese patent medicine companies have submitted Investigational New Drug (IND) applications to the US Food and Drug Administration (FDA), some of which are carrying out phase II or III clinical trials in the US (*https://clinicaltrials.gov/*).

The current review will focus on these Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad. The mechanisms and of these Chinese patent medicines and clinical trials indicating their efficacy in treating respiratory infectious diseases, cardiovascular and cerebrovascular diseases, cancers, and gynecological diseases will be highlighted and summarized. A search of the literature will be mainly conducted in the

Table 1. Product characteristics of Several Chinese patent medicines registered in some overseas countries and regions

Product name	Components	Main indications	Registration country	Year of approval
Huatuo Zaizao pill	Ligusticum chuanxiong, Tetradium ruticarpum, Borneolum and so on.	Cerebrovascular diseases	Russia	2010
Di'ao Xinxuekang capsule	Discorea nipponica Makino.	Cardiovascular disease	Netherlands	2012
Tongxinluo capsule	Panax ginseng, Hirudo nipponica Whitman, Scolopendra subspinipes mutilans, Eupolyphaga sinensis Walker, Buthus martensii Karsch, Cryptotympana pustulata Fabricius, Paeonia lactiflora, Borneolum, Santalum album, Burseraceae, Dalbergia odorifera, and Ziziphus jujuba Mill	Cardiovascular and cerebrovascular diseases	Vietnam	2013
Kangbingdu Koufuye	Radix Isatidis, Gypsum Fibrosum, Phragmites australis, Rehmannia glutinosa, Curcuma wenyujin, Anemarrhena asphodeloides Bunge, Acorus tatarinowii, Herba Pogostemonis and Fructus Forsythiae.	Influenza and wind heat cold	Canada	2015
Danshen Capsule	Salvia milthiorrhiza.	Mild menstrual pains	Netherlands	2016
Danning Pian (Biliflow)	Herba Taraxaci, Lysimachia christiniae Hance, Corydalis yanhusuo, Rheum palmatum, Mentha canadensis Linnaeus, Calculus Bovis Artifactus, Silymarin and Berberine.	Chronic cholecystitis and cholelithiasis	Canada	2016
Banlangen Keli	Radix Isatidis.	Common cold	United Kingdom	2017
Lemai Keli	Salviae miltiorrhizae, Tetradium ruticarpum, Paeonia lactiflora, Carthamus tinctorius, Cyperus Rotundus, Costustoot and Crataegus pinnatifida.	Cardiovascular and cerebrovascular diseases	Canada	2017
Xiaoyao Pian	Bupleurum chinense, Paeoniae Radix, Angelica sinensis, Atractylodes macrocephala, Poria Cocos, Glycyrrhiza uralensis and Mentha canadensis Linnaeus.	Irregular menstruation	Netherlands	2020

Table 2. Product characteristics of Several Chinese patent medicines which have been approves by the US Food and Drug Administration (FDA) carrying out Phase II or III clinical trails in the United States

Product name/Clinical registration No.	Conditions	Stage	Study Status	Start time
Lianhua Qingwen capsule				
NCT02867358	Acute uncomplicated influenza	Phase II	Ongoing	2016
Jinhua Qinggan granule				
NCT04723524	Mild-category patients of COVID-19	Phase II	Finished	2020
Compound Danshen Dripping Pill				
NCT03270787	Acute mountain sickness	Phase II	Finished	2016
NCT01659580	Angina pectoris	Phase III	Finished	2012
NCT02388984	Non-proliferative diabetic retinopathy	Phase III	Unknown	2013
NCT01825759	Coronary heart disease with essential hypertension	Phase III	Unknown	2013
NCT05295329	Diabetic patients with coronary microcirculation disturbance	Unknown	Not yet recruiting	2022
Huatuo Zaizao pill				
NCT01758536	Acute ischemic stroke	Phase IV	Unknown	2012
NCT04910256	Ischemic Stroke	Phase III	Unknown	2021
Tongxinluo Capsule				
NCT01919671	Acute ischemic stroke	Phase IV	Finished	2014
NCT04220372	Microvascular angina pectoris	Phase IV	Ongoing	2020
NCT01721590	Coronary artery disease	Phase IV	Finished	2012
NCT03792035	Acute myocardial infarction	Phase IV	Unknown	2019
NCT05309343	Cardiovascular diseases	Unknown	Recruiting	2021
NCT04026724	Angina pectoris	Unknown	Unknown	2019
NCT04022031	Atherosclerotic heart disease with angina pectoris	Unknown	Unknown	2019
Kanglaite injection				
NCT03101514	Head and neck cancer with radiotherapy	Phase II	Finished	2017
NCT02553187	Cancer cachexia	Phase IV	Unknown	2015
NCT03631459	Cancer cachexia	Unknown	Unknown	2018
NCT03986528	Advanced non-small cell lung cancer	Phase IV	Recruiting	2019
NCT00733850	Pancreatic cancer	Phase II	Finished	2008
Shenqi Fuzheng Injection				
NCT03456609	Carcinogenic fatigue	Unknown	Unknown	2018
NCT03455205	Cancer-related fatigue	Unknown	Unknown	2017
Guizhi Fuling Capsule				
NCT01588236	Primary dysmenorrhea	Phase II	Unknown	2012

databases of PubMed, Web of Science, Scopus, Springer, ScienceDirect, and the China Hospital Knowledge Database (CHKD). Overall, the hope is that this review will contribute to a better understanding of Chinese patent medicines by people around the world as TCM gains greater global acceptance.

2. Chinese patent medicines for respiratory infectious diseases

Respiratory infectious diseases are mainly caused by viruses or bacteria and are often contagious, posing a major public health problem worldwide. Over the past 3,000 years of Chinese history, TCM has worked well in treating respiratory infectious diseases. TCM has been used as the routine treatment of pandemic and endemic diseases including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), COVID-19, and seasonal epidemics caused by the influenza and dengue viruses (*13*). A point particularly worth noting is that several Chinese patent medicines such as Lianhua Qingwen capsules, Jinhua Qinggan granules, and Shufeng Jiedu Capsules have significant advantages in treating respiratory infectious diseases. Summarized here are recent advances in the efficacy (Table 3, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) and molecular mechanisms (Table 4, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) of these key common Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad.

2.1. Lianhua Qingwen capsules

Lianhua Qingwen capsules, a Chinese patent medicine, were originally developed to treat SARS in 2003 and were approved by the China Food and Drug Administration (CFDA) in 2004. Lianhua Qingwen capsules come from two classic and effective prescriptions, Maxing-Shigan-Tang and Yinqiao-San. The capsules have 13 herb components including Fructus Forsythiae (Lianqiao) and Flos Lonicerae Japonicae (Jinyinhua) (Figure 1). The main active ingredients of Lianhua Qingwen capsules have been identified and include quercetin, kaempferol, luteolin, β-sitosterol, indigo, wogonin, and tryptanthrin (*14*).

Over the last few decades, Lianhua Qingwen capsules have been widely used to treat viral influenza, pneumonia caused by coronaviruses, the common cold, and other diseases. The capsules are effective as a broad-spectrum antiviral, anti-bacterial, antipyretic and anti-inflammatory, they reduce coughing and expectoration, and they regulate immunity in the respiratory system (*15*). A series of clinical trials and basic studies on Lianhua Qingwen capsules in the treatment of SARS, influenza,

Figure 1. Composition of Lianhua Qingwen capsules that can be used to treat respiratory infectious diseases.

and COVID-19 have been conducted in China and other countries. Moreover, they have successfully entered a phase II clinical trial (NCT02867358) on treating acute uncomplicated influenza in the US (*https://clinicaltrials.gov/*). Lianhua Qingwen capsules are beginning to gain global acceptance. Thus far, Lianhua Qingwen capsules have gradually entered the international market and are successfully registered in 29 countries and regions.

SARS was responsible for the first pandemic of the 21st century, which was caused by SARS-COV in November 2002 in Guangdong Province, China (*16*). The disease was highly contagious and fatal, and the pandemic lasted until July 2003, affecting more than 8,000 people and causing 774 deaths in 26 countries on five continents. After the outbreak of SARS, Lianhua Qingwen capsules were quickly developed and used clinically, playing a significant role in China. Since SARS only lasted for 8 months, there was a lack of high-quality clinical trials on Lianhua Qingwen capsules.

Influenza is an acute respiratory infection caused by a negative-strand RNA virus of the Orthomyxoviridae family. There are three types of influenza viruses that infect humans: influenza A, B, and C. H1N1 and H7N9 are infamous subtypes of type A influenza. In 2009, a new H1N1 strain first identified in Mexico spread widely and caused millions of cases worldwide, with an estimated 18,500 laboratory-confirmed deaths (*17*). Lianhua Qingwen displayed anti-influenza activity clinically during the 2009 H1N1 pandemic in China. Wu *et al*. indicated that Lianhua Qingwen granules combined with a peramivir sodium chloride injection displayed marked potency in patients with influenza (*18*). It reduced the treatment time and increased levels of the inflammatory factors IL-6, CRP, and PCT. The overall efficacy in the experimental group was significantly higher than that in the control group (96.0% *vs.* 80%, $P = 0.014$). Yang *et al*. found that Lianhua Qingwen capsules inhibit early-stage replication of the influenza virus and prevent a severe inflammatory response (*19*). Levels of RANTES, IL-6, IL-8, IP-10, TNF-α, MCP-1, MIP-1β, and IFN-λ expression decreased *in vitro*, while *in vivo* results indicated that Lianhua Qingwen capsules did not reduce the lung viral load or mortality due to the influenza virus in mice, but the pathological changes in the lungs were alleviated with fewer inflammatory cells in the lungs. Moreover, Lianhua Qingwen capsules can be used as an adjunct to enhance the efficacy of oseltamivir in treating influenza. In addition, Gao *et al*. indicated that Lianhua Qingwen had a distinct metabolic influence on the treatment of influenza pneumonia, mainly targeting COX-2 in the arachidonic acid metabolism pathway (*20*).

COVID-19 broke out in 2019 and spread rapidly around the world, causing a global pandemic. As of August 15, 2022, nearly 600 million confirmed cases of COVID-19 have been documented globally. Unfortunately, there is still no specific drug that can cure COVID-19. Lianhua Qingwen capsules may be successful in preventing and treating infectious diseases. A meta-analysis of three trials (one randomized controlled trial (RCT) and two retrospective case-control studies) involving 245 COVID-19 patients indicated that Lianhua Qingwen capsules had significant efficacy in alleviating clinical symptoms such as a fever, cough, and fatigue and in curbing progression to severe or critical disease (*21*). Another meta-analysis of five RCTs involving 830 patients with mild or moderate COVID-19 indicated that the combination of Lianhua Qingwen capsules and conventional therapy was significantly associated with better clinical efficacy, a higher rate of improvement on chest computed tomography (CT), and a lower rate of conversion to severe cases (*22*).

However, the potential mechanism of Lianhua Qingwen capsules in treating COVID-19 remains unclear. In a pharmacology network-based study, Xia *et al*. investigated the potential therapeutic mechanisms of Lianhua Qingwen capsules in COVID-19 (*23*). They indicated that Lianhua Qingwen capsules made an indispensable contribution to preventing and curing COVID-19 by improving the inflammatory response and regulating cell apoptosis and the immune defence. They speculated that Akt1 might be a promising drug target for COVID-19. Another network pharmacology analysis indicated that Lianhua Qingwen capsules

modulated the inflammatory process, had antiviral action, and repaired lung injury caused by COVID-19 (*24*). Moreover, the capsules were also able to alleviate the cytokine storm and symptoms caused by abnormal angiotensin converting enzyme 2 (ACE2) (a SARS-CoV receptor, possibly the viral entry point in alveolar lung cells) expression. In addition, Li *et al*. found that Lianhua Qingwen inhibited SARS-CoV-2 replication in Vero E6 cells and reduced the production of pro-inflammatory cytokines (TNF-α, IL-6, CCL-2/MCP-1, and CXCL-10/IP-10) at the mRNA level, further resulting in the abnormal particle morphology of the virion in cells (*25*). These studies might provide theoretical substantiation of the use of Lianhua Qingwen capsules in the treatment of COVID-19. The exact mechanism and efficacy of Lianhua Qingwen capsules still need to be elucidated further using molecular biological techniques.

2.2. Jinhua Qinggan granules

Jinhua Qinggan granules, a Chinese patent medicine, were originally developed to treat H1N1 in 2009 and were approved by the CFDA in 2016. Jinhua Qinggan granules were created from the two classical TCM formulae Maxing-Shigan-Tang and Yinqiao-San, and they contain 11 herbs including Flos Lonicerae Japonicae (Jinyinhua), Gypsum Fibrosum (Shigao), and Ephedra Herba (Ma huang) (Figure 2). The main chemical components of Jinhua Qinggan granules include chlorogenic acid, mangiferin, forsythoside A, baicalin, arctiin, wogonoside, and ammonium glycyrrhizinate (*26*). Since they were developed, Jinhua Qinggan granules have become the main force in fighting respiratory infectious diseases. In the past, Jinhua Qinggan granules have been utilized in the treatment of H1N1 influenza, and they have displayed marked efficacy in alleviating symptoms and promoting recovery in patients with influenza (*27*). Since the outbreak of COVID-19, Jinhua Qinggan granules have displayed marked efficacy in the fight against SARS-CoV-2, and they have been recommended as a medication in the Diagnosis and Treatment Protocol for COVID-19 in China. Moreover, they have successfully entered a phase II clinical trial (NCT04723524) on treating COVID-19 in the US (*https://clinicaltrials.gov/*), which means that Jinhua Qinggan granules are beginning to gain global acceptance.

Shah *et al*. conducted a phase 2/3, double-blind, randomized, placebo-controlled trial to evaluate the efficacy and safety of treatment with Jinhua Qinggan granules in non-hospitalized patients with laboratory-confirmed mild COVID-19 (*28*). They found that Jinhua Qinggan granules displayed better clinical efficacy (82.67%) compared to a placebo (10.74%), and the recovery time from symptoms such as a cough, phlegm, a sore throat, and headaches in patients receiving Jinhua Qinggan granules was shorter than that in patients

Figure 2. Composition of Jinhua Qinggan granules that can be used to treat respiratory infectious diseases.

receiving a placebo (6 days *vs.* more than 11 days). However, there was no significant difference in SARS-CoV-2 negativity in both groups. In a retrospective study conducted at an old age home in Hong Kong, Lin *et al*. presented preliminary evidence that Jinhua Qinggan granules potentially reduce hospitalization and mortality in elderly patients with COVID-19 who were partially vaccinated and who were at risk of progression to severe disease (*29*). An *et al*. indicated that Jinhua Qinggan granules combined with Western medicine relieved the clinical symptoms of a fever and poor appetite in patients with COVID-19, reducing the use of antibiotics to a certain extent (*30*).

However, the potential mechanism of Jinhua Qinggan granules in treating COVID-19 remains unclear. Kageyama *et al*. found that the clinical benefits of the use of Jinhua Qinggan granules to treat COVID-19 might be associated with their rapid immunomodulatory effects on IL-6, IFN-γ, and the neutrophil/lymphocyte ratio (NLR) (*31*). Their findings indicated that Jinhua Qinggan granules might be suitable not only for suppressing disease onset in suspected and asymptomatic cases but also at preventing disease progression in patients with a mild to severe infection.

2.3. Shufeng Jiedu capsules

Shufeng Jiedu Capsules, a Chinese patent medicine, were originally developed to treat H1N1 in 2009 and were approved by the CFDA in the same year. They were created from a Chinese folk prescription and consist of 8 herbs including Rhizoma Polygoni cuspidati (Huzhang), Fructus Forsythiae (Lianqiao), and Radix

Figure 3. Composition of Shufeng Jiedu Capsules that can be used to treat respiratory infectious diseases.

Isatidis (Banlangen) (*32*) (Figure 3). Shufeng Jiedu Capsules are mainly used to treat upper respiratory tract infections such as the flu, swelling and pain in the throat, mumps, and strep throat with anti-inflammatory, immunomodulating, and antiviral properties.

Since 2009, Shufeng Jiedu Capsules have been used in China to treat H1N1 influenza based on governmental recommendations (NHC 2010), and they represent first-line TCM for the prevention and treatment of respiratory infectious diseases. Xia *et al*. conducted a meta-analysis of 13 RCTs involving 1,036 patients with acute exacerbations of chronic obstructive pulmonary disease (AECOPD) (*33*). Results suggested the positive effects of Shufeng Jiedu Capsules in combination with antibiotics and symptomatic treatments in reducing treatment failure and the duration of hospitalization and alleviating symptoms. Moreover, a phase III clinical study on Shufeng Jiedu Capsules to help reduce antibiotic use in AECOPD is being conducted in England (ISRCTN26614726) (*https://www.isrctn.com/)*.

Since the outbreak of COVID-19, Shufeng Jiedu Capsules have displayed marked efficacy in the fight against SARS-CoV-2, and they have been recommended as a medication in the Diagnosis and Treatment Protocol for COVID-19 in China. Moreover, Shufeng Jiedu Capsules have been registered in Hong Kong (Registration No. PR025534) and Macao (Registration No. MAC-C00004), China, for the treatment of COVID-19. Chen *et al*. indicated that Shufeng Jiedu Capsules combined with Arbidol to treat common COVID-19 reduced the duration of symptoms and increased clinical efficacy without causing serious adverse reactions (*34*). Zhang *et al*. indicated that Shufeng Jiedu Capsules are capable of alleviating a sore throat, coughing, fatigue and a fever in patients infected with Omicron by inhibiting viral replication (*35*). The clinical cure rate was significantly higher in patients receiving Shufeng Jiedu Capsules than in the control group (76.9% *vs.* 64.1%, $p = 0.032$).

Despite the definite evidence of effective use of Shufeng Jiedu Capsules in the treatment of pneumonia caused by the influenza virus and SARS-CoV-2, the underlying mechanism of action remains unknown. A series of network pharmacology studies and cell/animal experiments have been conducted to explore the mechanisms of Shufeng Jiedu Capsules. Shufeng Jiedu Capsules combined with oseltamivir significantly attenuated influenza A virus-induced lung damage by reducing IL-1β and IL-18 levels in serum and bronchoalveolar lavage fluid (BALF), and they inhibited the expression of NLRP3-associated components and viral titers in lung tissues of rats (*36*). In another *in vivo* study, Shufeng Jiedu Capsules significantly reduced the viral load in the lungs of mice infected with HCoV-229E, they decreased the inflammatory factors IL-6, IL-10, TNF-α, and IFN-γ in the lungs, and they increased the amount of CD^{4+} and CD^{8+} cells in the blood compared to the model group (*37*). Several network pharmacology analyses revealed that the preventive and therapeutic effect of Shufeng Jiedu Capsules on COVID-19 might involve modulation of the immune system and anti-inflammatory and anti-viral action *via* quercetin, luteolin, kaempferol, baicalein, and other active ingredients on key targets such as IL-6, IL-10, TNF, NF-κB, and PI3K-Akt (*38,39*).

3. Chinese patent medicines for cardiovascular and cerebrovascular diseases

Cardiovascular and cerebrovascular diseases are common diseases related to blood vessels of the heart and cerebrum and mainly include heart failure, atherosclerosis, myocardial infarction, arrhythmia, hypertension, and stroke. Although prevention and treatment have improved in recent years, cardiovascular and cerebrovascular diseases remain a leading cause of disability and mortality worldwide. Recent studies have suggested that TCMs such as Compound Danshen Dripping Pills (Fufang Danshen Diwan), Huatuo Zaizao pills, and Tongxinluo Capsules might be a candidate for the preventive treatment of those diseases. TCM treatment was most likely to be used by patients with a long history of CHD or with a history of stroke. Summarized here are the recent advances in the efficacy (Table 5, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) and molecular mechanisms (Table 6, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) of these Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad.

3.1. Compound Danshen Dripping Pills

Compound Danshen dripping pills are a Chinese

patent medicine that was approved by the CFDA in 1995, and they are commonly used to treat coronary heart disease (CHD), and especially angina pectoris, in China. They are also used to treat diabetic retinopathy and acute high-altitude exposure. Compound Danshen dripping pills were the first Chinese patent medicine to complete a phase II clinical trial (NCT00797953) on treating chronic stable angina in the US, and a phase III clinical trial (NCT03789552) on treating stable angina is underway (*https://clinicaltrials.gov/*). The pills are prepared from Salviae miltiorrhizae (Danshen), Panax notoginseng (Sanqi), and Borneolum (Bingpian) by modern techniques, and they activate blood circulation and eliminate blood stasis (Figure 4A). The main active ingredients of Compound Danshen dripping pills have been identified and include Danshensu, protocatechuic aldehyde, salvianolic acid, notoginsenoside, and ginsenoside (*40*).

Recently, a series of systematic reviews have been conducted to assess the clinical efficacy of Compound Danshen dripping pills in patients with CHD. Li *et al*. conducted a meta-analysis of 12 RCTs involving 2,574 patients with CHD to systematically evaluate the efficacy of Compound Danshen dripping pills combined with percutaneous coronary intervention (PCI) in patients with CHD (*41*). They indicated that Compound Danshen dripping pills combined with PCI markedly reduced the incidence of major adverse cardiac events (MACE), improved cardiovascular function, and inhibited inflammation in patients with CHD. Luo *et al*. conducted a meta-analysis of 13 systematic reviews involving 34,071 patients with angina or acute myocardial infarction (AMI) to summarize the evidence from systematic reviews on the efficacy and safety of Compound Danshen dripping pills in patients with CHD (*42*). They concluded that the potential benefits of Compound Danshen dripping pills in patients with CHD included alleviating symptoms, improving ECG results, and causing few adverse reactions. In addition, Liang *et al*. conducted a meta-analysis of 21 RCTs involving 2,356 patients with unstable angina pectoris to evaluate the clinical efficacy and safety of Compound Danshen Dripping pills and isosorbide mononitrate (ISMN) in the treatment of unstable angina pectoris in the elderly (*43*). They indicated that Compound Danshen dripping pills were superior to ISMN in the treatment of elderly patients with unstable angina pectoris based on a comparison of clinical efficacy, ECG efficacy, blood viscosity, and other indicators.

Although Compound Danshen dripping pills have performed well in the treatment of CHD, their mechanism had not been clearly discussed. In rats with acute myocardial ischemia, Compound Danshen dripping pills provided an excellent cardioprotective effect by reversing echocardiographic abnormalities, attenuating histopathological lesions, and ameliorating circulating myocardial markers and inflammatory cytokines by simultaneously modulating the MAPK, PI3K/AKT, and PPAR pathways (*44*). In a mouse model of microvascular dysfunction, pre-treatment with Compound Danshen dripping pills prevented lipid infusion-induced systemic microvascular disorders including coronary and peripheral microvascular dysfunction, and their possible mechanisms involved down-regulated FOXO1 and decreased leukocyte adhesion (*45*).

Figure 4. Compositions of Compound Danshen Dripping Pills (A) and Huatuo Zaizao pills (B) that can be used to treat cardiovascular and cerebrovascular diseases.

3.2. Huatuo Zaizao pills

Huatuo Zaizao pills, a Chinese patent medicine, are listed as the preferred drug to treat stroke in the Chinese Pharmacopoeia (Pharmacopoeia Commission of the People's Republic of China, 2015). They are commonly used to treat cerebrovascular diseases and promote rehabilitation after stroke. A phase III clinical trial (NCT04910256) that evaluated the efficacy and safety of Huatuo Zaizao pills in patients after an ischemic stroke has been conducted in the US (*https://clinicaltrials.gov/*). Moreover, Huatuo Zaizao pills have been registered as a drug and sold in 29 countries or regions around the world, and they have ranked among the top exports of Chinese medicine for more than 10 years consecutively. They are a proprietary prescription prepared from ingredients such as Ligusticum chuanxiong (Chuanxiong), Tetradium ruticarpum (Wuzhuyu), and Borneolum (Bingpian), and they act by alleviating disrupted micro-circulation and by preventing cerebral thrombosis (Figure 4B) (*46*).

Over the past few years, a series of studies on the pharmacognosy, pharmaceutics, and pharmacology of Huatuo Zaizao pills have mostly been published in the Chinese medical literature and have only been partly published in English. Despite the fact that Huatuo Zaizao pills are administered to promote the rehabilitation of stroke patients, the mechanisms of their pharmacological action are still unclear. Combined administration of Huatuo Zaizao pills and aspirin prolonged the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) and it decreased

whole blood viscosity (WBV) and plasma viscosity (PV) in rabbits, potently affecting hemorrheology and blood coagulation (*47*). Huatuo Zaizao pills treatment increases the expression of brain-derived neurotrophic factor (BDNF) and the level of neurogenesis in animals with cerebral ischemia-reperfusion (I/R) injury, which may be associated with functional recovery after stroke (*46*). In addition, Zhang *et al*. investigated the effect of Huatuo Zaizao pills on hippocampal synaptic function and amyloid-β (Aβ) deposition in transgenic mice with Alzheimer's disease (AD) (*48*). They indicated that Huatuo Zaizao pills ameliorated hippocampus-dependent memory deficits and improved synaptic dysfunction by reversing long-term potentiation (LTP) impairment in APP/PS1 AD transgenic mice. Moreover, Huatuo Zaizao pills reduced amyloid plaque deposition by regulating α-secretase and γ-secretase levels.

3.3. Tongxinluo Capsules

Tongxinluo Capsules are a Chinese patent medicine that was approved by the CFDA in 1996. They are commonly used to treat ischemic cerebrovascular diseases and angina pectoris in China. They mainly consist of 12 traditional Chinese herbs including Panax ginseng (Renshen), Hirudo nipponica Whitman (Shuizhi), Scolopendra subspinipes mutilans (Wugong), Eupolyphaga sinensis Walker (Tubiechong), Buthus martensii Karsch (Quanxie), Cryptotympana pustulata Fabricius (Chantui), Paeonia lactiflora (Chishao), Borneolum (Bingpian), Santalum album (Tanxiang), Burseraceae (Ruxiang), Dalbergia odorifera (Jiangxiang), and Ziziphus jujuba Mill (Suanzaoren). The capsules promote blood circulation and eliminate blood stasis (*49*) (Figure 5). Modern pharmacological studies have indicated that Tongxinluo capsules can improve the function of vascular endothelial cells, reduce blood viscosity, regulate blood lipids, improve hemorheology, and stabilize atherosclerotic plaques (*50*).

Recently, a series of large-scale RCTs have been conducted to evaluate the clinical efficacy of Tongxinluo capsules for cardiovascular and cerebrovascular diseases. Zhang *et al*. conducted a multi-center, randomized, double-blind, parallel-group, placebo-controlled clinical trial (CAPITAL) involving 1,212 patients with sub-clinical carotid atherosclerosis to determine whether Tongxinluo capsules were efficacious at retarding the progression of carotid atherosclerotic lesions (*51*). They indicated that treatment with Tongxinluo capsules in addition to routine therapy retarded the progression of mean intima-media thickness, plaque area, and vascular remodeling of the carotid arteries in patients with sub-clinical carotid atherosclerosis with a good safety profile. Li *et al*. conducted a meta-analysis of 16 RCTs involving 1,877 patients with AMI to evaluate the potential efficacy and safety of Tongxinluo capsules for secondary prevention in patients with AMI (*50*).

Figure 5. Composition of Tongxinluo Capsules that can be used to treat cardiovascular and cerebrovascular diseases.

They found that Tongxinluo capsules seemed to be beneficial for secondary prevention after AMI, allowing improvement of cardiac function (LVEF), regulation of lipid metabolism (TC, TG, and LDL), and inhibition of an inflammatory reaction (hs-CRP). The main adverse reaction was only gastrointestinal discomfort. Another meta-analysis suggested that Tongxinluo capsules as supplementation were associated with a significantly reduced risk of target vessel revascularization (TVR) or in-stent restenosis (ISR) after coronary revascularization and that the capsules might reduce the incidence of first or recurrent myocardial infarction (MI) and first or worsened heart failure (HF) within 12 months in patients with CHD without serious adverse reactions (*52*). Moreover, Tongxinluo capsules combined with atorvastatin effectively improved the clinical treatment of CHD, significantly reduced the frequency and duration of angina pectoris, decreased blood lipids, and improved inflammatory factors (*53*). In addition, the addition of Tongxinluo capsules to routine medication was beneficial to the treatment of a transient ischemic attack (TIA), significantly improving clinical efficacy and hemorheological characteristics, reducing blood viscosity, and promoting microcirculation (*54*).

Tongxinluo capsules have performed well in the treatment of cardiovascular and cerebrovascular diseases in clinical settings, and their mechanisms have also been actively studied. Tongxinluo capsules provided protection against blood-brain barrier (BBB) disruption after ischemic stroke (*55*). They inhibited the low-density lipoprotein receptor-related protein 1 (LRP-1) pathway, up-regulated the level of tight junction

(TJ) proteins such as occludin, claudin-5, and ZO-1, and subsequently protected against disruption of the BBB after cerebral ischemia in mice with permanent middle cerebral artery occlusion (pMCAO). In a rat model of cerebral ischemia/reperfusion (I/R) injury, Tongxinluo capsules effectively protected against I/R injury and reduced cell death *via* the Cx43/Calpain II/Bax/Caspase-3 pathway, helping to prevent and treat I/R injury (*56*). Moreover, in a rat model of intestinal I/R injury, pretreatment with Tongxinluo capsules significantly prevented the I/R-induced pathology of endothelial apoptosis, disruption of microvascular integrity, and the inflammatory reaction (57). Furthermore, Tongxinluo capsules might be as effective as simvastatin in stabilizing atherosclerotic plaques and they might provide an alternative therapy for atherosclerosis (*58*). They significantly reduce serum levels of lipids and inflammatory markers, attenuate the lipid content of plaque and the inflammatory reaction, and lower the incidence of plaque in a rabbit model of atherosclerosis.

4. Chinese patent medicines for cancers

Cancer has become a leading cause of death and an important obstacle to increasing life expectancy in every country of the world. Based on the Global Cancer Observatory (GLOBOCAN) registry, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020 worldwide (*59*). Moreover, an estimated 28.4 million new cancer cases are projected to occur in 2040, a 47% increase from the corresponding 19.3 million cases in 2020. Thus, cancer prevention and treatment remain a major challenge for the world in the coming years. In recent decades, TCM has been increasingly used and has become well-known for its significant role in preventing and treating cancer. It is widely used by TCM physicians and other health care providers to alleviate the symptoms of patients with cancer and to control the adverse reactions to and the toxicities of cancer therapies, thus improving patient QOL, preventing recurrence, and prolonging survival (*1*). A point particularly worth noting is that several Chinese patent medicines such as a Kanglaite injection and a Shenqi Fuzheng Injection have significant advantages in treating cancers. Summarized here are the recent advances in the efficacy (Table 7, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) and molecular mechanisms (Table 8, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) of several Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad. These Chinese patent medicines include a Kanglaite injection and a Shenqi Fuzheng Injection.

4.1. A Kanglaite injection

Figure 6. Compositions of a Kanglaite injection (A) and a Shenqi Fuzheng Injection (B) that can be used to treat cancers.

A Kanglaite injection, as an acetone extract of Semen Coicis Yokuinin (Figure 6A), is prepared as an herbal medicine using modern and advanced pharmaceutical technology. It is widely used to treat lung and liver cancer or complications of cancers, such as malignant pleural effusion, in China. In recent years, a Kanglaite injection has been approved by other countries such as Russia and the US. Since 2005, a Kanglaite injection has been approved as a prescription to treat cancer in clinical settings by the Ministry of Health of the Russian Federation. In addition, it has passed the review of the US FDA, and a phase III clinical trial is being conducted in the US.

A series of RCTs and systematic reviews have indicated that a Kanglaite injection is an effective adjunctive therapy for cancers. An overview was conducted by Lu *et al*. to map the evidence landscape based on 13 systematic reviews with meta-analyses published from 2004 to 2021 focusing on a Kanglaite injection to treat cancers and related conditions (*60*). They indicated that a Kanglaite injection was effective and safe as an adjunctive treatment for non-small cell lung cancer (NSCLC), malignant pleural effusion, and digestive system malignancies (such as hepatocellular carcinoma (HCC)). Adjunctive therapy with a Kanglaite injection improves clinical indices such as overall survival (OS), the disease response rate, and QOL, and it improves the values of immune indicators including the number of CD^{4+} lymphocytes and the CD^{4+}/CD^{8+} ratio. Moreover, a Kanglaite injection reduces the incidence of various adverse reactions caused by conventional chemotherapy drugs, such as gastrointestinal reactions, thrombocytopenia, and leukopenia. Another overview was conducted based on 20 systematic reviews with meta-analyses focusing on a Kanglaite injection to treat NSCLC (*61*). Results suggested that a Kanglaite injection whether combined with chemotherapy, radiotherapy, or targeted therapy, has an effect on the objective response rate (ORR) and QOL and decreases adverse reactions. Moreover, a Kanglaite injection combined with gefitinib

might increase the ORR, improve performance status, and increase the number of CD^{4+} cells and NK cells and the ratio of CD^{4+}/CD^{8+} but it did not reduce the toxicity of gefitinib or increase the number of CD^{3+} cells and CD^{8+} cells in patients with NSCLC (*62*).

A Kanglaite injection has performed well in the treatment of cancer in clinical settings, and its mechanism has also been actively studied. Shi *et al*. indicated that Kanglaite inhibited TNF-α-mediated epithelial mesenchymal transition (EMT) in colorectal cancer cell lines and a subcutaneous tumor model by inhibiting NF-κB signaling (*63*). Moreover, Kanglaite reversed the multidrug resistance (MDR) of human HCC by inducing apoptosis and cell cycle arrest *via* the PI3K/AKT signaling pathway (*64*). In addition, pre-treatment with Kanglaite increased the effects of cisplatin (DDP) on HepG2 cells, and it had a synergistic effect on the regulation of inflammation and chemo-resistance (*65*). The underlying mechanisms of pre-treatment with Kanglaite might involve the suppression of chemokine-like factor 1 (CKLF1) mediated NF-κB signaling and ATP-binding cassette drug efflux transporters in HepG2 cells.

4.2. A Shenqi Fuzheng Injection

A Shenqi Fuzheng injection is a Chinese patent medicine approved by the CFDA in 1999. It consists of two traditional Chinese herbs, Radix Astragali (Huangqi) and Codonopsis Pilosula (Dangshen) at a ratio of 1:1 (Figure 6B), and it is commonly used to improve immune function in chronic diseases including cancer and cerebrovascular diseases such as, angina, coronary heart disease, and heart failure (*2,66*). Recently, a number of trials have indicated that a Shenqi Fuzheng injection might play an important role in the treatment of various advanced cancers such as NSCLC and gastric cancer. Since 2017, it has passed the review of the US FDA, and a phase III clinical trial is being conducted in the US. This means that a Shenqi Fuzheng injection is beginning to gain global acceptance.

Over the past decade, a series of RCTs and systematic reviews have indicated that a Shenqi Fuzheng injection combined with chemotherapy, radiotherapy, or targeted therapy to treat various types of cancers is capable of improving clinical efficacy, immune function, and performance status and of reducing toxicity. A meta-analysis of 13 RCTs indicated that a Shenqi Fuzheng injection combined with chemotherapy yielded positive results in treating advanced gastric cancer in terms of the rate of complete remission (CR) and partial remission (PR), body weight, and decreased adverse reactions including grade 3-4 nausea and vomiting, grade 1-2 oral mucositis, grade 3-4 leucopenia, and grade 1-2 myelo-suppression (*67*). Another meta-analysis of 49 RCTs indicated that a Shenqi Fuzheng injection and conventional chemotherapy exhibited better efficacy, with a significantly better objective tumor response (*68*). Combined treatment with a Shenqi Fuzheng injection increased the number of NK, CD^{3+}, and CD^{4+} cells and the CD^{4+}/CD^{8+} ratio compared to conventional chemotherapy, suggesting that a Shenqi Fuzheng injection was efficacious in alleviating immune system damage caused by chemotherapy. Moreover, a Shenqi Fuzheng injection alleviated radiation-induced brain injury (*69*). It reduced impaired cognitive function, and especially memory impairment after brain radiation, by regulating the expression of inflammatory factors such as TGF-β1, TNF-α, and IL-10. A Shenqi Fuzheng injection also improved clinical efficacy and decreased radiation toxicity by regulating T-cell immune function in patients with NSCLC undergoing radiotherapy (*70*). In addition, a Shenqi Fuzheng injection combined with a first-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) markedly prolonged progression-free survival (PFS) and attenuated adverse reactions in patients with advanced NSCLC with EGRET-sensitive mutations (*71*). Compared to administration of an EGFR-TKI alone, combined treatment with a Shenqi Fuzheng injection resulted in a lower incidence of a rash and diarrhea in patients and was even better tolerated.

A Shenqi Fuzheng injection has performed well in the treatment of various advanced cancers in clinical settings, and its mechanisms have also been actively studied. A Shenqi Fuzheng injection might be useful in alleviating cancer-related fatigue (CRF) by inhibiting pro-inflammatory cytokines produced by peripheral immune cells, limiting the dysfunction of exhausted T cells, and improving anti-tumor immunity through the targets of PDL1, TIM3, and FOXP3 in tumor-bearing mice (*72*). Mitofusin-2 (Mfn2) is a mitochondrial GTPase that may be related to chemo-resistance. A Shenqi Fuzheng injection reversed DDP resistance through Mfn2-mediated cell cycle arrest and apoptosis in A549/DDP cells (*73*). Moreover, a Shenqi Fuzheng injection not only significantly improved physical status, survival, and spatial learning in mice treated with cranial radiation therapy (CRT), but it also attenuated all of the CRT-induced changes in brain tissues. It effectively attenuated irradiation-induced brain injury *via* inhibition of the NF-κB signaling pathway and microglial activation (*74*).

5. Chinese patent medicines for gynecological diseases

Gynecological disorders, including endometriosis, myoma uteri, infertility, primary dysmenorrhea, and premenstrual syndrome, are common phenomena among the female population. These disorders mostly generate a diversity of discomfort and inconvenience for women such as bothersome heavy menstruation, dysmenorrhea, and infertility; these disorders may not be genuine

threats to life, but they greatly affect women's QOL (*75*). TCM has been used to treat gynecological disorders for more than 2,000 years. The famous book Synopsis of the Golden Chamber written by Zhang Zhongjing during the Eastern Han Dynasty (25-220 A.D.) systematically recorded the prevention and treatment of women's pregnancy-related diseases, postpartum diseases, and other common diseases (*76*). A point particularly worth noting is that several Chinese patent medicines such as Guizhi Fuling Capsules have significant advantages in treating gynecological disorders. Summarized here are the recent advances in the efficacy (Table 9, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) and molecular mechanisms (Table 10, online data: *http://www.ddtjournal.com/action/getSupplementalData.php?ID=129*) of Guizhi Fuling Capsules. These capsules have passed the review of the US FDA. A phase II clinical trial (NCT01588236) of Guizhi Fuling Capsules for primary dysmenorrhea has been completed by the US FDA, which found them to be efficacious and safe (*77*).

Guizhi Fuling Capsules are a Chinese patent medicine approved by the CFDA in 1995, and they are widely used to treat gynecological conditions in China. The capsules were developed from Guizhi Fuling Pills, a classic and effective prescription that was first recorded in the famous book Synopsis of the Golden Chamber written by Zhang Zhongjing during the Eastern Han Dynasty (25-220 A.D.). According to the Chinese Pharmacopoeia (Pharmacopoeia Commission of the People's Republic of China, 2015), the capsules consist of five ingredients: Guizhi (Cinnamon Cortex), Shaoyao (Paeoniae Radix), Mudanpi (Moutan Cortex), Taoren (Persicae Semen), and Fuling (Poria Cocos) (Figure 7). The main active ingredients of Guizhi Fuling Capsules have been identified and include paeonol, coumarin, cinnamic alcohol, cinnamic acid, paeoniflorin, and amygdalin (*78*). In modern medicine, Guizhi Fuling Capsules are commonly used to treat endometriosis, myoma uteri, primary dysmenorrhea, menopausal syndrome, and other gynecological diseases caused by an estrogen and progesterone imbalance.

Over the past decade, a series of RCTs and systematic reviews have indicated that Guizhi Fuling Capsules are an effective adjunctive therapy for gynecological diseases. A systematic review of 30 RCTs involving 3,586 patients was conducted to evaluate the efficacy and safety of Guizhi Fuling Capsules/Pills in the treatment of chronic pelvic inflammatory disease. Results indicated that Guizhi Fuling Capsules/Pills combined with Western medicine were more effective than Western medicine alone in terms of clinical efficacy, the rate of recurrence, anti-inflammation, and plasma viscosity (*79*). Another systematic review of 38 RCTs involving 3,816 patients indicated that the Guizhi Fuling Formula plus mifepristone may be more effective than mifepristone alone in the treatment of

Figure 7. Composition of Guizhi Fuling Capsules that can be used to treat gynecological diseases.

uterine fibroids (*80*). In addition, a meta-analysis of 10 RCTs involving 1,052 patients indicated that the Guizhi Fuling Formula as an adjuvant therapy to mifepristone appeared to have additional benefits in preventing the recurrence of endometriosis and improving pregnancy among women with endometriosis (*81*).

Guizhi Fuling Capsules/Pills have performed well in the treatment of various advanced cancers in clinical settings, and their mechanisms have also been actively studied. Guizhi Fuling Capsules displayed promising efficacy in a mouse model of endometrial hyperplasia induced with estradiol (*82*). The capsules attenuated estrogen-induced endometrial hyperplasia in mice by triggering ferroptosis *via* the p62-Keap1-NRF2 pathway. Guizhi Fuling Pills also alleviated insulin resistance in polycystic ovary syndrome (PCOS) with an underlying mechanism of regulating intestinal flora to control inflammation (*83*). Moreover, Guizhi Fuling Pills inhibited the over-activation of autophagy and apoptosis of ovarian granulosa cells in rats with PCOS by activating the PI3K/AKT/mTOR pathway, thus improving ovarian function and restoring ovulation (*84*). In addition, a metabonomic study had been conducted by Lang *et al*. to investigate the efficacy and mechanism of action of Guizhi Fuling capsules in rats with dysmenorrhea induced with oxytocin (*85*). They found that Guizhi Fuling capsules were efficacious in rats with dysmenorrhea by regulating multiple metabolic pathways including sphingolipid metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism, amino acid metabolism, lipid metabolism, and energy metabolism.

6. Conclusion

An indispensable part of TCM, Chinese patent medicines have played an important role in preventing and treating diseases in China. Most Chinese patent medicines are derived from ancient TCM prescriptions and are clinically efficacious. Since they are easy to use, easy to store, and cost-effective, Chinese patent medicines have been generally accepted and widely used in Chinese clinical practice as a vital medical

resource. In recent years, as TCM has developed and it has been accepted around the world, many Chinese patent medicine companies have gained market access and successfully registered several Chinese patent medicines as over-the-counter (OTC) or prescription drugs in regions and countries that primarily use Western medicine such as the EU, Russia, Canada, Singapore, and Vietnam. Moreover, several Chinese patent medicines have obtained FDA approval and are undergoing phase II or III clinical trials in the US.

Therefore, the current review has focused on several Chinese patent medicines that have been successfully registered or that have been submitted for registration abroad. Summarized here are recent advances in the efficacy and molecular mechanisms of these Chinese patent medicines for respiratory infectious diseases (Lianhua Qingwen capsules, Jinhua Qinggan granules, and Shufeng Jiedu Capsules), cardiovascular and cerebrovascular diseases (Compound Danshen Dripping Pills, Huatuo Zaizao pills, and Tongxinluo Capsules), cancers (a Kanglaite injection and a Shenqi Fuzheng Injection), and gynecological diseases (Guizhi Fuling Capsules). The hope is that this review will contribute to a better understanding of Chinese patent medicines by people around the world.

Since 1996, the Chinese Government has issued calls and proposals for the global acceptance of TCM. Chinese patent medicines are becoming increasingly popular in the international market, which represents a breakthrough for the global acceptance of TCM. Given their confirmed efficacy and safety, these Chinese patent medicines can play an increasingly important role in global healthcare, but there is still a long way to go for the global acceptance of TCM. Currently, challenges to the registration of Chinese patent medicines overseas mainly lie in control of the quality of raw materials, the manufacturing process, and evidence of safety and efficacy. To ensure the consistent and acceptable quality of final products, Chinese patent medicine companies should pay attention to all of the links in the production chain. Moreover, high-quality clinical data in accordance with international standards, as well as identification of definite mechanisms of action, are absolutely essential to the further development and acceptance of Chinese patent medicines around the world. In short, the hope is that more Chinese patent medicines will enter the international market and benefit people around the world in the future.

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Original Article

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Screening for microbial potentiators of neutral lipid degradation in CHO-K1 cells

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SUMMARY A cell-based assay was conducted to screen microbial culture broths for potentiators of neutral lipid degradation in Chinese Hamster Ovary K1 cells. A total of 5,363 microbial cultures from fungi and actinomycetes were screened in this assay. Brefeldin A (**1**) from fungal cultures was found to promote the degradation of triacylglycerol (TG) with an EC_{50} of 2.6 μM. Beauveriolides I (**2**), III (**3**), beauverolides A (**4**), B (**5**), and K (**6**) from fungal cultures showed potentiating effect on cholesteryl ester (CE) degradation with EC_{50}s ranging from 0.02 to 0.13 μM. Among these compounds, **2** and **6** exhibited the strongest activities (EC_{50}, 0.02 μM). From actinomycete cultures, oxohygrolidin (**7**) (EC_{50} for TG and CE, > 1.7 and 0.8 μM, respectively) and hygrolidin (**8**) (EC_{50} for TG and CE, 0.08 and 0.004 μM, respectively) promoted degradation of CE more preferably than TG.

Keywords Neutral lipid degradation, screening, microbial potentiator, brefeldin A, beauveriolide, hygrolidin

1. Introduction

The synthesis and degradation of lipids are finely controlled not only to prevent intracellular accumulation of lipids but to provide energy supply and structural components of cell membranes. Lipid is stored in the final form of neutral lipids, such as triacylglycerol (TG) for long-chain fatty acids and cholesteryl ester (CE) for cholesterol (*1*). However, multiple factors, such as increased consumption of high-fat diet and lack of physical exercise, can determine the lipid storage capacity. In the end, excessive accumulation of stored TG and CE causes metabolic diseases, namely obesity, fatty liver, and atherosclerosis (*2*). Our laboratory has a long history of searching for and discovering various compounds involved in neutral lipid synthesis from microbial resources using mammalian cells (*3-5*). Biaryl dihydronaphthopyranone atropisomers produced by a fungus, dinapinones A1 (**9**) (DPA1) and A2 (**10**) (DPA2) (Figure 1), were originally discovered as inhibitors of neutral lipid accumulation. DPA2 showed potent inhibition of neutral lipid accumulation, whereas DPA1 showed almost no activity. Intriguingly, a 1:1 mixture of DPA1 and DPA2 was found to exhibit the most potent inhibition of neutral lipid accumulation (*6,7*). Further study of the mechanism of action revealed that, instead of inhibiting the synthesis of neutral lipids, DPA promoted neutral lipid degradation along with inducing autophagy (*8*).

Inhibitors of lipid synthesis primarily work as a preventive effect to stop or retard the progress of metabolic diseases in most cases. A number of inhibitors have been reported and some are practically used as preventive drugs. As a typical example, statins, which are hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, block hepatic cholesterol synthesis to lower the serum low-density lipoprotein (LDL) level and to retard the progression to atherosclerosis (*9*). Conversely, potentiators of accumulated neutral lipid degradation are rarely discovered. Such potentiators function as treatment drugs rather than preventive drugs for metabolic diseases involved in neutral lipid accumulation in the human body. Although discovering such potentiators seems more difficult and challenging than discovering inhibitors, our finding of dinapinones prompted us to establish an assay system and perform screening of potentiators from microbial cultures. The results are described in the present study.

2. Materials and Methods

2.1. General

NMR analyses were performed using a 400 MHz

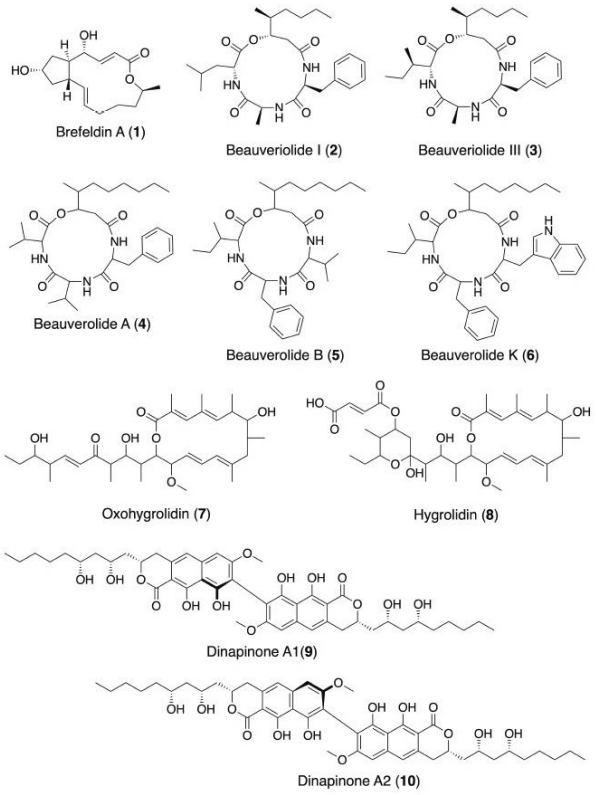

Figure 1. Structures of active compounds.

spectrometer (Agilent Technologies, Santa Clara, CA, USA) and 600 MHz spectrometer (Bruker, Karlsruhe, Germany). MS analyses were performed using an AccuTOF LC-plus JMS-T100LP system (JEOL, Tokyo, Japan) and Waters Xevo G2-XS Qtof Acquity UPLC H-class plus (Waters Corporation, Milford, MA, USA).

2.2. Materials

[1-^{14}C]Oleic acid (1.85 GBq/mmol) and [1-^{14}C] oleoyl-CoA (1.85 GBq/mmol) were purchased from PerkinElmer (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Ham's F-12 medium was purchased from Nacalai Tesque (Kyoto, Japan). Plastic microplates (48-well) were purchased from Corning (Corning, NY, USA). Agar was purchased from Shimizu Food (Shizuoka, Japan). $CoCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $Fe_2(SO_4)_3 \cdot nH_2O$, glucose, KH_2PO_4, K_2HPO_4, $Mg_3(PO_4)_2 \cdot 8H_2O$, $MgCl_2 \cdot 4H_2O$, polypeptone, soluble starch, sucrose, $ZnSO_4 \cdot 7H_2O$, bovine serum albumin (BSA) (fatty acid free), and penicillin (10,000 U/mL) and streptomycin (10,000 mg/mL) solution were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). $CaCO_3$, $MgSO_4 \cdot 7H_2O$, and KBr were purchased from Kanto Chemical (Tokyo, Japan). Peptone was purchased from Life Technologies Corporation (Detroit, MI, USA). Ehrlich meat extract was purchased from Pharmaceutical Industrial (Tokyo, Japan). Solulys was purchased from Oriental Yeast (Tokyo, Japan). Yeast extract was purchased from Becton Dickinson (Sparks, MD, USA). EDTA was purchased from Dojindo (Kumamoto, Japan). Brefeldin A, beauveriolides I and III, and dinapinones (A1, A2, and DPA) were available from our compound library (*10-12*). Other beauverolides A, B, and K and hygrolidins were isolated from our culture broths.

2.3. Purification of active compounds from culture broths

Beauverolides (**4**-**6**): Fungal BF-0452 strain was seeded in medium containing 2.0% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% agar, and tap water, pH 6.0 for 3 days at 27°C in a rotary shaker. The seed culture was then inoculated into production medium containing 2.0% sucrose, 1.0% glucose, 0.5% solulys, 0.5% Ehrlich meat extract, 0.1% KH_2PO_4, 0.3% $CaCO_3$, 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$, 0.1% agar, 1.0% trace metal solution (1 mg/mL of each $FeSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoCl_2 \cdot 2H_2O$ in water), and tap water, pH 6.0. Fermentation was performed at 27°C for 14 days under static condition. The culture broth (1.8 L) was extracted with ethanol (1.8 L). Ethanol extracts were concentrated to remove ethanol, and the aqueous solution was extracted with ethyl acetate (3.6 L), yielding crude materials (227 mg). The extracts were then dissolved in a small volume of methanol, applied to an ODS column (11 g, i.d. 15 × 150 mm, 100-200 mesh, Fuji Silysia Chemical Ltd., Aichi, Japan) and eluted stepwise with 40%, 60%, 80%, and 100% CH_3CN solvents (2 fractions per 150 mL each). The 80%-2 CH_3CN eluents containing active materials were evaporated *in vacuo* to yield brownish materials (18 mg). The materials were purified by high performance liquid chromatography (HPLC) under the following conditions: column, Develosil C30 UG5 (Nomura Chemical Co., Ltd., Aichi, Japan) (i.d. 20 × 250 mm); eluent, 90% CH_3CN; flow, 6.0 mL/min; detection, UV at 210 nm. Compounds **4**-**6** were eluted as a peak with retention times of 16.8 min, 18.8 min, and 18.1 min, respectively. Each peak fraction was collected and concentrated to yield **4** (0.6 mg), **5** (3.2 mg), and **6** (1.1 mg) as white powders.

Hygrolidins (**7**-**8**): Actinomycete KM68-21 strain was grown in medium containing 1.0% soluble starch, 0.4% yeast extract, 0.2% peptone, and tap water, pH unadjusted at 27°C for 7 days in a rotary shaker. The culture was inoculated into new medium with additional 0.1% $CaCO_3$, 0.004% $Fe_2(SO_4)_3 \cdot nH_2O$ and 0.01% KBr at 27°C for 7 days in a rotary shaker. Culture broth (3 L) was centrifuged (3,000 rpm, 10 min) and the mycelia was collected and then extracted with 70% ethanol (3 L). Ethanol extracts were filtered to remove mycelia and then concentrated to remove ethanol. The remaining

aqueous solution was extracted with ethyl acetate (1.8 L). The organic layer was collected and dried under pressure to produce a brownish crude extract (388 mg). This crude extract was dissolved in a small amount of methanol, applied to an ODS column (17.5 g, i.d. 30 × 130 mm, 100-200 mesh, Fuji Silysia Chemical Ltd.), and eluted stepwise with 0%, 20%, 40%, 60%, 80%, and 100% CH_3CN solvents (120 mL each). The 80% and 100% CH_3CN eluents containing active materials were evaporated *in vacuo* to yield pale brown materials (38 mg and 44 mg, respectively). The materials from the 80% fraction were subjected to HPLC under the following conditions: column, Capcell pak C18 (Osaka Soda Co., Ltd., Osaka, Japan) (i.d. 4.6 × 250 mm); eluent, 20 minutes linear gradient from 75%-95% CH_3CN; flow, 1.0 mL/min; detection, UV at 210 nm. Compounds **7** and **8** were eluted as a peak with retention times of 10.0 min and 16.4 min, respectively. Each peak fraction was collected and concentrated to yield **7** (6.3 mg) and **8** (2.0 mg) as white powders. The 100% fraction was subjected to HPLC (column, Capcell pak C18 (i.d. 4.6 × 250 mm); eluent, 88% CH_3CN; flow, 1.0 mL/min; detection, UV at 210 nm). Under these HPLC conditions, **8** was eluted as a peak with retention time of 10.0 min, collected, and concentrated to yield **8** (4.6 mg) as a white powder.

2.4. Identification of active compounds

Compounds **1-3** were identified from our database using Ultra-Fast Liquid Chromatography (UFLC) (Prominence, Shimadzu, Kyoto, Japan) retention time and UV spectrum. Other compounds **4-8** were identified from the spectral data, including ^{1}H-NMR, UV and/or MS data, and the search results of Scifinder® and/or Dictionary of Natural Products (Supplementary Figures S1-S3, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=127*). These data were also identical with published data (*13-17*). The structures of active compounds are shown in Figure 1.

2.5. Cell culture

Chinese Hamster Ovary K1 cells (CHO-K1 cells) were maintained at 37°C and 5.0% CO_2 in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin (100 mg/mL) according to a previously described method (*18*).

2.6. Assay of neutral lipid degradation in CHO-K1 cells

Assays for CE and TG degradation using CHO-K1 cells were performed according to established methods with some modifications (*8*). Cells (1.25×10^5 cells) were seeded in a 48-well plastic microplate in 250 µL of maintained medium (described above) and allowed to recover overnight in 37°C and 5.0% CO_2; 5.0 µL of [^{14}C]oleic acid (1 nmol, 1.85 kBq in 10% ethanol/phosphate-buffered saline (PBS) solution) was then added to each well of the cell culture. The cells were incubated for 24 hours at 37°C and 5.0% CO_2 to allow for accumulation of [^{14}C]TG and [^{14}C]CE within the cells. Medium were then removed and cells were washed twice with 250 µL of Buffer A (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing 2.0 mg/mL BSA (fatty acid free) and then washed once with 250 µL of Buffer B (Buffer A without BSA) to remove the remaining [^{14}C]oleic acid. Fresh medium (250 µL) was then added along with 2.5 µL of sample and control (in methanol). After 12-hour incubation in 37°C and 5.0% CO_2, cells were washed twice with 250 µL of PBS. The cells were then lysed using 250 µL of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) SDS, and neutral lipids were extracted following the method of Bligh and Dyer (*19*). [^{14}C]TG and [^{14}C]CE were separated on a TLC plate (silica gel F254, 0.5-mm thick, Merck KGaA, Darmstadt, Germany) and then analyzed with a bioimaging analyzer (FLA7000; Fujifilm, Tokyo, Japan). Neutral lipid degradation activity (%) was defined as ([^{14}C]TG or [^{14}C]CE of sample/[^{14}C]TG or [^{14}C]CE of control) × 100. The EC_{50} value was defined as the sample concentration causing 50% degradation of neutral lipids (TG or CE).

2.7. Assay of neutral lipid synthesis in CHO-K1 cells

Assays for TG and CE synthesis using CHO-K1 cells were conducted according to established methods with some modifications (*8*). The IC_{50} value was defined as the sample concentration causing 50% inhibition of neutral lipid synthesis.

2.8. Sterol *O*-acyltransferase (SOAT) enzyme assay using microsomes prepared from CHO-K1 cells

SOAT assay using microsomes prepared from CHO-K1 cells was conducted using our established method (*20*). The IC_{50} value was defined as the sample concentration causing 50% inhibition of SOAT.

3. Results

3.1. Establishment of cell-based lipid degradation assay for screening

A cell-based screening method was conducted based on our neutral lipid degradation assay (*8*). In the previous study on DPA, three mammalian cell lines, CHO-K1, HeLa, and HepG2 cells, were tested for neutral lipid (TG and CE) accumulation. As a result, CHO-K1 cells were found to have the highest ability to incorporate [^{14}C]oleic acid into both CE and TG. Accordingly, we chose this cell line in our screening system. CHO-K1 cells were incubated with [^{14}C]oleic acid for 24 hours to

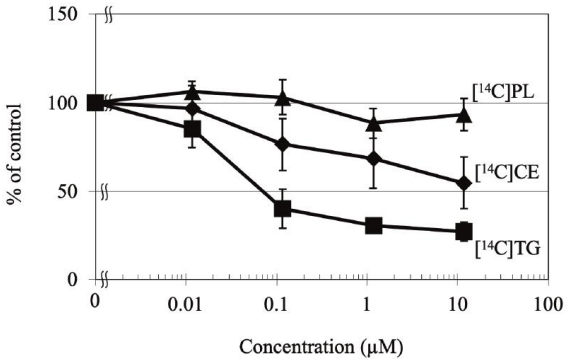

Figure 2. Effect of DPA (1:1 mixture of 9 and 10) on neutral lipid degradation. CHO-K1 cells were incubated for 24 hours with [^{14}C] oleic acid to accumulate [^{14}C]TG and [^{14}C]CE. After removing free [^{14}C]oleic acid, cells were treated with DPA 0, 0.12, 1.2, or 12 μM for 12 hours. Cells were then lysed. Cellular [^{14}C]TG (■), [^{14}C]CE (♦), and [^{14}C]PL (▲) were separated on TLC and quantified using an image analyzer. The results obtained were plotted as % of control (without drugs). Values represent means ± SD (n = 3).

accumulate [^{14}C]TG and [^{14}C]CE. After free [^{14}C]oleic acid was removed, cells were treated with a sample and control (in methanol) (time 0) and incubated for 12 hours. Accumulated [^{14}C]TG and [^{14}C]CE were naturally degraded after 12-hour incubation in the control to 63% and 60% from time 0, respectively. In the screening for lipid degradation potentiators, the amounts of [^{14}C]TG and [^{14}C]CE that remained following 12 hours of incubation were regarded as 100% (control value). Hit samples should promote degradation of [^{14}C]TG and/or [^{14}C]CE to less than 60% of the control with no morphological abnormalities in CHO-K1 cells under microscopy and no effect on phospholipid level ([^{14}C]phospholipid (PL)). EC_{50} values are defined as the effective concentration of a sample causing 50% promotion of each neutral lipid degradation.

First, the effects of DPA (1:1 mixture of **9** and **10**), DPA1 (**9**), and DPA2 (**10**) were confirmed in the assay. As shown in Figure 2, DPA strongly enhanced the TG degradation with an EC_{50} of 0.07 μM, whereas CE degradation was moderately enhanced with an EC_{50} of > 12 μM (approximately 20 μM). Almost no effect on PL degradation implied that DPA affected only neutral lipid degradation without any cytotoxic effect on CHO-K1 cells. DPA2 (**10**) showed a similar effect on the TG degradation with higher EC_{50} of 1.0 μM (TG) and weak effect on CE degradation (72% of control at 12 μM) (Table 3). DPA1 (**9**) itself showed no effect on neutral lipid degradation. These findings are fundamentally consistent with our previous study (*8*). In this screening, DPA was used as a positive control compound.

3.2. Screening result for neutral lipid degradation potentiators from microbial sources

A total of 5,363 microbial culture broths were screened for neutral lipid degradation potentiators in CHO-K1 cells with only 0.35% of them categorized as hit samples (Table 1). The hit rate from fungal cultures (hit rate 0.59%) was 7-fold higher compared to actinomycete cultures (0.08%). Out of 19 active culture broths (Table 2), 14 fungal broths promoted degradation of [^{14}C]TG. From these broths, brefeldin A (**1**) (*21*) was identified as an active compound by database analyses of the retention time and the UV spectrum using UFLC. From one of the fungal cultures, known beauveriolide I (**2**) (*22*) was identified as a potentiator of [^{14}C]CE degradation. Structurally related beauverolides A, B, and K (**4**-**6**) (*13,14*) were also isolated from the culture broth of soil isolate fungus BF-0452. Two broths from actinomycetes contain hygrolidins that promoted degradation of [^{14}C]CE more preferably than [^{14}C]TG. Known oxohygrolidin (**7**) (*17*) and hygrolidin (**8**) (*16*) were isolated from the culture broth of marine-derived actinomycete KM68-21.

Table 1. Screening result of microbial potentiators of neutral lipid degradation

Origin	Sample number	Hit sample (rate %)		Hit per-origin (rate %)
		[^{14}C]TG	[^{14}C]CE	
Fungi	2,837	14 (0.49%)	3 (0.10%)	17 (0.59%)
Actinomycetes	2,526	0 (0.00%)	2 (0.08%)	2 (0.08%)
Total	5,363	14 (0.26%)	5 (0.09%)	19 (0.35%)

Table 2. Hit strain and microbial potentiators of neutral lipid degradation

Hit strain	Active compounds	Potentiator
Fungal strain		
BF-0398, BF-0460, BF-0487, BF-0546, BF-0562, BF-0586, BF-0589, BF-0626, BF-0763, BF-0808, BF-0899, BF-0938, BF-0939 and BF-0973.	**1**	TG degradation
BF-0450	**2**	CE degradation
BF-0452 and BF-0453	**4, 5** and **6**	CE degradation
Actinomycetes strain		
KM59-1	**7**	CE degradation
KM68-21	**7** and **8**	CE degradation

3.3. Effect of microbial potentiators on neutral lipid degradation in CHO-K1 cells

A total of eight known compounds (**1**-**8**, Figure 1) were obtained as potentiators of neutral lipid degradation in the screening system. The enhancing activity of neutral lipid (TG and CE) degradation in CHO-K1 cells by these compounds is summarized in Table 3. Brefeldin A (**1**), a lactone-containing fungal metabolite, promoted [^{14}C]TG degradation with an EC_{50} of 2.6 μM, but had no effect on CE degradation. Thus, **1** is a potentiator

Table 3. Effect of microbial potentiators on neutral lipid degradation and synthesis

Compound	Cell-based lipid degradation		Cell-based lipid synthesis		Enzyme-based CE synthesis (SOAT)
	EC_{50} (μM)[a]		IC_{50} (μM)[a]		IC_{50} (μM)[a]
	[^{14}C]TG	[^{14}C]CE	[^{14}C]TG	[^{14}C]CE	[^{14}C]CE
Brefeldin A (**1**)	2.6	>34	1.6	>34	>34
Beauveriolide I (**2**)	>2.0	0.02	>2.0	0.01	0.05
Beauveriolide III (**3**)	>2.0	0.13	>0.2	0.04	0.19
Beauverolide A (**4**)	>1.8	0.10	>1.8	0.70	0.03
Beauverolide B (**5**)	>1.8	0.06	>1.8	0.02	0.03
Beauverolide K (**6**)	>1.5	0.02	>1.5	0.01	0.04
Oxohygrolidin (**7**)	>1.7	0.80	>1.7	0.63	0.30
Hygrolidin (**8**)	0.08	0.004	>0.14	0.0002	0.33
Dinapinone A1 (**9**)	>12	>12	>12[b]	>12[b]	>12
Dinapinone A2 (**10**)	1.0	>12	0.65[b]	>12[b]	>12
Dinapinone A	0.07	>12	0.054[b]	0.18[b]	>12

[a]Data are expressed as means (n ≥ 3). [b]from reference (*8*).

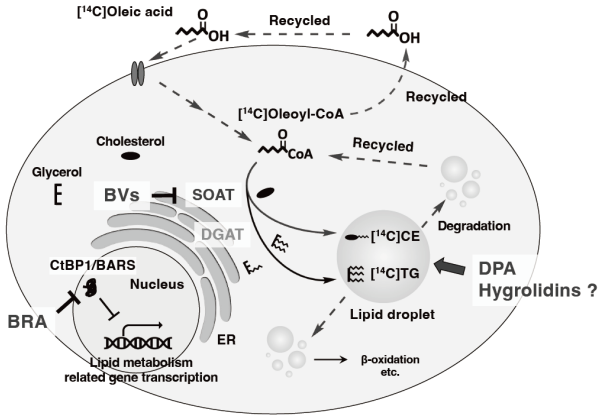

Figure 3 Graphical summary of microbial potentiators of neutral lipid degradation. CE; cholesteryl ester, TG; triacylglycerol, SOAT; sterol *O*-acyltransferase, DGAT; diacylglycerol acyltransferase, ER; endoplasmic reticulum, CtBP1/BARS; C-terminal-binding protein/brefeldin A-induced ADP-ribosylated substrate, BRA; brefeldin A, BVs; beauveriolides, DPA; dinapinone A.

selective for TG degradation.

Five beauveriolides and beauverolides including beauveriolide III (**3**) (*11,12*), **2**-**6** were found to exhibit strong [^{14}C]CE degradation activity. Compounds **2** and **6** showed the strongest with EC_{50} of 0.02 μM, followed by **5**, **4**, and **3** with EC_{50}s of 0.06 μM, 0.10 μM, and 0.13 μM, respectively. Sixteen-membered macrolide **8** exhibited strong potentiation of both [^{14}C]CE (EC_{50}, 0.004 μM) and [^{14}C]TG (EC_{50}, 0.08 μM) degradation, whereas the other macrolide **7** only potentiated [^{14}C]CE degradation with a higher EC_{50} of 0.08 μM.

4. Discussion

In this lipid degradation assay, CHO-K1 cells were incubated with [^{14}C]oleic acid overnight to incorporate a high amount of [^{14}C]oleic acid into [^{14}C]TG and [^{14}C]CE accumulated in lipid droplets (*8*). After removal of free [^{14}C]oleic acid, the accumulated [^{14}C]TG and [^{14}C]CE still remained at approximately 60% after further 12-hour incubation. This finding indicates that the CHO-K1 cell line is suitable for use in neutral lipid degradation assays.

Fungal brefeldin A (**1**) was identified as a potentiator of [^{14}C]TG degradation in our screening assay. Bartz *et al.* reported similar findings; in CHO-K2 cells pretreated with [^{3}H]oleic acid, **1** (7 μM) promoted 63% breakdown of stored [^{3}H]neutral lipids after 12-hour incubation (*23*). Brefeldin A (**1**) is well known to interfere with protein transport by preventing accumulation of coatomer proteins on the Golgi apparatus, which facilitates vesicle fusion from the endoplasmic reticulum (*24,25*). Accordingly, other studies reported that **1** induced accumulation of neutral lipid (*26-28*). However, Bartz *et al.* proposed that the lipid degradation potentiator effect of **1** is not linked to Golgi apparatus disruption and that, instead, **1** inactivates C-terminal-binding protein/brefeldin A-induced ADP-ribosylated substrate (CtBP1/BARS) by stimulating its mono-ADP-ribosylation. CtBP1/BARS works as a gene transcriptional co-repressor in nucleus. Retained CtBP1/BARS function leads to the up-regulation of genes that regulate lipid storage (*23*).

Fungal beauveriolides I (**2**) and III (**3**) are well known as inhibitors of SOAT enzymes involved in CE synthesis (*29*). In the present screening program, beauveriolides and beauverolides (**2**-**6**) were isolated as potentiators of CE degradation. To investigate CE synthesis (by SOAT activity), **2** to **6** inhibited the synthesis with analogous IC_{50}s (Table 3, middle column) to EC_{50}s in the present cell-based degradation assay (Table 3, left column). In a SOAT enzyme assay using cells microsomes, **2** to **6** also exhibited potent SOAT inhibition. These data indicated that [^{14}C]CE is degraded to free cholesterol and [^{14}C]oleic acid (also provided from [^{14}C]TG degradation), which are reused to produce [^{14}C]CE *via* SOAT during the degradation assay. Beauveriolides and beauverolides may block the

[^{14}C]CE recycling by inhibiting SOAT activity, leading to ostensible potentiation of [^{14}C]CE degradation. A variety of microbial SOAT inhibitors have been reported (*3-5,18*), but most of them were not discovered in the present screening system.

We evaluated both fungal and actinomycete culture broths in the present screening system and found that fungi were more preferable than actinomyces as a resource for this screening (Table 1). The sole actinomycete-derived potentiators that we found are hygrolidins, which are members of two diene-containing 16-membered macrolides with diverse chains. Compound **8** with a fumarate-linking tetrahydropyran in the side ring was found to be the strongest potentiator of both CE and TG degradation. Compound **7** with an alkenyl side chain exhibited markedly weaker activity compared to **8**. This group of macrolides are well known as V-ATPase inhibitors that damage the function of lysosomes (*30,31*). The mechanism of lipid droplet degradation in mammalian cells remains elusive; certain proteins located on lipid droplets (such as perilipines) regulate the incorporation of lipase into lipid droplets, and autophagy (lipophagy) is involved in lipid droplet degradation (*32-35*). Thus, lysosomes are not directly involved in lipid droplet degradation. Indeed, **7** and **8**, V-ATPase inhibitors, did not inhibit lipid droplet degradation, but conversely potentiated the degradation. The mechanism by which hygrolidins potentiate neutral lipid breakdown requires further investigation. In addition, both compounds exhibited weak SOAT inhibitory activities in the enzyme-based assay (Table 3), which may partially contribute to the potentiating effect of lipid degradation in our assay.

In conclusion, we conducted an assay of lipid droplet degradation using CHO-K1 cells and screened for potentiators from microbial cultures. All potentiators identified are known compounds with known mechanism of actions (Figure 3). These compounds will help to unveil the incompletely explored mechanism of lipid droplet degradation in mammalian cells.

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Original Article

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Silkworm arylsulfatase in the midgut content is expressed in the silk gland and fed *via* smearing on the food from the spinneret

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SUMMARY We found the activity of arylsulfatase in the midgut contents of the silkworm, *Bombyx mori*. We identified a 60-kDa protein that comigrates with the activity on a column chromatography following ammonium sulfate precipitation. Based on its partial amino acid sequence, we searched for its coding gene using Basic Local Alignment Search Tool (BLAST) and identified *KWMTBOMO05106*. Transcriptional data suggest a specific expression of the gene in middle silk glands. The majority (80%) of arylsulfatase activity was found in the silk glands, concurring the specific transcription in the silk gland. Observing the feeding behaviour of the silkworm, we found that silkworms smear a mucus secretes from the spinneret on the food pellet as they feed on. Arylsulfatase activity was also detected in the food pellet bitten by the silkworm as well as in the gut content. Furthermore, arylsulfatase activity was not detected either in the food pellet and in the gut content when silkworms had obstructed the spinneret. These results suggest that arylsulfatase is secreted from the silk glands and may contribute to digestive function.

Keywords silkworm, enzyme, secretion, arylsulfatase

1. Introduction

In previous work, we have proposed the utility of the silkworm as an experimental animal for evaluating the therapeutic potential of pharmaceuticals (*1*). We have reported that the therapeutic potential of existing therapeutic agents can be quantitatively assessed in a silkworm bacterial infection model, obtaining 50% effective dose (ED_{50}) values comparable to those in mammalian models (*2,3*). In addition, exploration using soil bacterial culture supernatants (*4*) has led to the discovery of novel antibiotics, such as lysocin E (*5,6*) and ASP2397 (*7*), which have been shown to have a therapeutic effect in mice models. This is partially because the pharmacokinetic parameters of antimicrobials are well matched between the silkworm and mammalian models (*8,9*). With regard to metabolism, which has a major impact on the pharmacokinetics of compounds, it has been shown that in the silkworm, as in humans, hydroxylation by cytochrome P450s occurs, followed by conjugation reactions and excretion (*8,10*). With regard to the conjugation reactions in the silkworm, glycosyltransferases(*11,12*), glutathione transferases (*13*), and sulphotransferases (*14*) have been identified. Although these conjugation enzymes have been studied in the silkworm, little work has been performed on the metabolism of the conjugated compounds, although a group of conjugation enzymes involved in human pharmacokinetics has been identified in the silkworm.

A typical example of the metabolism of conjugated compounds is the reaction by arylsulfatases. Arylsulfatases are a group of enzymes that catalyze the desulfation of aromatic sulfates (*i.e.*, aryl sulfates) (*15*). In the human gut, arylsulfatases of enterobacterial origin are known to affect the metabolism and kinetics of steroid hormones that are sulfate-conjugated and excreted with the bile (*16,17*). A deficiency of arylsulfatase in humans causes metachromatic leukodystrophy due to the accumulation of sulfatides, resulting in central and peripheral nerve damage (*18*). Arylsulfatases are conserved across animal phyla (*15*), and the presence of arylsulfatase invertebrates has been reported in the literature (*19*). In *Caenorhabditis elegans*, a deficiency of arylsulfatase, which is involved in the sulfation of steroid hormones, has been shown to prolong lifespan (*20*), but little is known about its physiological function. Also in the silkworm,

arylsulfatase expression in the brain and other organs has been reported, yet its physiological function is unknown (*21*). We performed an assay using the model compound 4-methyl umbelliferyl sulfate and found arylsulfatase activity in the silkworm gut contents.

In the present study, we purified the arylsulfatase in the intestinal tract and identified the primary structure of the protein. We further showed that this enzyme is secreted from the silk gland onto the food pellet during feeding, which may indicate that the enzyme contributes to metabolizing ingested materials. These results shed light on the hitherto unknown physiological function of arylsulfatase in the silkworm

2. Materials and Methods

2.1. Raring of silkworm

Silkworm eggs (Hu•Yo × Tsukuba•Ne) were purchased from Ehime Sanshu (Ehime, Japan) and hatched larvae were fed artificial food, Silkmate 2S (Katakura Industries, Co., Ltd. Tokyo, Japan) at 27˚C until the 5th instar stage.

2.2. Arylsulfatase assay

4-methyl umbelliferyl sulfate (1.3 mM) was mixed with a sample in a total of 150 µL of 50 mM Tris buffer pH 7.9 and incubated at 30˚C for 30 min. After adding saline 350 µL and methanol 500 µL, the samples were centrifuged at 4˚C, 14,000 rpm for 5min. The 200 µL of supernatant was mixed with 3 mL of 1.6 M glycine buffer (pH 11) and the fluorescence of umbelliferon, desulfated from 4-methyl umbelliferyl sulfate, was measured with the fluorometer (Hitachi F4500, Tokyo, Japan).

2.3. Purification of arylsulfatase from silkworm digestive content

Silkworm intestine content was collected from the 5th instar larva (Day 3 to 6) and homogenized (Fr. I). The samples were centrifuged at 4˚C, 16,000× *g* for 20 min, and precipitants were collected (Fr. II). The Fr. II was homogenized with 50 mM ammonium sulfate and supernatants were collected by centrifugation at 4˚C, 16,000× *g* for 20 min (Fr. III). Next, ammonium sulfate was added to the supernatant (final 3.5 M) and incubated at 4˚C for overnight. And then, centrifuged at 4˚C, 16,000× *g* for 20 min and dissolved in 50 mM Tris-HCl (pH 7.9) (Fr. V). To prepare Fr. VI, the samples were mixed with an equal volume of acetone and froze at – 20˚C for 1 h and centrifuged at 125,000 × *g*, 4˚C for 30 min. The precipitants were dissolved in 50 mM Tris-HCl (pH 7.9) with 20% glycerol by sonication (Sonifire OPC × 15 sec) and centrifuged again at 125,000× *g*, 4˚C for 30 min. The supernatants (Fr. VI) were applied onto diethylaminoethyl (DEAE)-cellulose column chromatography and collected flow through fr. (Fr. VII). Further, MonoS column chromatography was performed by a linear gradient of 0 to 0.5 M ammonium sulfate.

2.4. Gene expression analysis by quantitative RT-PCR

Total RNA was prepared from each tissue using the RNeasy mini kit (Qiagen, Venlo, Netherlands) and after DNase treatment, cDNA was prepared by reverse transcription reaction (High-Capacity RNA-to-cDNA Kit, Applied Biosystems) using 50 ng of total RNA. The prepared cDNA was used as a template for quantification of the arylsulfatase gene by real-time PCR (Thunderbird SYBR qPCR Mix from Toyobo (Japan)) by using 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences used for quantification were 5'-CCACTTAGCAACAGCGAGGA-3' and 5'-TCAGTTCGTGATTCGCCGAT -3'.

2.5. Bioinformatics analysis

Sequence reads data of RNA-Seq analysis in silkworm organs (DRR142721, DRR142719, DRR077426, DRR077427, and DRR12883) were downloaded from Sequence Read Archive (SRA). RNA-Seq analyses were performed using CLC Genomics Workbench ver. 21 (Qiagen, Venlo, Netherlands). Reads were aligned to the p50T strain genome (Accession No. GCF_014905235.1) allowing a minimum length fraction of 0.8 and a minimum similarity fraction of 0.8. Transcripts per million (TPM) values were normalized by trimmed mean of M values (TMM) method.(*22*) Calculation of phylogenic tree was performed at the site *http://www.phylogeny.fr/simple_phylogeny.cgi* by one-click mode (*23*). Perdition of signal peptide of protein was performed by SignalP ver. 6.0 (*24*).

3. Results

3.1. Purification and identification of arylsulfatase from the silkworm intestine

We measured the activity of arylsulfatase by the fluorescence intensity of umbelliferon produced by the enzymatic activity from the non-fluorescent substance 4-methylumbelliferone sulfate (*25*). As a result, arylsulfatase activity was identified in the silkworm gut contents. As this activity was recovered in centrifuged pellets, the responsible enzyme appears insoluble. We therefore searched for conditions where the enzyme could be solubilized and found that 0.5 M ammonium sulfate could solubilize the enzyme (Table 1). When the concentration of ammonium sulfate was increased to 3.5 M, activity was detected in the precipitate. Partial purification of the enzyme by acetone precipitation and DEAE cellulose chromatography was then performed,

Table 1. Purification table of arylsulfatase from silkworm digestive content

Fraction		Sulfatase activity (U × 10^3)	Protein (mg)	Specific activity (U × 10^3/mg)	Yield (%)
I	Gut content	910	8200	0.11	100
II	Gut content ppt	860	4200	0.20	95
III	Gut content ppt wash	670	4000	0.17	74
IV	Soluble sulfatase	680	650	1.0	75
V	3.5M $(NH_4)_2SO_4$ ppt	600	600	1.0	66
VI	Acetone treatment ppt	400	360	1.1	44
VII	DEAE flow through	150	140	1.1	16

and activity was recovered in the flow-through fraction of DEAE cellulose column chromatography. The yield from the intestinal contents was 16%, with a 10-fold increase in specific activity (Table 1). Furthermore, the DEAE flow-through fraction was separated by Mono-S column chromatography, yielding a 60-kDa protein whose activity pattern matched the band intensity in SDS-PAGE.

Further analyzing the DEAE cellulose fraction, we performed Mono-S column chromatography using the DEAE cellulose fraction, and a major activity peak appeared (Figure 1A). This active fraction was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and several proteins were detected. Among them, a 60-kDa protein matched the pattern activity of arylsulfatase (Figure 1B). We then analyzed the partial amino acid sequence of the 60-kDa protein. The protein was extracted from the gel slice containing this band and the N-terminal amino acid sequence was analyzed by Erdmann degradation. As a result, TKRKSSNIVLIVADDL was identified. A BLAST search using this sequence in the Silkbase site (*https://silkbase.ab.a.u-tokyo.ac.jp/*), a database constructed by high-precision whole-genome sequencing, revealed a 100% match with the amino acid sequence encoded by the KWMTBOMO05106 gene (Figure 2A). Currently, five genes encoding arylsulfatases have been identified in the silkworm genome database (Figure 2A, Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=125*). This protein showed the highest homology to human arylsulfatase B and will henceforth be denoted as silkworm arylsulfatase B (Figure 2C). The amino acid sequence predicted from genome database sequencing (*26*) indicates that a signal sequence (predicted by SignalP 6.0) comes N-terminus region by following the amino acid residues that match the identified partial sequence. The amino acid sequence of the active domain, which is commonly conserved in arylsulfatases, was also conserved in this protein (*i.e.*, silkworm arylsulfatase B). Analysis of organ-wise expressions of this gene using silkworm RNA-Seq data registered in the SRA database showed that expression levels were high in the silk gland but low in the midgut (Figure 2D). This expression pattern was also confirmed by qRT-PCR (Figure 2E). Furthermore, when arylsulfatase activity was measured

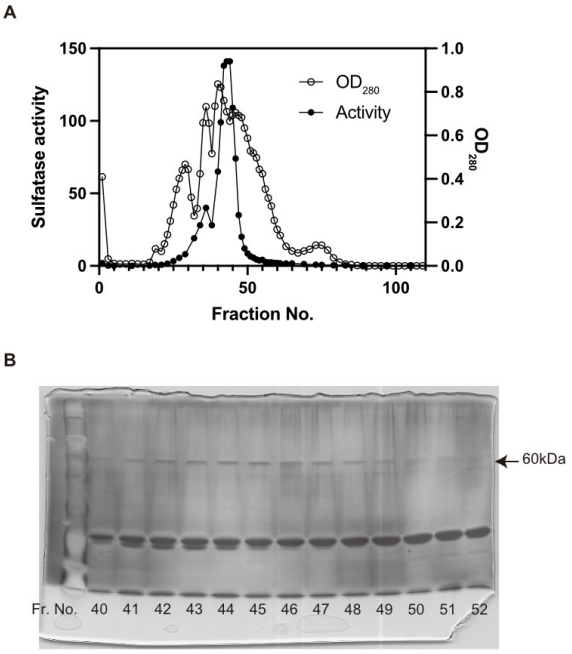

Figure 1. Elution profile of arylsulfatase activity in MonoS column chromatography. A. Protein and sulfatase activity elusion pattern from MonoS column chromatography. Protein concentrations were measured optical density of 280 nm of eluted fractions. Sulfatase activity was defined as the amount of umbelliferon production (nmol) in 20 μL aliquot of the assay sample. **B**. Silver staining image of SDS-PAGE for fractions 40 to 52 of MonoS column chromatography.

for each organ, the major activity was found in silk glands (Table 2).

3.2. Dynamics of silkworm arylsulfatase B

During feeding, silkworms secrete materials from the spinnerets to the feeding area of mulberry leaves (*27*). Our observation of silkworm feeding behavior confirmed that silkworms secrete mucus from the spinnerets during feeding (Figure 3A and Supplementary Movie, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=126*). We also found that arylsulfatase activity appeared on the mulberry leaves, when bitten by the silkworm (Figure 3B). Furthermore, we found that the spinnerets obstructed with a sticky bond resulted in the loss of arylsulfatase activity in the gut (Figure 3C).

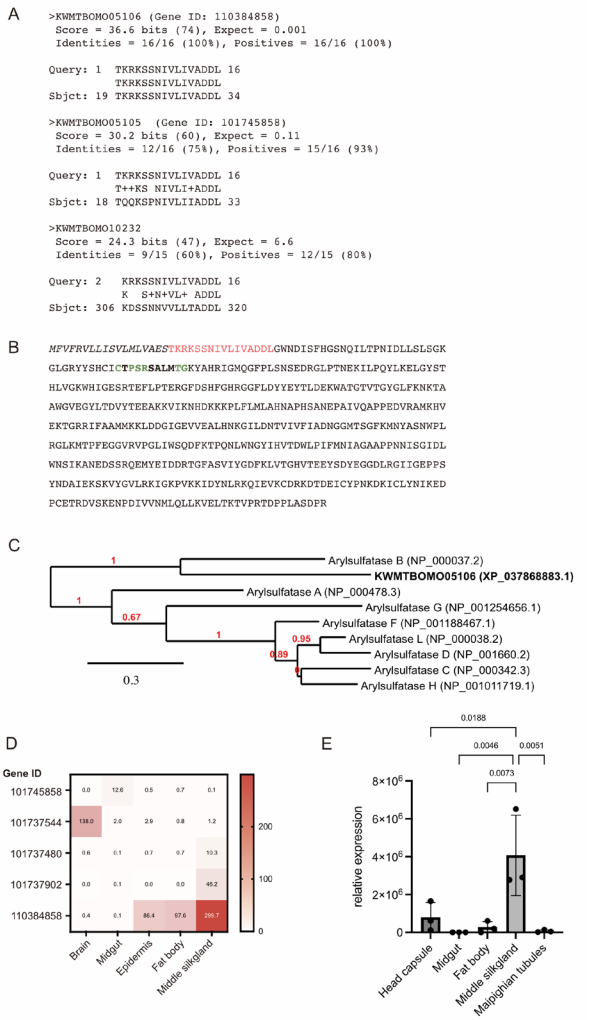

Figure 2. Identification and expression analysis of arylsulfatase in silkworm gut content. A. The result of blastP search using obtained amino acid sequence. **B**. Amino acid sequence of the product of the KWMTBOMO05106 gene. Red letters of amino acid sequence indicated identified by partial amino acid determined by Edman sequence. Green letters stand for the conserved motif of sulfatase activity. Italic letters indicated signal peptide estimated by SignalP 6.0. **C**. Phylogenic tree of KWMTBOMO05106 gene product compared with human arylsulfatases. **D**. RNA-Seq result of the genes annotated as arylsulfatase B in silkworm genome. **E**. The mRNA expression of the KWMTBOMO05106 gene (Gene ID: 110384858) in various organs was analyzed by RT-PCR.

4. Discussion

We have shown that arylsulfatase activity is present in the silkworm intestinal tract, and it seems most likely that the arylsulfatase is produced in the silk glands, secreted from the spinneret during feeding, and then ingested into the gut. In this study, it was shown for the first time that a metabolic enzyme (*i.e.*, silkworm arylsulfatase B) for xenobiotics is expressed in the silk gland and secreted from the spinnerets and transported to the intestinal tract. As this enzyme is expressed in the silk gland, it may be possible that the presence of the enzyme may influence the composition of the silk glands, but so far there are no reports of sulfated

Table 2. Majority of arylsulfatase activity existed in the silk gland

Tissue	Sulfatase activity (U/tissue)
Silk gland	8,000
Muscle	2,600
Intestine content	2,000
Head	490
Hemolymph	250
Midgut	190
Fat body	120

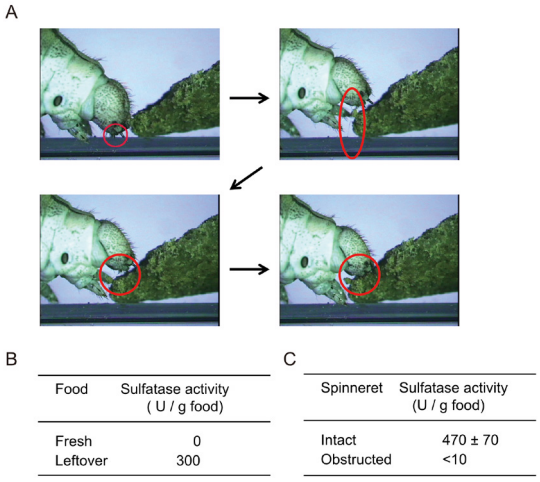

Figure 3. Silkworm Arylsulfatase B was secreted from the spinneret and transferred into the gut *via* feeding. A. Silkworm secret mucus from the spinneret and feeds on mucus-coated food. **B**. Arylsulfatase activity in food left uneaten by silkworms. **C**. Arylsulfatase activity in the intestine is reduced by obstruction of the spinneret (n = 3, data are shown as mean ± S.D.).

compounds in the silk glands that can be degraded by arylsulfatase. It is therefore likely that the physiological function of arylsulfatase B may be a digestive enzyme, and it works after being secreted from the spinneret, but not in the silk gland. In the literature, enzymes in mucus secreted from the spinneret are known to inhibit the biosynthesis of green lead volatiles (an attractant for other insects) (*27*). In plants, sulfate conjugates are produced in flavonoid metabolism by sulfotransferases. Thus, it may be interesting to focus on the interaction between the secreted arylsulfatase and plant-derived substances. In addition to arylsulfatase, other enzymes such as α-glycosidase, lipase, and lysozyme were highly expressed in the silk gland (Supplementary Table S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=125*). This suggests the presence of a group of enzymes with functions similar to arylsulfatase B. Nevertheless, silkworms covered the spinneret with sticky bonds showed no change in weight gain when fed artificial feed or mulberry leaves. Therefore, the significance of this enzyme for short-term growth may be limited, and the nutritional impact of the enzyme in the silkworm needs further

investigation.

In humans, arylsulfatase is involved in the metabolism of steroid hormones. Similarly, sulfotransferases catalyze sulfate conjugation reactions of juvenile hormones, ecdysone, and 20-hydroxysteroids in several insect species (*28*), but sulfate conjugates are rarely detectable in the body of insects (*29*). Based on the present finding, it may be because the sulfated steroid hormones in the intestine are rapidly metabolized to non-sulfated form by the arylsulfatase in the intestinal tract.

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Brief Report

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WRQ-2, a gemcitabine prodrug, reverses gemcitabine resistance caused by hENT1 inhibition

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SUMMARY Gemcitabine is widely used in the clinic as a first-line antitumor agent. However, intrinsic and acquired resistance hinders its wide clinical application. In this study, a gemcitabine prodrug nominated as WRQ-2 was designed and synthesized by conjugating gemcitabine with the indole-3-methanol analogue OSU-A9 through a carbamate linkage. WRQ-2 exhibited high cytotoxicity against six cancer cell lines (HeLa, A549, MDA-MB-231, HuH-7, MGC-803, and HCT-116) with IC_{50} values in low micromolar range. WRQ-2 reversed the resistance of HeLa cells to gemcitabine caused by hENT1 inhibition. Compared to gemcitabine, WRQ-2 induced a higher degree of DNA damage and apoptosis in the presence of hENT1 inhibitor. Our study suggests that compound WRQ-2 is a potential gemcitabine prodrug and worth of further antitumor activity investigation.

Keywords Gemcitabine, prodrug, drug resistance, OSU-A9, cancer

1. Introduction

Pyrimidine nucleoside analogs are an important class of chemotherapeutic agents with excellent cytotoxic activity against a wide range of cancers (*1,2*). Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is one of the representative drugs which was first synthesized by Hertel *et al*. in 1988 and is the first-line drug for pancreatic cancer and non-small cell lung cancer since it was approved by the FDA in 1996 (*3,4*). Gemcitabine functions as a prodrug that needs to be activated intracellularly before exerting antitumor effect (*5*). Gemcitabine is transported into the cell mainly *via* human equilibrative nucleoside transporter 1 (hENT1) (*6,7*) and then, it is phosphorylated by deoxycytidine kinase (dCK) to gemcitabine monophosphate (dFdCMP), and dFdCMP is successively phosphorylated by nucleoside monophosphate and diphosphate kinase to gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) (*8,9*). dFdCTP can replace the site of deoxycytidine and competitively bind into the DNA strand which results in the cessation of DNA strand synthesis, DNA breakage and cell death (*10,11*). In addition, gemcitabine can lead to depletion of dNTPs pool by inhibiting cytidine-5'-triphosphate (CTP) synthase (*12*).

Although gemcitabine plays important roles in the clinical treatment of cancers, its wide use is greatly hindered due to intrinsic or acquired drug resistance. There are three main mechanisms leading to drug resistance, (*i*) downregulation of dCK, the rate-limiting enzyme for the first step of phosphorylation, resulting in insufficient conversion to the dFdCDP and dFdCTP forms (*13,14*); (*ii*) lack of nucleoside transport proteins responsible for bringing gemcitabine into tumor cells, including hENT1 and concentrative nucleoside transporter 1 (CNT1), which makes it difficult for gemcitabine to enter tumor cells; (*iii*) overexpression of cytidine deaminase (CDA), which is able to metabolize gemcitabine to the inactive 2',2'-difluoro-2'-deoxyuridine (dFdU) (*15*). Therefore, there is an urgent need to improve the efficiency of gemcitabine. To overcome the problems of drug resistance and poor metabolic stability, prodrugs of gemcitabine with different structural features are widely developed *via* structural modification of either 4-amino or 5'-hydroxyl groups to attenuate drug resistance and improve efficiency (*16,17*).

Indole-3-carbinol (I3C) is a broadly existing phytochemical that has been proven in animal studies to prevent carcinogenic effects and inhibit the proliferation of human breast, colon, prostate, and endometrial cancer cells (*18*). Furthermore, mechanistic evidence suggests that I3C was able to induce growth arrest and apoptosis by regulating a broad range of signaling pathways associated with cell cycle regulation and survival (*19,20*). OSU-A9, an I3C analogue, possesses apoptosis-inducing

capacity which is much more potent than that of the parental I3C. In addition, OSU-A9 retains the pleiotropic potency of I3C against multiple signaling pathways associated with growth arrest and apoptosis, while normal cells are less sensitive (*21*).

On the other hand, in addition to using as monotherapy, gemcitabine has shown good synergistic effects in combination with other anticancer drugs such as kinase inhibitors (*22,23*) and histone deacetylase inhibitors (*24,25*). For example, in 2016, Jiang *et al*. combined gemcitabine and ubenimex to design novel interacting prodrugs, which released gemcitabine intracellularly and showed desirable cytotoxicity (*26*). As previously mentioned, 4-amino acids are affected by CDA metabolism, which reduces the efficacy of gemcitabine, and this challenge can be solved by modifying 4-amino group to construct prodrugs. The structures of 4-amino-based gemcitabine prodrugs mainly include amides and carbamates, which can be readily hydrolyzed in tumor cells to release free gemcitabine. Based on the results of existing studies, we designed and synthesized a novel gemcitabine prodrug, named as WRQ-2, to link OSU-A9 with the 4-amino group of gemcitabine through a carbamate bond. WRQ-2 displayed comparable antitumor activity to gemcitabine and attenuate the drug resistance (Figure 1).

2. Materials and Methods

2.1. Cell lines and culture

A549 (human lung cancer cell line), MDA-MB-231 (human breast cancer cell line), HuH-7 (human hepatoma cell line), and MGC-803 (human gastric cancer cell line) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. HeLa (human cervical cancer cell line) cells were cultured in modified Eagle medium (MEM, Gibco) supplemented with 10% fetal bovine serum. HCT-116 (human colorectal cancer cell line) cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 10% fetal bovine serum. These cells were obtained from the National Infrastructure of Cell Line Resource (Beijing, China).

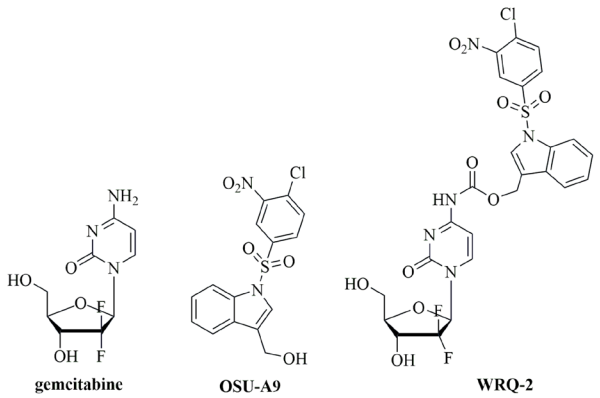


Figure 1. The structures of gemcitabine, OSU-A9, and the prodrug WRQ-2.

2.2. Cell proliferation assay

Cancer cells inoculated in 96-well plates (5×10^3 per well) were exposed to the indicated drugs for 72 h. In the assay for gemcitabine (Energy Chemical, Shanghai, China) resistance, tumor cells were pre-incubated with dipyridamole (10 μM; Energy Chemical), which is a well-documented hENT1 inhibitor, three hours before exposure to various concentrations of WRQ-2 or gemcitabine for 72 h. Then, the medium was removed and the wells were washed with phosphate-buffered saline (PBS, Gibco). Fifteen microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) were added to each well at a working concentration of 5 mg/mL. After 4 h incubation, the medium was removed and 150 μL of DMSO was added to each well to dissolve the crystals. The absorbance was measured at 490 nm on a microplate reader (Tecon, Switzerland).

2.3. Cell apoptosis assay

Apoptosis rates were assessed using the Annexin V-FITC/PI apoptosis detection kit (Solarbio, Beijing, China) according to the manufacturer's instructions. HeLa cells were inoculated into 6-well tissue culture plates (4×10^5 cells/well). After 36 h of drug treatment, cells were collected, washed with PBS, and resuspended in 1 mL of binding buffer. Then, 5 μL Annexin V-FITC and 5 μL PI were added to the binding buffer and incubated for 15 min at room temperature and protected from light. Cells were analyzed by flow cytometry (Beckman Coulter, Bria, California, USA) within 1 hour.

2.4. Western blot

HeLa cells were seeded in a six-well transparent plate (5×10^5 cells/well). Different concentrations of compounds WRQ-2 or gemcitabine were added to cells followed by incubation for 24 h. PBS was utilized to harvest and wash the cells three times. After that, RIPA cell lysis buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to lyse the cells on ice for a half-hour, followed by centrifugation of the cell lysates at 12,000× g for 15 min at 4°C. After collecting the supernatant from the centrifuge, bicinchoninic acid (BCA, Thermo Fisher Scientific) protein assay was conducted to determine the protein concentration. Subsequently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of protein (20 μg). After transferring the proteins to poly(vinylidene fluoride)

membranes, the proteins were blocked with 5% milk. After incubation with the primary antibody at 4°C for 12 h, the membranes were washed with TBST three times and incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence reagent and detected by an ECL Western blotting detection system (Bio-Rad, Hercules, California, USA).

2.5. Statistical analyses

Data were expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests. The value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS software.

3. Results and Discussion

3.1. Chemistry

The synthesis route of WRQ-2 was outlined in Scheme 1. The compound 1 was obtained following the method reported by Che *etc*. (*27*). Two starting materials condensed under the condition of K_2CO_3 in anhydrous dichloromethane (DCM). The aldehyde group of compound 1 was reduced by NaBH4 to afford the compound 2.

On the other hand, the double tert-butyldimethylsilyl (TBS) protected gemcitabine (compound 3) was obtained by using the method reported by Li, *etc*. (*28*). Compound 2 and 3 condensed to form the carbamate under the condition of triphosgene to give the compound 4. Finally, two TBS groups of compound 4 were removed by tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) to afford the targeted compound WRQ-2 (Appendix).

3.2. Cell proliferation inhibition

The effect of WRQ-2 on cell viability against A549, MDA-MB-231, HuH-7, MGC-803, HeLa, and HCT-116 cells was determined using an MTT assay with gemcitabine as the positive control. WRQ-2 showed significant antiproliferative activity against all six tumor cells with IC_{50} values in the μM to the sub-μM range (Table 1), comparable to gemcitabine, which indicated that the carbamate bond of WRQ-2 might be cleaved intracellularly to release gemcitabine.

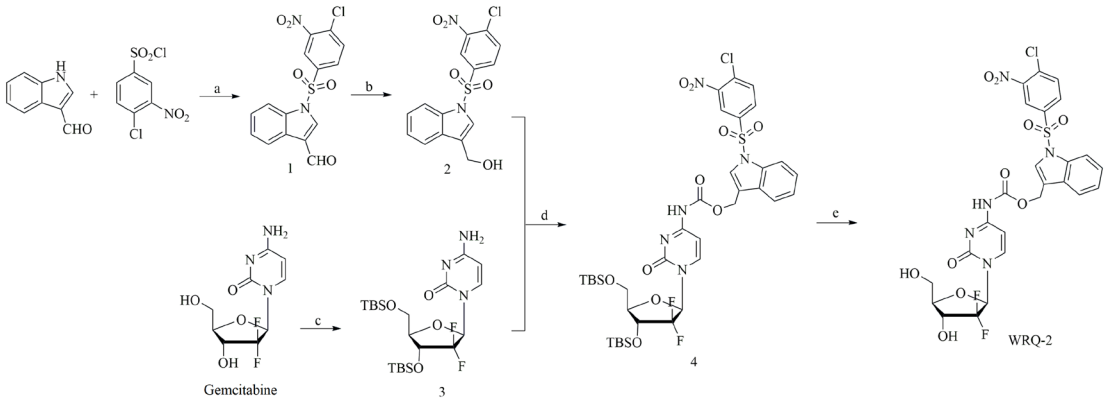
To investigate whether the compound WRQ-2 could reverse gemcitabine drug resistance suffering from low expression of hENT1, the cytotoxic effects of WRQ-2 and gemcitabine were examined by incubating HeLa cells with the hENT1 inhibitor dipyridamole at a concentration of 10 μM. The results showed that the cytotoxicity of gemcitabine was significantly diminished when cells were pretreated with dipyridamole, with IC_{50} values increased from 0.13 to 1.76 μM, by approximately 10-fold. In contrast, the cytotoxicity of WRQ-2 was not noticeably affected (0.17 μM for single treatment *vs*. 0.25 μM for co-treatment) which suggested that WRQ-2 may have the potential to reverse gemcitabine drug resistance caused by low expression of hENT1.

3.3. Induction of DNA damage in HeLa cells

The metabolite of gemcitabine can replace the site of deoxycytidine in the DNA strand by competitive

Table 1. Cytotoxic activity of compound WRQ-2 and gemcitabine on tumor cells

Tumor cells	*In vitro* cytotoxicity IC_{50} (μM)	
	WRQ-2	gemcitabine
HeLa	0.17 ± 0.03	0.13 ± 0.01
MGC-803	0.07 ± 0.01	0.04 ± 0.01
HCT-116	0.79 ± 0.09	0.51 ± 0.18
A549	1.05 ± 0.09	0.82 ± 0.06
HuH-7	1.43 ± 0.11	0.93 ± 0.13
MDA-MB-231	2.58 ± 0.27	3.02 ± 0.48



Scheme 1. Reagents and methods. (a) K_2CO_3, anhydrous dichloromethane, room temperature, 20 h; **(b)** $NaBH_4$, MeOH, 0°C to room temperature, 12 h; **(c)** *t*-butyldimethylsilyl chloride, imidazole, anhydrous *N*,*N*-dimethylformamide, room temperature, 24 h; **(d)** triphosgene, triethylamine, anhydrous dichloromethane; **(e)** tetrabutylammonium fluoride, tetrahydrofuran, room temperature, 0.5 h.

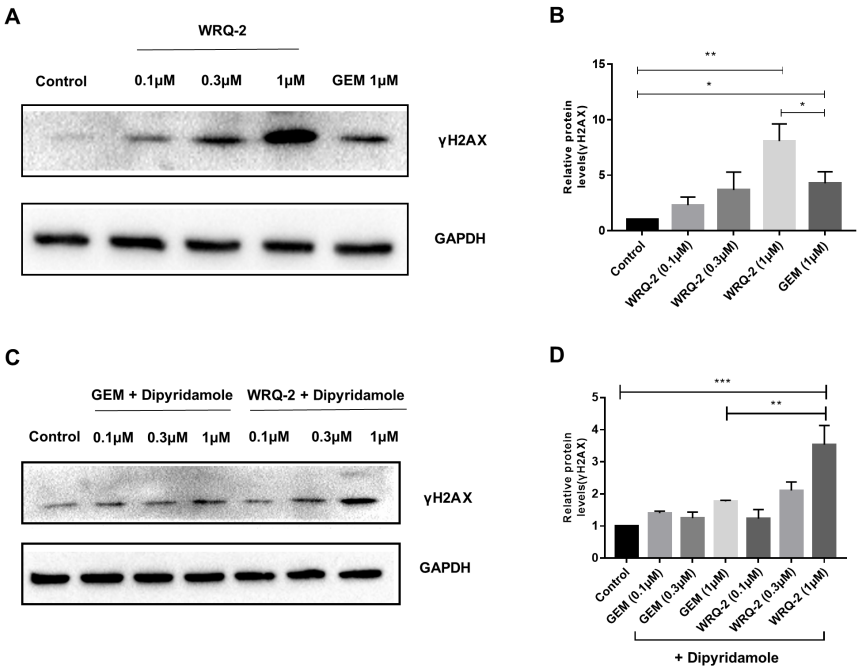


Figure 2. WRQ-2 and gemcitabine increase γH2AX protein expression in HeLa cells. (A, B) γH2AX expression was measured using western blot analysis in HeLa cells treated with WRQ-2 or gemcitabine for 24h**. (C, D)** Cells were pretreated with dipyridamole for 3 h and then exposed to gemcitabine or WRQ-2 for 24 h. γH2AX expression was measured using western blot analysis. GEM, gemcitabine. *p<0.05, **p< 0.01.

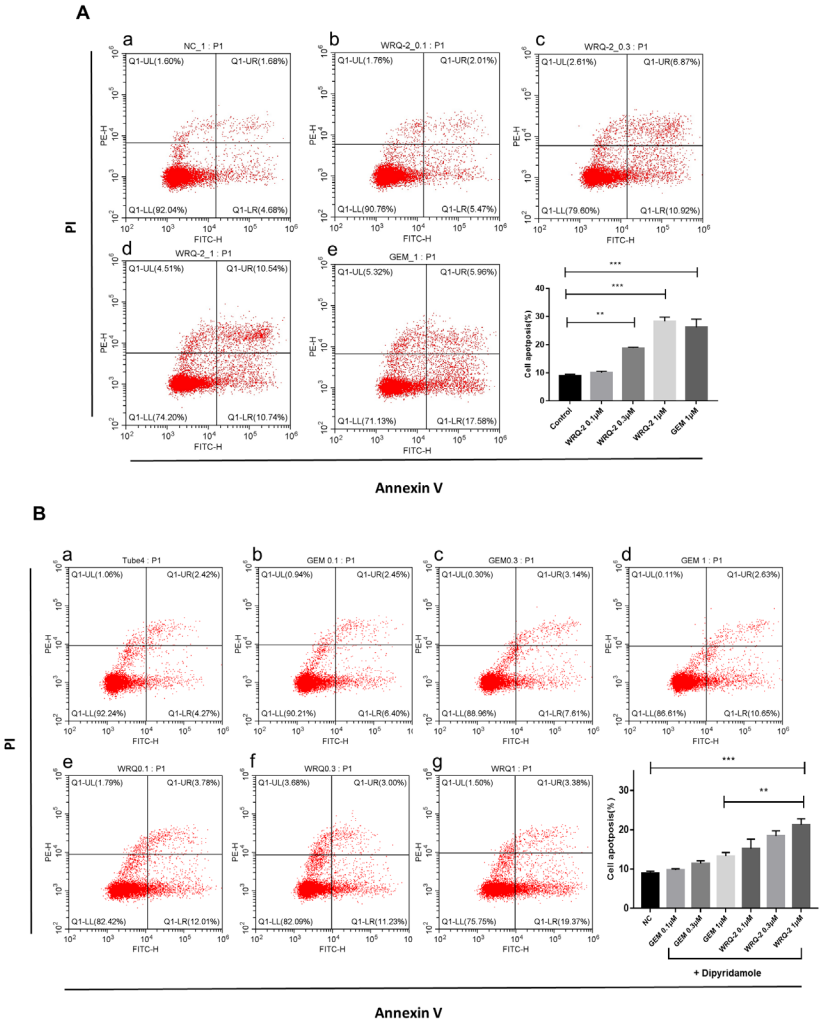


Figure 3. WRQ-2 and gemcitabine induce apoptosis in HeLa cells. (A) After incubation of cells with different concentrations of WRQ-2 or gemcitabine for 36 h, HeLa cells were stained with Annexin V/PI and the apoptosis rate was measured by flow cytometry analysis. **a**, vehicle; **b**, 0.1 μM WRQ-2; **c**, 0.3 μM WRQ-2; **d**, 1 μM WRQ-2; **e**, 1 μM gemcitabine. (**B**) HeLa cells were first pretreated with 10 μM dipyridamole for 3 h. After incubation with different concentrations of WRQ-2 or gemcitabine for 36 h, cells were stained with Annexin V/PI and the apoptosis rate was measured by flow cytometry analysis. **a**, vehicle; **b**, 0.1 μM gemcitabine + 10 μM dipyridamole; **c**, 0.3 μM gemcitabine + 10 μM dipyridamole; **d**, 1 μM gemcitabine + 10 μM dipyridamole; **e**, 0.1 μM WRQ-2 + 10 μM dipyridamole; **f**, 0.3 μM WRQ-2 + 10 μM dipyridamole; **g**, 1 μM WRQ-2 + 10 μM dipyridamole. GEM, gemcitabine. **p<0.01, ***p<0.001.

incorporation into the DNA strand, resulting in the cessation of DNA strand synthesis and DNA damage. Accordingly, we examined the changes of the expression level of γH2AX, a marker of DNA strand damage. As shown in Figures 2A and 2B, WRQ-2 significantly increased the level of γH2AX, stronger than gemcitabine at the concentration of 1 μM.

When cells were pretreated with dipyridamole, WRQ-2 still significantly induced an elevated expression of γH2AX, while gemcitabine induced much lower level (Figures 2C and 2D). These results suggested that WRQ-2 was capable of inducing more DNA damage than gemcitabine at the same dose in a hENT1 independent manner.

We noted that WRQ-2 increased the level of γH2AX expression in HeLa cells, suggesting that WRQ-2 may be able to induce apoptosis in HeLa cells. We analyzed the apoptosis induced by WRQ-2 and gemcitabine in HeLa cell lines. HeLa cells were exposed to WRQ-2 (0.1, 0.3, or 1μM) and gemcitabine (1 μM) at different concentrations for 36 h. Apoptosis of HeLa cells was subsequently detected by AnnexinV/PI staining and flow cytometry. As shown in Figure 3A, WRQ-2 could dose-dependently induced apoptosis in HeLa cells, and the degree of apoptosis was similar to that of gemcitabine at the concentration of 1 μM. When cells were pretreated with dipyridamole, the apoptosis-inducing effect of gemcitabine was inhibited, while WRQ-2 were less affected (Figure 3B). These results suggest that WRQ-2 can initiate apoptotic signaling in the presence of hENT1 inhibition.

4. Conclusion

In conclusion, a novel gemcitabine prodrug, WRQ-2, was rationally designed by conjugating with the antitumor compound OSU-A9. Compared with gemcitabine, WRQ-2 showed similar antiproliferative activity against six tumor cell lines with IC_{50} values in the μM to the sub-μM range. Furthermore, the cell proliferation inhibition capacity of WRQ-2 was not obviously affected by the inhibition of hENT1, whose low expression is one of the main reasons for gemcitabine resistance. In the presence of hENT1 inhibitor, the ability of gemcitabine to induce cellular DNA damage and apoptosis became weaker, while WRQ-2 was less affected, suggesting that WRQ-2 may enter cells in a hENT1 independent manner to exert antitumor effects. These results suggest that WRQ-2 is a promising gemcitabine prodrug that deserves further preclinical antitumor evaluation.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Appendix

For experimental work, chemical reagents and solvents are purchased from Energy Chemical and were used directly without any purification. The purity of the compounds was confirmed by thin-layer chromatography (TLC). Spots were visualized under ultraviolet lamp. ^{1}H NMR spectra in DMSO or $CDCl_3$ solution were respectively recorded at CDRI, Lucknow on NMR spectrometer (400 MHz, Bruker-400 Ultra shield TM) using TMS [$(CH_3)_4Si$] as internal standard. Splitting patterns are nominated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded at CDRI, Lucknow on Mass spectrometer (Waters Synapt).

1-((4-chloro-3-nitrophenyl)sulfonyl)-1H-indole-3-carbaldehyde (1)

Indole-3-carbaldehyde (1 g, 6.8 mmol, 1.0 eq.) and 4-chloro-3-nitrobenzenesulfonyl chloride (3.52 g, 13.6 mmol, 2.0 eq.) were mixed in dry DCM. To this solution was added of K_2CO_3 (2.84 g, 20.4 mmol, 3.0 eq.). The mixture was refluxed for 20 h. The crude was purified by PE : EtOAc = 10:1 to obtain compound 1 2.2 g with yield of 88%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.13 (s, 1H), 8.47 (d, J = 2.5 Hz, 1H), 8.30 (d, J = 8.0 Hz, 1H), 8.18 (s, 1H), 8.06 (dd, J = 8.5 Hz, 2.5 Hz, 1H), 7.94 (d, J = 8.5 Hz, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.46-7.49 (m, 1H), 7.40-7.43 (m, 1H). EI-MS (m/z) Calcd for $C_{15}H_{10}ClN_2O_5S^+$ $[M+H]^+$ 365, found: 365.

(1-((4-chloro-3-nitrophenyl)sulfonyl)-1H-indol-3-yl) methanol (2)

Compound 1 (2 g, 5.4 mmol, 1.0 eq.) was dissolved in MeOH at 0°C, To this solution was added of $NaBH_4$ (0.2 g, 5.4 mmol, 1.0 eq.). After the mixture was stirred at 0 °C for 30 min, it was transferred to room temperature and stirred for 12 h. After extraction with EtOAc, concentrate solvent *in vacuo* to obtain compound 2 (1.95 g, 97%) which was used in the next step without purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.74 (d, J = 2.2 Hz, 1H), 8.25 (dd, J = 8.6, 2.2 Hz, 1H), 7.99 (dd, J = 8.4, 6.3 Hz, 2H), 7.71-7.64 (m, 2H), 7.39 (t, J = 7.4 Hz, 1H), 7.31 (t, J = 7.4 Hz, 1H), 5.18 (t, J = 5.4 Hz, 1H), 4.62 (d, J = 5.4 Hz, 2H). EI-MS (m/z) Calcd for $C_{15}H_{12}ClN_2O_5S^+$ $[M+H]^+$ 367, found: 367.

4-amino-1-((2R,4R,5R)-4-((tert-butyldimethylsilyl) oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)-3,3-difluorotetrahydrofuran-2-yl)pyrimidin-2(1H)-one (3)

Gemcitabine (1 g, 3.8 mmol, 1.0 eq.) was dissolved in 25

mL of DMF. To this solution was added of imidazole (0.78 g, 11.4 mmol, 3.0 eq.) and of TBSCl (1.5 g, 9.5 mmol, 2.5 eq.). The mixture was stirred at 25°C for 24 h. The crude was purified by EtOAc : PE = 3:1 to obtain compound 4 (1.74g, 93%). ^{1}H NMR (400 MHz, DMSO-d^6) δ 7.54 (d, *J* = 7.5 Hz, 1H), 7.41 (d, *J* = 4.0 Hz, 2H), 6.17 (t, *J* = 8.0 Hz, 1H), 5.77 (d, *J* = 7.5 Hz, 1H), 4.31 (dd, *J* = 20.7, 12.2 Hz, 1H), 3. 95- 3.88 (m, 2H), 3.77 (dd, *J* = 12.1, 2.9 Hz, 1H), 0.89 (d, *J* = 6.5 Hz, 18H), 0.13-0.06 (m, 12H). EI-MS (m/z) Calcd for $C_{21}H_{40}F_2N_3O_4Si_2^+$ $[M+H]^+$ 492, found: 492.

(1-((4-chloro-3-nitrophenyl)sulfonyl)-1H-indol-2-yl) methyl (1-((1R,3R,4R)-3-((tert-butyldimethylsilyl) oxy)-4-(((tert-butyldimethylsilyl)oxy)methyl)-2,2-difluorocyclopentyl)-2-oxo-1,2-dihydropyrimidin-4-yl)carbamate (4)

A solution of triphosgene (134 mg, 0.45 mmol, 1.0 eq.) in dry DCM (5 mL) was added dropwise to a stirred solution of compound 3 (555 mg, 1.13 mmol, 2.5 eq.) and anhydrous Et_3N (313 μL, 2.25 μmol, 5.0 eq.) in dry DCM (10 mL) and stirred at 20°C for 2 h. A solution of compound 2 (414 mg, 1.13 mmol, 2.5 eq.) in dry DCM (5 mL) was added dropwise, and the solution stirred at 20°C for 24 h. The reaction mixture was condensed and the residue purified by chromatography, eluting with PE/EtOAc (v/v = 1:2) to get the pure compound 4. Yield: (380 mg, 38%) as a colorless oil. ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.66 (d, *J* = 2.2 Hz, 1H), 8.19 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.96-7.88 (m, 4H), 7.63 (d, *J* = 7.8 Hz, 1H), 7.34 (t, *J* = 7.8 Hz, 1H), 7.25 (t, *J* = 7.8 Hz, 1H), 7.02 (d, *J* = 7.5 Hz, 1H), 6.11 (t, *J* = 7.3 Hz, 1H), 5.24 (s, 2H), 4.33-4.22 (m, 1H), 3.90-3.87 (m, 2H), 3.71 (dd, *J* = 12.2, 2.8 Hz, 1H), 0.81 (s, 9H), 0.79 (s, 9H), 0.01-0.00 (m, 12H). EI-MS (m/z) Calcd for $C_{37}H_{49}ClF_2N_5O_{10}SSi_2^+$ $[M+H]^+$ 884, found: 884.

(1-((4-chloro-3-nitrophenyl)sulfonyl)-1H-indol-2-yl)methyl (1-((1R,3R,4R)-2,2-difluoro-3-hydroxy-4-(hydroxymethyl)cyclopentyl)-2-oxo-1,2-dihydropyrimidin-4-yl)-l4-azanecarboxylate (WRQ-2)

Compound 4 (88.3 mg, 0.1 mmol, 1.0 eq.) was dissolved in 5 mL THF, TBAF (1.0 M in THF, 300 μL, 3.0 eq.) was added and the mixture reacted at room temperature for 30 min. After the reaction, the solvent was concentrated in vacuum and the crude product was purified by DCM: MeOH = 20:1 to obtain the final product WRQ-2 as a white solid (60.3 mg, 92 %). ^{1}H NMR (400 MHz, DMSO-d^6) δ 8.40 (d, *J* = 2.6 Hz, 1H), 8.25 (d, *J* = 7.7 Hz, 1H), 7.95-7.93 (m, 2H), 7.80 (dd, *J* = 9.1, 2.6 Hz, 1H), 7.71 (d, *J* = 7.8 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.31 (t, *J* = 7.4 Hz, 1H), 7.12 (d, *J* = 7.6 Hz, 1H), 6.92 (d, *J* = 9.1 Hz, 1H), 6.33 (brs, 1H), 6.16 (t, *J* = 7.4 Hz, 1H), 5.35 (s, 2H), 4.20 (dd, *J* = 21.2, 12.7 Hz, 1H), 3.89 (dt, *J* = 8.5, 2.9 Hz, 1H), 3.83-3.80 (m, 1H), 3.66 (dd, *J* = 12.7, 3.5 Hz, 1H). EI-MS (m/z) Calcd for $C_{25}H_{21}ClF_2N_5O_{10}S^+$ $[M+H]^+$ 656, found: 656.

Brief Report

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Membrane translocation of vinculin after UVA exposure facilitates melanosome trafficking

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SUMMARY Skin pigmentation is among the defenses against ultraviolet (UV) radiation. During formation of skin pigmentation, melanosomes that are transported to the cell membrane and released are internalized by keratinocytes. We here examined whether vinculin, the origin of actin fibers, is involved in this intracellular transport of melanosomes by using melanocytes with suppressed vinculin expression. Using fluorescence immunostaining, the migration of vinculin to the cell membrane due to exposure to 365-nm LED light was examined. The intracellular distribution of melanosomes after irradiation was weighted toward the pericellular region compared with non-irradiated cells. With the suppression of vinculin expression, the amount of extracellularly released melanin decreased. We conclude that the membrane migration of vinculin after UVA exposure is involved in the intracellular transport of melanosomes.

Keywords vinculin, melanocyte, melanosome trafficking, actin filament, ultraviolet

1. Introduction

Sunlight reaching the Earth's surface includes ultraviolet (UV), visible, and infrared wavelengths. Of these, the UVB and UVA wavelengths are most relevant to skin homeostasis, as both have high energy and trigger exposed skin to produce various responses (*1,2*). UVA and UVB exposure causes erythema, sunburn, and pigmentation (*3*). Such skin responses protect organisms from the harmful effects of UV light, and skin pigmentation is a typical response. After UV exposure, various bioactive factors that enhance melanin formation in melanocytes are produced. Melanin is synthesized within mature melanosomes. Mature melanosomes are transported near the cell membrane and then incorporated into keratinocytes (*4*). Intracellular transport of melanosomes has been reported as a mechanism of microtubule trafficking and transport on actin fibers as in other cell organelles (*5,6*). However, factors determining the destination of melanosomes remain unknown.

While investigating proteins that are preferentially altered after UVB exposure, we found that UVB exposure increased the amounts of vinculin at the cell membrane. Vinculin connects the cell membrane to actin fibers (*7*). Actin fibers are used as rails for the transport of melanosomes after UVB exposure, and inhibition of actin fiber formation inhibits the transport of melanosomes to the cell membrane (*8*).

We here examined whether wavelengths in the UVA region, which have a significant effect on pigmentation, are involved in vinculin translocation to the cell membrane in addition to those in the UVB region. Furthermore, to clarify the function of vinculin transferred to the cell membrane after UVB exposure, we verified our hypothesis that actin fibers, which are formed after vinculin is transferred to the plasma membrane, are involved in melanosome trafficking.

2. Materials and Methods

2.1. Cell lines

The mouse melanoma cell line B16 was supplied by Health Science Research Resources Bank. The B16 cells were cultured in DMEM medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Moretate Biotech, Bulimba, Australia). Human melanocytes and cultured media were purchased from Cell Applications (San Diego, CA).

2.2. Sample preparation

A total of 10^6 cells of B16 cells were seeded in a 10-cm petri dish. After culturing the cells for 24 h, they were

exposed to UVA at 20 W m^{-2} by using a 365-nm LED light source. At 24 h after UVA irradiation, the cells were lysed with RIPA buffer and centrifuged at 10,000× *g* and 4°C for 30 min. The supernatants were stored at −80°C for western blot analysis.

2.3. Immunocytochemistry

B16 cells were seeded on culture slides (Corning Inc., Corning, NY). After culturing the cells for 24 h, they were exposed to UV at 20 W m^{-2} by using the 365-nm LED light source. At 24 h after UV irradiation, the cells were fixed with 4% paraformaldehyde for 10 min. After blocking the cells with normal goat serum (1:50) for 30 min at room temperature, they were incubated with rabbit anti-vinculin antibody (1:100; Bethyl Laboratories, Montgomery, TX) overnight at 4°C, followed by secondary Hilyte-488-conjugated anti-rabbit goat antibody (1:500; BioSource International, Camarillo, CA) for 1 h at room temperature. The cells were then stained for actin filaments with rhodamine-conjugated phalloidin for 1 h at room temperature and stained for nucleic acid with Hoechst 33342 (Dojindo, Kumamoto, Japan). Fluorescence was observed under a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan).

2.4. RNAi-induced vinculin knockdown assay

INTERFERin (Polyplus-transfection, Illkirch, France) was used to transfect vinculin siRNA (AGAAUCCAAGUGAACACUA(dTdT), Bioneer Corporation, Daejeon, Republic of Korea) into human melanocytes. At 72 h after transfection, vinculin expression was measured through western blot analysis.

2.5. Western blot analysis

Cell extracts (10 μg) were reduced with mercaptoethanol at 95°C for 10 min and then separated on a 12% polyacrylamide gel. Proteins were blotted on a nitrocellulose membrane (ClearTrans Nitrocellulose Membrane; Fujifilm Wako Pure Chemical, Osaka, Japan) by using a semi-dry blot system (NA-1512S, Nihon eido, Tokyo, Japan). The membranes were blocked with 2% skimmed milk. The blocked membranes were incubated with rabbit anti-vinculin antibody (1:1,000) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (1:5000; Fujifilm Wako Pure Chemical) and then with horseradish peroxidase-conjugated anti-rabbit IgG goat antibody (1:10,000; BioSource International) or HRP-conjugated goat anti-mouse IgG antibody (1:2500; BioSource International). The protein bands were subsequently visualized with a ImmunoStar Zeta by using a luminograph apparatus (Atto, Tokyo, Japan).

2.6. Microscopic observation of melanosome localization in B16 melanoma cells

B16 cells were seeded onto a 6-well plate (TrueLine, San Jose, CA). After incubation for 24 h, the cells were exposed to UVA at 20 W m^{-2} by using the 365-nm LED light source for 15 min. At 24 h after UVA irradiation, the distribution of melanosomes in B16 cells were observed using a microscope (BZ-X700, Keyence).

2.7. Release of melanosomes from human melanocytes

Vinculin siRNA-transfected human melanocytes were treated with 100 nM α-MSH, then the cells were irradiated with UVA at 20 W m^{-2} by using the 365-nm LED light source for 15 min. To evaluate the effect of actin fibers on melanin transport, a similar experiment was conducted using cytochalasin D to inhibit actin fiber formation. After 24 h of UVA irradiation, melanosome release was measured spectrophotometrically at 475 nm.

2.8. Statistical analysis

We analyzed the results *via* Tukey's test in R (version 4.1.2, R Development Core Team), and p values < 0.05 were considered to indicate statistical significance.

3. Results and Discussion

We previously reported that exposure to 312-nm UV light, which is in the UVB region (*7*), changes vinculin localization at the plasma membrane. By using fluorescence immunostaining, we here confirmed that exposure to 365-nm LED light induces the migration of vinculin to the cell membrane (Figures 1A and 1B). Vinculin, stained green, was observed in the cell plasma before irradiation (Figure 1A) but migrated to the cell membrane (Figure 1B) and co-localized with actin after exposure. Vinculin, an adhesion apparatus-lining protein, localizes to both the integrin-mediated cell extracellular

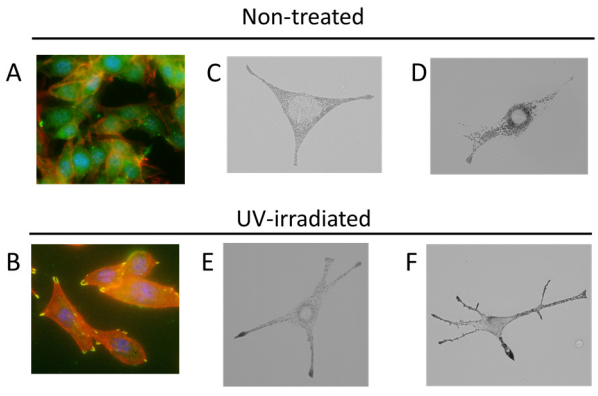

Figure 1. Distribution of vinculin and melanosomes in B16 cells after UVA irradiation. Immunohistochemistry of vinculin in melanocytes treated without (**A**) and with (**B**) UVA radiation. Vinculin is stained with Hylite-488 (green), actin filament is stained with rhodamine-conjugated phalloidin (red), and nuclei are stained with Hoechst (blue). The localization of melanosomes in cells treated with (**C**, **D**) and without UVA radiation (**E**, **F**) is shown.

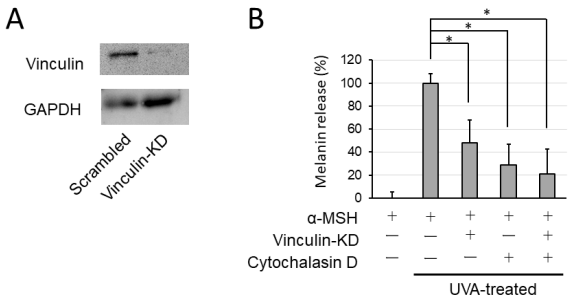

Figure 2. Effect of vinculin expression on melanin release from melanocytes. Western blot analysis of siRNA-mediated suppression of vinculin expression (**A**). Assessment of melanin release from melanocytes (**B**). α-MSH-stimulated melanocytes; melanin release in the absence of UVA irradiation was defined as 0% and that after UVA exposure was defined as 100%. Vinculin-KD indicates cells in which vinculin expression was suppressed with siRNA. Cytochalasin D was also used as an inhibitor of actin fiber formation. Bars indicate means ± SEM ($n = 3$). *: $p < 0.05$.

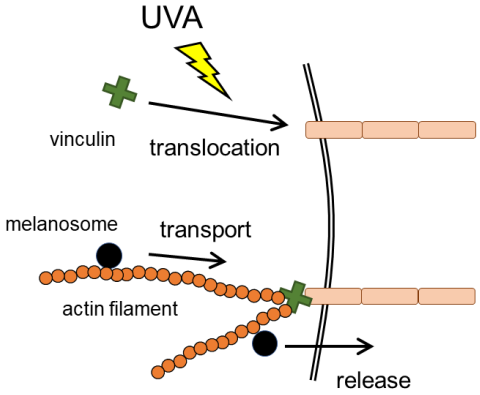

Figure 3. Involvement of vinculin migration and melanosome trafficking after UV exposure. This schematic diagram shows vinculin in membrane migration and melanosome trafficking after UV exposure.

matrix adhesion apparatus and cadherin-mediated cell-cell adhesion apparatus (*9-11*). After binding to actin, vinculin localizes to the force-associated adhesion apparatus to regulate cell adhesion and extension. In this study, exposure to wavelengths in the UVA region caused distribution of vinculin to the cell membrane. Vinculin was uniformly distributed around the cell periphery, with no directionality observed in its localization. This suggests that membrane migration of vinculin causes melanocytes to remain in place rather than enhancing their motility. Moreover, a decrease in cell motility may be consistent with the decrease in melanocyte motility after UV exposure.

Additionally, melanosome localization in the 365-nm LED-exposed cells was observed under a bright-field optical microscope (Figures 1C-1F). In cells cultured without 365-nm LED light, melanosomes were distributed throughout the cells (Figure 1C and 1D), while in some irradiated cells, melanosomes were localized at dendritic sites within the cells (Figures 1E and 1F). Intracellularly formed actin filaments also serve as transport rails for intracellular organelles (*12*). In melanocytes, melanosomes mature at the nuclear periphery because of UV exposure. The motor proteins kinesin and dynein present in the melanosome membrane are involved in the transport of these mature melanosomes; microtubules act as transport rails (*5*). The melanosomes transported to the cell membrane periphery are transferred to actin fibers, which act as new transport rails. They are then transported by myosin-Va, an actin-dependent motor protein, to the cell membrane, from where they are transported to the dendrite terminals (*6*). The pericellular localization of UV-exposed melanosomes was predicted to be due to vinculin migration to the plasma membrane, which promoted actin fiber formation. To determine whether changes in 365-nm LED light-induced intracellular melanosome trafficking involve vinculin translocation to the plasma membrane, we examined changes in the extracellular release of melanin from human melanocytes in which vinculin expression was suppressed with siRNA. This suppressed vinculin expression was evaluated through western blotting. At 48 h after transfection with vinculin siRNA, the density of the vinculin band reduced to approximately 30% (Figure 2A). Next, the human melanocytes were treated with α-MSH to mature intracellular melanosomes. These melanocytes were then exposed to 365-nm LED light, and the amount of melanosomes migrating to the membrane due to LED exposure was evaluated by measuring the amount of melanin released in culture media (Figure 2B). To examine the effect of actin filament formation on melanosome trafficking and membrane migration of melanosomes, we treated cells with cytochalasin D, an inhibitor of actin filament formation. The 365-nm LED light increased the amount of extracellularly released melanin, whereas downregulation of vinculin expression reduced the amount of melanin released to approximately 49%. Similarly, cytochalasin D suppressed melanin release to approximately 28%. Melanin release was suppressed at about the same rate in both vinculin knockdown cells with cytochalasin D treatment and those without. These results indicate that membrane translocation of vinculin after 365-nm LED light exposure increases the number of actin fibers at the cell membrane, thereby increasing melanosome release. Additionally, suppression of vinculin expression in melanocytes decreased the extracellular release of melanosomes, suggesting that vinculin is involved in the intracellular trafficking of melanosomes. In addition, UV exposure regulates the expression of β-catenin, the origin of actin fibers, at the plasma membrane (*13*). This report supports that UV light increased the formation of actin fibers from the cell membrane.

In conclusion, vinculin was translocated to the cell membrane after UVA exposure, indicating its involvement in the intracellular trafficking of melanosomes (Figure 3). In addition, melanosomes

transported to the vicinity of the cell membrane were released extracellularly, thereby facilitating the supply of melanosomes to keratinocytes. Membrane translocation of vinculin increases the number of adhesion points to surrounding cells *via* cadherins. This increase may be involved in the defense of organisms against UVA light through an increase in the number of melanin-supplying keratinocytes or melanin-supplying pathways, which in turn enhances skin pigmentation.

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Valemetostat: First approval as a dual inhibitor of EZH1/2 to treat adult T-cell leukemia/lymphoma

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SUMMARY Adult T-cell leukemia/lymphoma (ATL) is a mature T-cell lymphoma with a poor prognosis. Accumulating trimethylation of histone H3 lysine 27 (H3K27me3) caused by upregulated function of either enhancer of zeste homologue 2 (EZH2) or its homolog EZH1 plays an essential role in the maintenance of transcriptional repression in ATL. Selective inhibition of EZH2 may complementarily induce EZH1 activation, so dual targeting EZH1/2 is a rational strategy in developing potent antitumor agents. Valemetostat is the first dual EZH1/2 inhibitor approved for treatment of aggressive ATL in Japan in September 2022. Several other dual EZH1/2 inhibitors such as HH2853, HM97594, and HM97662 have also demonstrated potential in treating malignant tumors. Dual targeting EZH1/2 may have promising antitumor action in hematological malignancies and solid tumors.

Keywords valemetostat, adult T-cell leukemia/lymphoma, EZH1, EZH2

Adult T-cell leukemia/lymphoma (ATL) is a mature T-cell lymphoma that is highly prevalent in southwestern Japan, the central and southern United States, and other regions due to chronic infection with human T-cell lymphotropic virus type 1 (HTLV-1) (*1,2*). In Japan, there are approximately one million HTLV-1 carriers in whom the estimated lifetime risk of developing ATL is 6-7% for men and 2-3% for women, and an estimated 1,000 deaths from ATL annually (*3,4*). ATL is classified into two indolent types (smoldering and favorable chronic) and three aggressive types (acute, lymphoma, and unfavorable chronic). Newly diagnosed aggressive ATL is mainly treated with conventional chemotherapy such as CHOP (a combination of cyclophosphamide, doxorubicin, vincristine, and prednisolone) or a CHOP-like regimen, followed by allogeneic hematopoietic stem cell transplantation (allo-HSCT) if criteria are met (*3*). In the past few years, mogamulizumab and lenalidomide has been used as salvage therapy for relapsed or refractory ATL (*5*). Although various treatment options are now available, the prognosis for ATL is still dismal, with a 4-year survival rate of 16.8% for acute ATL, 19.6% for lymphoma-ATL, 26.6% for unfavorable chronic ATL, 62.1% for favorable chronic ATL, and 59.8% for smoldering ATL (*3*). A novel strategy is urgently required for treatment of ATL.

Valemetostat, a dual inhibitor of enhancer of zeste homolog (EZH) 1 and EZH2 developed by Daiichi Sankyo, was approved for treatment of patients with relapsed or refractory ATL in Japan in September 2022 (*6*). This approval was based on the results of an open-label, single-arm, phase 2 trial in which 25 patients with relapsed or refractory ATL who had received a median of 3 prior lines of therapy had an overall response rate (ORR) of 48.0%, including complete remission in 5 patients and partial remission in 7 (*7*). Of note, 24 patients that had previous received mogamulizumab treatment had an ORR of 45.8% (complete remission in 4 patients and partial remission in 7) (*7*). Treatment-emergent adverse events including thrombocytopenia, anemia, alopecia, dysgeusia, neutropenia, lymphopenia, leukopenia, decreased appetite, and pyrexia were manageable and tolerated (*7*). Larger scale clinical studies are warranted to further investigate the efficacy and safety of valemetostat in the treatment of relapsed or refractory ATL.

In addition to dysregulation of transcription due to a genetic abnormality, abnormal epigenetic regulation of transcription plays an important role in carcinogenesis and cancer development. Trimethylation of histone H3 lysine 27 (H3K27me3), which is catalyzed by polycomb repressive complex 2 (PRC2), is a suppressive histone mark of chromatin condensation and gene silencing (*8*). In ATL, H3K27me3-mediated gene repression was reported to be correlated with poor prognostic markers (*9*). EZH1 or EZH2 functions as an enzymatically active

core subunit of PRC2 to methylate Lys-27 on histone 3 (H3K27) by transferring a methyl group from the cofactor *S*-adenosyl-*L*-methionine (*8*). The oncogenic role of EZH2 has attracted considerable attention since overexpression or somatic mutations (gain-of-function) of EZH2 has been found in many solid cancers and hematologic malignancies (*10-13*). Different types of EZH2 inhibitors that diminish the abundance of H3K27me3 and thus unleash the expression of tumor suppressive genes have been synthesized and tested for cancer treatment thus far. Tazemetostat is an EZH2 inhibitor that was approved for treatment of metastatic or locally advanced epithelioid sarcoma and relapsed follicular lymphoma in the US in 2020 (*14,15*). That said, some H3K27me3-high malignancies are reported to be relatively tolerant to EZH2 inhibitors (*16*). Yamagishi *et al*. noted a relationship between mutual interference and the compensatory function of co-expressed EZH1 and EZH2 in malignant lymphomas, suggesting a rationale for dual targeting EZH1 and EZH2 (*16*). A study has indicated that EZH1/2 dual inhibitors were superior to an EZH2 selective inhibitor in suppressing trimethylation of H3K27 and tumor cell proliferation both *in vitro* and *in vivo* (*17*). In ATL, EZH1 and EZH2 were found to be independently required for tumor cell proliferation (*16*), indicating the necessity of targeting both EZH1/2 in treatment of ATL.

The efficacy and safety of valemetostat in treating relapsed/refractory peripheral T-cell lymphoma (PTCL) and B-cell lymphoma is currently being investigated in two phase II clinical trials (*18*). Valemetostat displayed acceptable safety and encouraging preliminary efficacy in treatment of relapsed/refractory PTCL in a phase I study (*19*). Forty-five patients had an ORR of 55.6%, including a complete response in 11 and a partial response in 14 (*19*). Valemetostat is also being evaluated to treat tumors characterized by an SMARCB1/INI1 deficiency (a SWI/SNF mutation), such as malignant rhabdoid tumors, epithelioid sarcoma, or synovial sarcoma as are frequently observed during childhood and adolescence, in a phase I trial (*20*). Besides valemetostat, several other dual EZH1/2 inhibitors such as HH2853, HM97594, and HM97662 have demonstrated potential in treating malignant tumors. HH2853 is currently being tested for its efficacy and safety in treating relapsed or refractory non-Hodgkin's lymphoma in a phase 1/2 trial (*21-23*). Dual targeting EZH1/2 may have promising antitumor action in hematological malignancies and solid tumors.

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Molecular and immunological diagnosis of Monkeypox virus in the clinical laboratory

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SUMMARY The 2022 monkeypox outbreak outside Africa is ongoing. Cases have been reported in Hong Kong and Chongqing, China. In order to better prevent and control the potential spread of monkeypox virus in China, the development of sensitive and reliable detection commercial kits is imminent. This correspondence reviews the existing laboratory assays and related technologies for nucleic acid (PCR) and serological assays for the diagnosis of monkeypox virus to provide reference for the management and decision-making departments. Due to the serological cross-reactivity of orthopoxviruses, PCR is the laboratory test of choice to confirm monkeypox virus infection. We recommend a dual-target PCR approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence.

Keywords Monkeypox, diagnosis, PCR, *Orthopoxvirus*

The monkeypox outbreak has been recognized as a Public Health Emergency of International Concern (PHEIC) by the World Health Organization (WHO) on 23 July 2022 (*1*). Data as of 02 December 2022, it has currently caused 82, 062 cases and 65 deaths and has spread to more than 100 countries, 98.8% in locations that have not historically reported monkeypox (*2*). Before monkeypox, WHO classified only six outbreaks as PHEIC, including the most recent pandemic COVID-19 (the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)) that caused 644 million infections and 6.64 million deaths. Monkeypox can be transmitted to humans or certain animals. Human-to-human transmission of monkeypox is primarily through close contact with contaminated objects, such as skin lesions, body fluids, respiratory droplets and beddings. Interestingly, in the 2022 monkeypox outbreak, most cases were among men who had sex with men (MSM), and the disease displayed characteristics of a sexually transmitted infection (STI), with symptoms often confined to the anogenital region (*3*). Currently, the WHO estimates the global pandemic risk of monkeypox as "moderate", but as "high" in Europe. Given the current China "dynamic zero COVID-19" strategy, the risk of monkeypox spreading in China is still low (*4*). However, the risk of monkeypox epidemics should not be underestimated.

Monkeypox virus belongs to the genus *Orthopoxvirus* and the family Poxviridae, the same as infamous variola virus (smallpox). The monkeypox virus genome is approximately 197 kb of double-stranded DNA containing 190 closely packed genes (*5*). There are two clades of monkeypox virus, the Congo Basin (Central African) clade and the West African clade, and they exhibit approximately 0.5% genome sequence differences and circulate in different regions of Africa (*6*). In humans and cynomolgus monkeys, the Congo Basin clade is more virulent than the West African clade, with estimated human case fatality rates of 10.6% and 3.6% respectively (*7*). The current outbreak is caused by a less virulent West African clade of monkeypox virus.

Because the clinical presentation of this outbreak may have been atypical, accurate differential virological diagnosis should be performed in patients with STI-associated rashes, even the rashes are localized and not (yet) spread. Laboratory diagnosis is particularly important in such a circumstance. As with SARS-CoV-2, timely and valid laboratory testing is a key approach that can be deployed quickly enough to provide prompt feedback on any public health interventions. So far, more than 30 Chinese *in vitro* diagnosis (IVD) companies have obtained the European Union conformite europeenne (CE) certification for monkeypox virus diagnostic products. This article aims to analyze the characteristics and clinical application scenarios of detection methods.

1. Diagnosis for monkeypox virus nucleic acid

Monkeypox diagnosis can be confirmed by the detection

of virus DNA through polymerase chain reaction (PCR) and next-generation sequencing in a clinical specimen or the detection of monkeypox virus through the CRISPR-Cas12a-based approach (*8*). However, CRISPR-like technologies currently have no commercial diagnostic products for other pathogens, even for the widespread COVID-19, they are mostly still in the research stage. The real-time PCR is the preferred method for routine diagnosis of monkeypox virus with low cost and high speed.

The real-time PCR assay was used to target the conserved regions of the DNA polymerase gene *E9L*, *B7R*, DNA-dependent RNA polymerase subunit rop18, extracellular envelope protein gene *B6R* and *F3L* gene (Table 1) (*9-12*). This PCR assay, if poorly designed, may have false-negative results (below limit of detection) or reduced analytical sensitivity. The monkeypox virus is not a new virus and there are several published assays available, and this is a very different scenario from that of COVID-19 in early 2020 (*13*). Poxviruses have a lower mutation rate than RNA viruses due to the 3'-5' exonuclease proofreading activity of the viral DNA polymerase in Poxvirus's DNA genome replication (*14*). The monkeypox virus genome is typical of orthopoxviruses, in which the orthopoxvirus species homology genes are located in the center of the linear genome, and genes reflecting differences in tissue tropism, host range, and virulence

Table 1. published PCR assays to detect *Orthopoxvirus* and monkeypox virus

Targeted lineage	Gene target	primers and probes	Sequence (5'-3') and fluorescence labeling	Reference
Orthopoxvirus	I7L	F	TAATACTTCGATTGCTCATCCAGG	(*27*)
		R	ACTTCTCACAAATGGATTTGAAAATC	
		P	FAM-TCCTTTACGTGATAAATCAT-NFQ MGB	
	rpo18	F	CTGTAGTTATAAACGTTCCGTGTG	(*28*)
		R	TTATCATACGCATTACCATTTCGA	
		P	FAM-ATCGCTAAATGATACAGTACCCGAA T* CTCTACT p	
	E9L-NVAR	F	TCAACTGAAAAGGCCATCTATGA	(*11,29,30*)
		R	GAGTATAGAGCACTATTTCTAAATCCCA	
		P	TET-CCATGCAATATACGTACAAGATAGTAGCCAAC-QSY7	
Monkeypox	F3L	F	CTCATTGATTTTTCGCGGGATA	(*19,31*)
		R	GACGATACTCCTCCTCGTTGGT	
		P	FAM-CATCAGAATCTGTAGGCCGT-MGBNFQ	
	F3L	F	CATCTATTATAGCATCAGCATCAGA	(*32*)
		R	GATACTCCTCCTCGTTGGTCTAC	
		P	JOE-TGTAGGCCGTGTATCAGCATCCATT-BHQ1	
	N3R	F	AACAACCGTCCTACAATTAAACAACA	(*31*)
		R	CGCTATCGAACCATTTTTGTAGTCT	
		P	FAM-TATAACGGCGAAGAATATACT-MGBNFQ	
	B6R	F	ATTGGTCATTATTTTTGTCACAGGAACA	(*11,29,30*)
		R	AATGGCGTTGACAATTATGGGTG	
		P	MGB/DarkQuencher-AGAGATTAGAAATA-FAM	
	B7R	F	ACGTGTTAAACAATGGGTGATG	(*33*)
		R	AACATTTCCATGAATCGTAGTCC	
		P	TAMRA-TGAATGAATGCGATACTGTATGTGTGGG-BHQ2	
	G2R_G	F	GGAAAATGTAAAGACAACGAATACAG	(*9,28*)
		R	GCTATCACATAATCTGGAAGCGTA	
		P	FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-BHQ1	
Monkeypox West African-specific	G2R_WA	F	CACACCGTCTCTTCCACAGA	(*9,28*)
		R	GATACAGGTTAATTTCCACATCG	
		P	FAM-AACCCGTCGTAACCAGCAATACATTT-BHQ1	
Monkeypox Congo Basin-specific	C3L	F	TGTCTACCTGGATACAGAAAGCAA	(*9*)
		R	GGCATCTCCGTTTAATACATTGAT	
		P	FAM-CCCATATATGCTAAATGTACCGGTACCGGA-BHQ1	
Variola virus	B12R	F	ATGTTCAAGCTGTTAATATCAATCTCG	(*32*)
		R	TTTGCCACTGAACCATTCTATCAT	
		P	FAM-CTGTCGGAGCCACAGTTTCGAGACG-BHQ1	
	A38R	F	TCTGTACTATGTGTTAAAAGATTCTACAA	(*33*)
		R	AATGTATCTGTTATAGTCAGCATACCC	
		P	FAM-CGTTGATGGACACCACGTTTGTTATTA-BHQ1	
Cowpox virus	D11L	F	AAAACTCTCCACTTTCCATCTTCT	(*33*)
		R	GCATTCAGATACGGATACTGATTC	
		P	JOE-CCACAATCAGGATCTGTAAAGCGAGC-BHQ1	
Vaccinia virus	B10R	F	GGCAATGGATTCAGGGATATAC	(*33*)
		R	ATTTATGAATAATCCGCCAGTTAC	
		P	Cy5-CAATGTGTCCGCTGTTTCCGTTAATAAT-BHQ3	
Varicella-zoster virus	ORF38	F	AAACCGCACATGATAACGC	(*32*)
		R	GATTAGGACCATCCCCCG	
		P	TAMRA-ACAATGAGTAGTGGCTTTATGGCGAG-BHQ2	
Human DNA	RNase P	F	AGATTTGGACCTGCGAGCG	(*29,30*)
		R	GAGCGGCTGTCTCCACAAGT	
		P	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	

F: forward; R: reverse; P: probe; T* marks the position of the quencher.

are located in the terminal regions (*15*). The genome terminus contains an inverted terminal repeat of the same sequence but in opposite directions, and the genome is very low in guanine and cytosine (33%) (*16*). To help the scientific community quickly obtain monkeypox genomic information, the China National Center for Bioinformation (CNCB)-National Genomics Data Center (NGDC) has established an open-access information resource on monkeypox virus (MPXV): the Monkeypox Virus Resource (MPoxVR; *https://ngdc.cncb.ac.cn/gwh/poxvirus/*). MPoxVR has created BLAST, genome annotation, variant identification, and variant annotation, and these four commonly used online tools can be freely accessed (*17*). Another research team illustrated the frequency of variation at different positions in the monkeypox virus genome in the nucleotide (nt) context (*18*).

The Chinese technical plan for monkeypox prevention and control (2022 version) provides primers and probes for the monkeypox virus target gene F3L (*19*), which can be used for the detection of monkeypox virus by laboratory's self-established detection methods. Positive results obtained by PCR testing for orthopoxvirus also require confirmation of monkeypox virus by PCR and/or sequencing (*20*). We recommend a dual-target approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence (*21*). More than 30 monkeypox virus nucleic acid kits (developed by Sansure Bioretch, Shanghai ZJ Bio-Tech, Guangzhou Wondfo, Beijing Innovita, Hangzhou Dean Biotechnology, and Hotgen Biotech *etc*.) have obtained European Union CE certification, meaning that these kits can be marketed in European Union countries and countries that recognize European Union CE certification. But none of these products have been approved by the Chinese or US FDA. Roche Diagnostics has announced that it has developed three multiplex PCR solutions specific for orthopoxviruses and monkeypox virus clades to help researchers make rapid diagnoses. To standardize the technical review of monkeypox virus nucleic acid kits, the China Medical Device Evaluation Center organized and compiled the key points for the development of monkeypox virus detection methods (*https://www.cmde.org.cn/*). The China National Institute of Metrology has successfully developed two pseudoviruses, B6R wild type and F3L mutant, which are reference materials of monkeypox virus. The reference materials can be used as measurement standard for the development and performance evaluation of monkeypox virus detection kits, and provides traceability.

In addition, the type and quality of the specimen, and the type of laboratory testing are important for the confirmation of monkeypox infection. Samples from skin lesions (*22,23*) – fluid from vesicles and pustules, and dry crusts are the best diagnostic samples for monkeypox. During sample collection, a swab will be swabbed vigorously over the rash lesions and on more than one lesion. If monkeypox is suspected, professionals should collect appropriate samples and transport them safely to the appropriate laboratory. Packaging and shipping of the specimens should follow national and international requirements. Patient blood samples should not be routinely collected because PCR blood tests are usually inconclusive due to the short duration of viremia relative to symptoms onset. Prolonged shedding of viral DNA from the upper respiratory tract after resolution of skin lesions challenges current infection prevention and control guidelines (*24*).

Monkeypox virus culture should be carried out in a Biosafety Level 3 (BSL-3) laboratory, and monkeypox related inactivation materials should be operated in a Biosafety Level 2 (BSL-2) laboratory. Therefore, sample tubes containing guanidine salts (guanidine isothiocyanate or guanidine hydrochloride, *etc*.) or surfactants can be used to ensure viral inactivation for biosafety clinical detection. Staff are advised to be vaccinated if possible.

2. Monkeypox virus antigens and antibodies detection

The mainstream immunoassay method for the rapid detection of monkeypox virus antigens and antibodies is the colloidal gold method. Beijing Hotgen Biotech announced that the antigen detection kit of monkeypox virus (colloidal gold method) has been certified by the UK MHRA. Types of samples for monkeypox antigen detection are skin lesion swabs (swabs of lesion tops, exudate of rash or growth) and lesion crusts. In 2005, investigators found that anti- monkeypox virus IgM test performed between day 5 and day 77 post rash onset was 94.8% sensitive and 94.5% specific in both vaccinated and unvaccinated patients. In patients unvaccinated or monkeypox virus naïve, assay of anti- monkeypox virus IgG from day 14 onwards was 100% sensitive and 88.5% specific (*25*). Orthopox-specific IgM can be detected by in-depth serological assays in cynomolgus macaques detected during the 6-day incubation period before the rash development (*26*). The anti- monkeypox virus IgG in these animals produced 23 distinct recognizable *Orthopoxvirus* proteins. A diagnosis of monkeypox virus infection is possible if IgM and IgG antibodies are detected in the serum of an unvaccinated individual with a history of rash and severe illness. Antigen and antibody assays cannot make a monkeypox confirmation because of the serological cross-reactivity of orthopoxviruses, but serologic testing may be feasible in areas where monkeypox virus is endemic. Currently, testing is only recommended if a patient has a rash consistent with monkeypox. The diagnosis of monkeypox cannot rely solely on plasma/serum antibody test, and a serological detection can be used to further investigate past infection for epidemiological tracing.

3. Conclusion

Due to the serological cross-reactivity of orthopoxviruses, antigen and antibody detection methods cannot make the monkeypox-specific confirmation. PCR is the laboratory test of choice because of its high accuracy and sensitivity. At present, there is no monkeypox PCR or serological test kit approved for marketing in China. Due to the severe situation of the global monkeypox epidemic worldwide, it is necessary to accelerate the pace of product development. We recommend a dual-target PCR approach in which one assay targets a conserved sequence of the *Orthopoxvirus* genus and the other targets a monkeypox virus specific sequence.

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Xpert MTB/RIF Ultra in the auxiliary diagnosis of tuberculosis among people living with human immunodeficiency virus

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SUMMARY Clinical diagnosis of tuberculosis (TB) in people living with the human immunodeficiency virus (HIV) poses a challenge. The Xpert MTB/RIF Ultra (Ultra) has displayed greater sensitivity at diagnosing tuberculosis and rifampicin resistance compared to the Xpert MTB/RIF (Xpert). However, whether Ultra is able to facilitate an auxiliary diagnosis of TB in patients with an HIV-TB co-infection remains unclear. Accordingly, the current study evaluated the use of Ultra in patients with an HIV-TB co-infection by summarizing relevant studies. The sensitivity and specificity of Ultra and Xpert at diagnosing patients with an HIV-TB co-infection have been summarized and compared. The performance of Ultra in diagnosing extrapulmonary tuberculosis was also summarized. Although a large-cohort, multi-center study needs to be conducted to assess Ultra's ability to detect TB in AIDS patients in the future, the current evidence supports the use of Ultra for the assessment of patients with an HIV-TB co-infection.

Keywords Xpert MTB/RIF Ultra, HIV, tuberculosis, diagnosis

People living with human immunodeficiency virus (PLHIV) are susceptible to tuberculosis (TB) due to their deficient immunity, and this leads to accelerated disease progression (*1*). However, cases of an HIV-TB co-infection are difficult to diagnose due to their subtle clinical manifestations and low rate of detection by conventional diagnostic assays (*2*). Therefore, a rapid and precise method of auxiliary diagnosis of an HIV-TB co-infection is urgently required. The Xpert MTB/RIF (Xpert) assay is a classic rapid nucleic acid amplification test (NAAT) that rapidly measures *Mycobacterium tuberculosis* and rifampicin resistance and that has a high level of sensitivity. However, Xpert has been reported to have inferior performance in diagnosing an HIV-TB co-infection compared to TB alone (*3*). To solve this problem, the Xpert MTB/RIF Ultra (Ultra) was updated by Cepheid (Sunnyvale, USA). As a new-generation rapid molecular test, Ultra has a larger chamber for DNA amplification and 2 multicopy amplification targets for TB (IS6110 and IS1081, for a lower limit of detection of 16 CFU/mL), which renders it more sensitive than Xpert (*4*). Thus, Ultra may improve the detection of TB among PLHIV. However, the ability of Ultra to detect TB among PLHIV has not yet been adequately investigated. Summarized here is the diagnostic performance of Ultra in patients with an HIV-TB co-infection.

To summarize the sensitivity and specificity of Ultra's performance, there are both advantages and disadvantages in Ultra's diagnosis of TB among AIDS patients. On the one hand, Ultra is more sensitive at diagnosing TB among AIDS patients. Berhanu *et al*. reported that Ultra's sensitivity was 11.7% higher (88.2% *vs*. 76.5%) in adult HIV-positive patients compared to Xpert (*5*). Ultra's sensitivity has been found to be superior to that of Xpert only in patients with an HIV-TB co-infection. Similar results have been reported by Dorman *et al*., who found that Ultra was 13% more sensitive than Xpert (90% *vs*. 77%) (*6*). Moreover, Ultra has also displayed higher sensitivity in children with HIV. A comparative study reported that Ultra's sensitivity was 22.2% higher (88.9% *vs*. 66.7%) in children with an HIV-TB co-infection than Xpert (*7*). In 2020, a two-cohort study was conducted by Mishra *et al*. in South Africa (*8*). They reported positive results only in HIV-TB cases in cohort A, where Ultra's sensitivity increased by 14% (81% *vs*. 67%), but there was little difference in sensitivity in HIV-negative patients (87% *vs*. 89%). They reported in cohort B that, unlike in cohort A, Ultra's sensitivity decreased in HIV-positive cases (87% *vs*. 92%), and Ultra's sensitivity decreased by 13% (94% *vs*. 81%) in HIV-negative patients.

On the other hand, Ultra's specificity in diagnosing

Table 1. Sensitivity and specificity: Xpert MTB/RIF Ultra *vs*. Xpert MTB/RIF

Cohorts	Samples (*n*)	Xpert MTB/RIF Ultra		Xpert MTB/RIF		*Ref.*
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	
HIV-positive adults	48	(81; 59-95)	(78; 58-92)	(67; 44-86)	(100; 88-100)	(*8*)
	121	(87; 47-99)	(71; 55-84)	(92; 64-99)	(86; 75-94)	(*8*)
	147	(88.2; 72.5-96.7)	(94.7; 88.8-98)	(76.5; 58.8-89.3)	(100; 96.8-100)	(*5*)
	115	(77; 68-84)	/	(90; 83-95)	/	(*6*)
HIV-positive children	9	(88.9; 51.8- 99.7)	/	(66.7; 29.9-92.5)	/	(*7*)

This table shows the performance of Xpert MTB/RIF Ultra and Xpert MTB/RIF in diagnosing HIV according to the original papers listed. The tests were compared in the same studies.

Table 2. Diagnostic performance of Xpert MTB/RIF Ultra in detecting extrapulmonary tuberculosis

Specimens	HIV-positive patients	Sensitivity (%)	Specificity (%)	*Ref.*
CSF	41 (98%)	(76.5; 62.5-87.2)	(100; 97.6-100)	(*11*)
CSF	129 (100%)	(70; 47-87)	/	(*12*)
CSF	103 (17%)	(64.3; 38.8-83.7)	(100; 43.9-100)	(*10*)
Lymphadenitis	77 (58%)	(91; 76-98)	(67; 50-81)	(*13*)
Pleural fluid	9 (6.5%)	(37.5; 23.8-51.2)	(98.8; 96.5-100)	(*14*)

This table shows: (i).The performance of Xpert MTB/RIF Ultra with respect to various specimens from people living with HIV according to the original papers listed; (ii).Xpert MTB/RIF Ultra has a potential advantage at diagnosing tuberculous meningitis in patients with HIV.

patients with an HIV-TB co-infection was reported to be lower than that of Xpert. Berhanu *et al*. reported that Ultra's specificity was 94.7% compared to 100% for Xpert (*5*). Although Ultra's sensitivity yielded conflicting results in a two-cohort study, Ultra's specificity was 22% (78% *vs*. 100%) and 15% (71% *vs*. 86%) lower in patients with an HIV-TB co-infection than that of Xpert (*8*). In short, Ultra has a higher sensitivity than Xpert at detecting TB in HIV-positive populations, but Ultra has a lower specificity than Xpert (Table 1). The discrepancy may be due to an insufficient sample size and may be resolved by another study with a larger sample.

M. tuberculosis is known to usually infect the lungs, but it can also infect other organs. However, due to the difficulty in obtaining extra pulmonary samples and the paucibacillary nature of the disease, the diagnosis of extrapulmonary TB (EPTB) is challenging (*19*). Many other studies have concentrated on Xpert's performance in diagnosing EPTB. A recent Cochrane study reported that Ultra had better performance, including a higher sensitivity and lower specificity, than Xpert in diagnosing EPTB (*9*). However, limited studies have thus far focused on Ultra's effectiveness in diagnosing patients with an HIV-EPTB co-infection. A search of PubMed yielded only 5 studies on Ultra's effectiveness in diagnosing patients with an HIV-EPTB co-infection (Table 2). Three of those studies on Ultra involved cerebrospinal fluid (CSF). One reported that Ultra had a sensitivity 76.5% and specificity of 100% in diagnosing patients with an HIV-EPTB co-infection, another reported that Ultra had a sensitivity of 64.3% and a specificity of 100%, and yet another reported that Ultra had a sensitivity of 70% (specificity was not reported) (*10-12*). Minnies *et al*. reported that Ultra had a sensitivity of 91% and specificity of 67% in diagnosing lymphadenitis in patients with an HIV-EPTB co-infection (*13*). Another prospective study reported that Ultra had a sensitivity of 37.5% and specificity of 98.8% when testing pleural fluid from patients with an HIV-EPTB co-infection (*14*). Recently, Boloko *et al*. reported that the Ultra was able to test pre-processed blood from suspected cases of HIV-associated TB (*15*). They provided additional prognostic information that may include other available markers. A retrospective cohort study reported Ultra's accuracy in diagnosing TB in various samples from adult patients (n = 183 (4.8%)). Kaswala *et al*. reported that Ultra's sensitivity when testing pus was 92.0%. However, Ultra had the lowest sensitivity when testing CSF (38.5%) (*16*). In a nutshell, Ultra can detect pathogens in various types of specimens, but studies with larger cohorts and at multiple centers should be conducted, and especially in the HIV-TB population.

Management and control of resistant TB relies on timely and correct diagnosis. Unlike the previous generation of the assay (Xpert), Ultra can detect rifampicin resistance by using melting temperature analysis and 4 probes that target the rpoB gene (*4*). However, Ultra and Xpert have similar performance in terms of sensitivity and specificity in detecting rifampicin resistance. Dorman *et al*. reported that Ultra and Xpert had a similar sensitivity (95% *vs*. 95%) and specificity (98% *vs*. 98%) in people living with HIV. In that study, most patients were from Belarus, China, India, and Georgia. The Ile491Phe mutation that Ultra

was able to detect was not found in patients originally from these regions, resulting in similar findings for both assays (*8*). As such, a multi-center, large-cohort study of patients with an HIV-TB co-infection may need to soon be conducted to confirm the detection of resistance.

Ultra has been reported to have an increased sensitivity and decreased specificity in diagnosing patients with an HIV-TB co-infection. However, few studies reported on testing for rifampicin resistance with Ultra in patients with an HIV-TB co-infection. Recently, Martyn *et al*. reported that Ultra's Ct values could also indicate patients with tuberculous meningitis at increased risk of death (*17*). Shapiro *et al*. reported that tongue swab testing may complement non-sputum samples for diagnosis of TB in PLHIV (*18*). However, studies at more centers and with larger cohorts need to be conducted to determine Ultra's accuracy when testing various specimens.

In summary, Ultra has a higher sensitivity at detecting an HIV-TB co-infection than Xpert. Moreover, Ultra could potentially be used to detect an HIV-EPTB co-infection in the future, and especially one involving tuberculous meningitis. However, Ultra had a lower specificity at detecting an HIV-TB co-infection than Xpert. In addition, Ultra offers no benefits over Xpert in detecting rifampicin resistance among PLHIV, but further studies are needed.

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Most dentists approve of oral health check-ups for local residents at community pharmacies and desire collaboration with community pharmacists

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SUMMARY Community pharmacies are required to play a role in maintaining the health of local residents. Since September 2015, a national policy in Japan has allowed saliva tests to be used for oral health check-ups at community pharmacies. In the present study, we aimed to reveal dentists' perceptions and expectations for oral health check-ups provided at community pharmacies. A questionnaire survey was administered to dentists at 1,000 randomly selected dental clinics in Tokyo, Japan; 257 responses (25.7%) were included in the analysis and 85.2% of respondents approved of oral health check-ups at community pharmacies. Most respondents who approved of oral health check-ups expected that community pharmacists would recommend that local residents visit a dental clinic (85.8%) and provide a report to dentists (60.3%) after the check-up. Furthermore, 79.0% of respondents desired collaboration with community pharmacies. These findings suggested the potential for oral health check-ups to facilitate collaboration between community pharmacies and dental clinics aimed at maintaining and improving oral health of local residents.

Keywords health support, oral disease, oral health promotion, questionnaire survey

It is known that periodontal disease, a serious oral disease, is related to systemic diseases such as diabetes mellitus (*1*) as well as medical conditions such as stroke (*2,3*). Therefore, maintaining oral health is important for preventing various health problems. However, in Japan, periodontal disease is the second most common major disease behind hypertension (*4*). Additionally, only 52.9% of adults have regular dental check-ups (*5*), which may delay the detection of oral diseases.

Community pharmacies have an important role to play in maintaining the health of local residents by providing advice about lifestyle choices and by recommending visits to healthcare facilities. A majority of pharmacists in Australia (*6,7*) and England (*8*) stated that it was part of their role to deliver oral health advice to the community and to provide oral healthcare service to local residents. In contrast, most Japanese community pharmacists are not proactive in providing consultations or giving advice about oral health problems to local residents. Since September 2015, a Japanese national policy has allowed saliva tests to be used to conduct oral health check-ups at community pharmacies. The results of these tests can indicate the risk of dental caries and periodontal disease. In our previous study, we reported that local residents were very satisfied with the oral health check-ups and information about oral self-care provided by community pharmacies in addition to showing their potential to improve oral self-care and dental care-seeking behavior (*9*). Our findings suggested that community pharmacies can contribute to the maintenance and promotion of oral health by providing oral health check-ups to local residents. However, that study did not investigate what dentists thought of oral health check-ups at community pharmacies. Therefore, in the present study, we conducted a questionnaire survey of Japanese dentists in order to understand their views on oral health check-ups at community pharmacies.

The questionnaire survey was conducted from July 11 to August 9, 2019. The survey targeted dentists at 1,000 randomly selected dental clinics in Tokyo, which has the largest number of community pharmacies and dental clinics in Japan. The anonymous self-administered questionnaire covered the following contents: 1) the basic characteristics of the participants, 2) their views on oral health check-ups at community pharmacies, and 3) what they wanted community pharmacists to do after

oral health check-ups. An information sheet was attached to each questionnaire that outlined the aims of the study and explained the oral health check-ups provided at community pharmacies. The questionnaires were sent and returned by postal mail. Return of the completed questionnaire was considered to indicate consent to participate in the study. The study protocol was approved by the research ethics committee of the Faculty of Pharmacy, Keio University (approval number: 190613-2).

We received responses from 259 (25.9%) dentists, 2 of whom were excluded due to lack of responses to most of the questions; therefore, 257 responses (25.7%) were included in the analysis. Most of the respondents (72.4%) had over 20 years of experience practicing dentistry.

Only 5.1% of the respondents were aware that oral health check-ups could be provided at community pharmacies (Figure 1A), but 85.2% of respondents approved of this practice (Figure 1B). The primary reason was that oral health check-ups might improve oral health awareness among local residents (86.3%) (Figure 1C). Additionally, the respondents thought that the oral health check-ups would lead to early detection of oral diseases (70.3%), improve collaboration between dental clinics and community pharmacies (59.4%), and increase visits to dental clinics (58.0%).

Most of the respondents who approved of oral health check-ups wanted community pharmacists to recommend that local residents visit a dental clinic after the check-up (85.8%) (Figure 2A). In addition, 60.3% wanted the community pharmacy to provide a report to dentists about the results of the check-up as well as the medications that local residents are prescribed. Furthermore, 79.0% of the respondents desired collaboration with community pharmacies aimed at maintaining and improving the oral health of local residents (Figure 2B).

This is the first study to investigate the views of Japanese dentists regarding oral health check-ups performed at community pharmacies. The results revealed that very few of the dentists surveyed knew that oral health check-ups could be performed at community pharmacies. This might be because there are few community pharmacies or drugstores that actually provide oral health check-ups. However, 85% of the dentists surveyed approved of providing oral health check-ups at community pharmacies. The respondents agreed that the oral health check-ups might improve oral health awareness and lead to early detection of oral diseases, which suggests that the benefit of the check-ups for local residents may have led to the high level of approval among the dentists. This finding might encourage community pharmacists to provide the oral

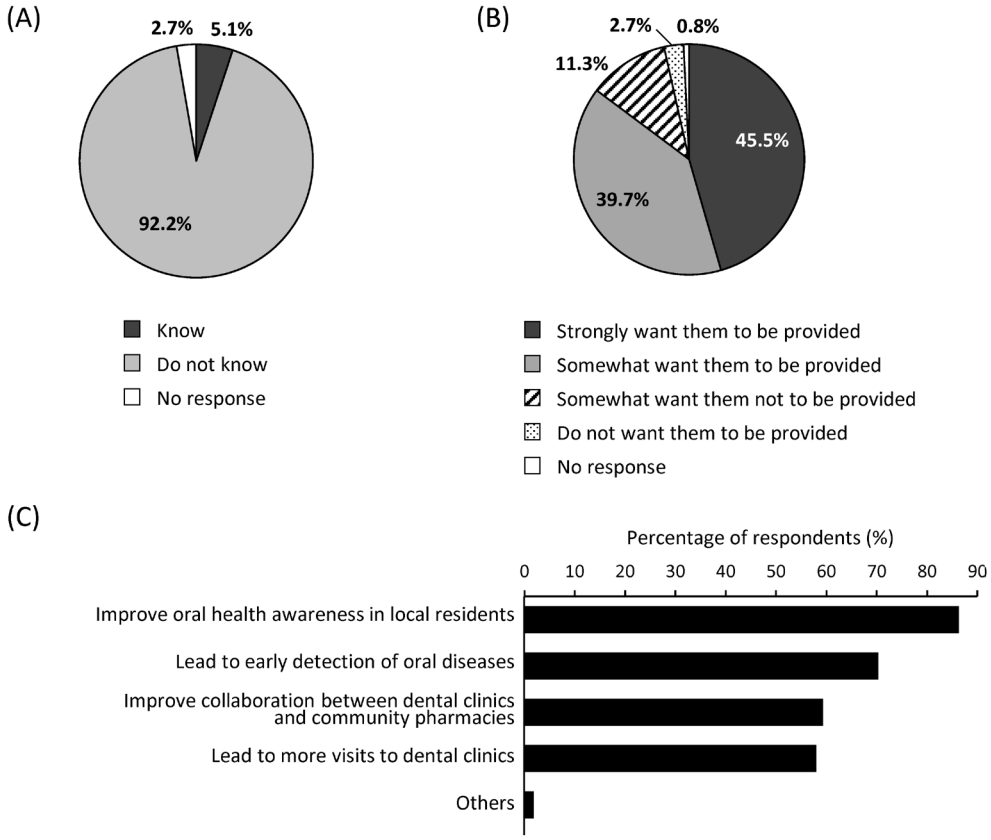

Figure 1. Views on oral health check-ups at community pharmacies. (A) Awareness of oral health check-ups at community pharmacies (n = 257). **(B)** Level of expectation for oral health check-ups at community pharmacies (n = 257). **(C)** Reasons for wanting oral health check-ups to be provided at community pharmacies. Only those who responded "strongly want them to be provided" or "somewhat want them to be provided" in Figure 1B answered this question (n = 219, multiple answers allowed).

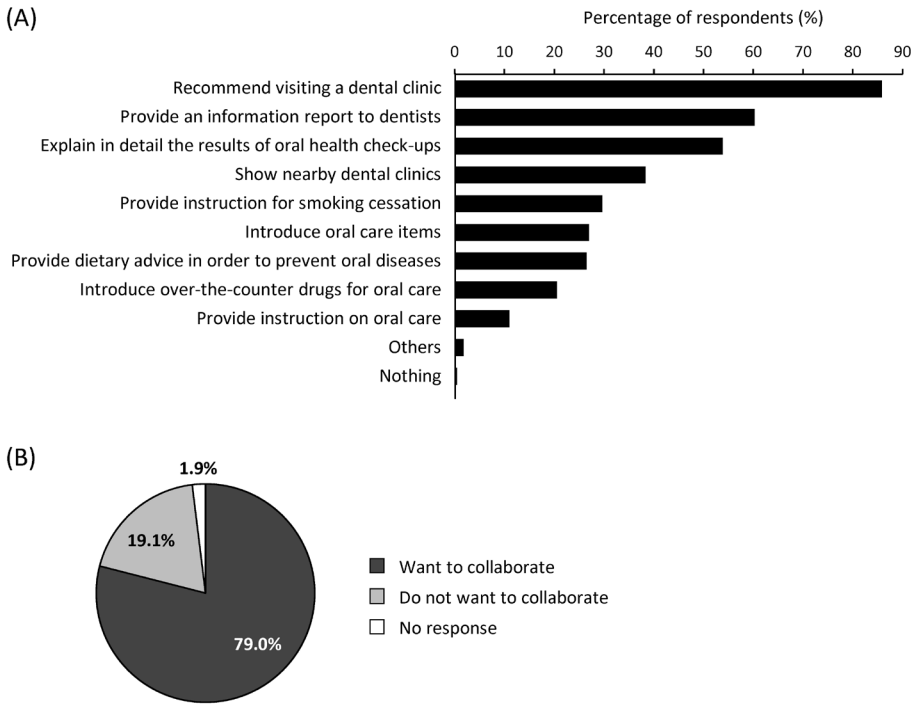

Figure 2. Expectation for community pharmacists aimed at oral health promotion. (A) What dentists want community pharmacists to do after oral health check-ups. Only those who responded "strongly want them to be provided" or "somewhat want them to be provided" in Figure 1B answered this question (n = 219, multiple answers allowed). **(B)** Views on collaboration with community pharmacies aimed at maintaining and improving the oral health of local residents (n = 257).

health check-up service in the future.

Most of the respondents wanted community pharmacists to recommend that local residents visit a dental clinic after the check-up. This suggests that dentists expect community pharmacists to detect local residents at high risk of oral diseases and advise them to visit a dental clinic for a more thorough examination by an expert. In an opinion piece, Wilson and Soni highlighted the potential of pharmacies to encourage hard-to-reach individuals to become dental attenders (*10*). Meanwhile Sturrock *et al*. suggested that community pharmacies can connect with patients who are not currently dental attenders by promoting pharmacy-based oral health services (*11*). We previously showed that oral health check-ups at community pharmacies had the potential to improve the dental consultation behavior of local residents (*9*). The expectations of the dentists surveyed are consistent with the above perceptions. Furthermore, the present study indicated that reports provided to dentists about the results of the check-ups and the medications that local residents are prescribed were useful. This may be because some medications can affect oral health; for example, xerostomia and gingival hyperplasia are due to anticholinergic agents and calcium channel blockers, respectively. However, at present, collaboration between community pharmacists and dentists in Japan is poor. Consequently, community pharmacists are required to proactively coordinate with dentists. Given that most of the dentists surveyed desired collaboration with community pharmacies aimed at maintaining and promoting the oral health of local residents, further study is needed to determine how best to facilitate collaboration between community pharmacists and dentists.

A limitation of this study is that it focused only on dentists working in Tokyo. It is possible that dentists working in other areas, particularly outside of major metropolitan areas, will have different opinions, so further study is needed over a broader area.

In conclusion, the majority of dentists surveyed approved of oral health check-ups performed at community pharmacies. They wanted community pharmacies to recommend that local residents visit a dental clinic as well as to share medical information about local residents after the oral health check-up. These findings suggest the potential for oral health check-ups to facilitate greater collaboration between community pharmacies and dental clinics aimed at maintaining and improving the oral health of local residents.

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Letter to the Editor

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Cystohepatic duct can be the bridge of calculous cholecystitis complicating cholangitis and obstructive jaundice

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SUMMARY Cholestasis and obstructive jaundice can be extrahepatic or intrahepatic. Here we present one case with calculous cholecystitis who presenting with repeated obstructive jaundice and without bile duct dilation. The patient received laparoscopic cholecystectomy, and cystohepatic duct was identified intraoperatively, there was no cholestasis or obstructive jaundice postoperatively. Cystohepatic duct is a rare biliary anomaly observed in 0.7% of all surgical cases and in 1.5% of all cadaveric dissections. The cystohepatic duct can be the bridge of calculous cholecystitis complicating cholangitis and obstructive jaundice, here we for the first time presented this entity.

Keywords Cystohepatic duct, calculous cholecystitis, obstructive jaundice, cholecystectomy

Letter to the Editor,

Cholestasis and obstructive jaundice can be extrahepatic or intrahepatic. Extrahepatic obstructive jaundice is usually attributed to obstructed biliary system at the level of the common bile duct. The common causes of obstruction are stone, tumor and stenosis. The intrahepatic obstructive jaundice present with obstructive jaundice but without dilation of biliary system.

Several patients with repeated calculous cholecystitis and obstructive jaundice are encountered, and this kind of patients presented with no dilation of biliary system. Here we present one patient whose abdominal pain were mild or even without abdominal pain, and when antibiotic therapy had been administrated, obstructive jaundice relieved. The patient received laparoscopic cholecystectomy. Cystohepatic duct was identified intraoperatively and there was no cholestasis or obstructive jaundice postoperatively.

A 74-year-old male had a 7-year history of repeated right upper quadrant pain and medium cholestasis and obstructive jaundice. The patient was then diagnosed as calculous cholecystitis in local hospital. The patient was then admitted to our hospital, and received a routine abdominal contrast-enhanced computed tomography (CT) scan preoperatively. This abdominal contrast-enhanced CT scan showed multiple gallbladder stones, and the diameter of the biggest stone was 2 cm, without dilation of common bile duct. The total bilirubin was 68 μmol/L, and glutamic-pyruvic transaminase was 49 U/L. The gallbladder wall was thick and a little oedematous.

Antibiotic therapy was then administrated, and cholestasis and obstructive jaundice relieved gradually. Subsequently the patient received laparoscopic cholecystectomy. After the cystic duct and cystic artery were identified and cut off respectively, the gall bladder was detached from the cholecystic bed. When the detaching proceeded to the bottom of gallbladder, a bile duct connecting the wall of gallbladder and cholecystic bed was encountered (Figure 1A). The diameter of this cystohepatic duct was 3 mm. The cystohepatic duct was then clipped with hemolock and cut off subsequently after being identified (Figure 1B). The patient recovered uneventfully and was discharged at day 2 postoperatively. The patient presented no cholestasis or obstructive jaundice during follow-up.

The cystohepatic duct was not noticed preoperatively because this kind of duct was small and not presented frequently. After the surgery, we reviewed the images of CT carefully and located this cystohepatic duct which connected the wall of bottom of gallbladder and cholecystic bed (Figure 1C).

Obstructive jaundice results from cholestasis. Cholestasis can be extrahepatic or intrahepatic. Obstructive jaundice is amenable to surgical treatment when the obstruction is extrahepatic.

Extrahepatic obstructive jaundice usually presents with cholestasis and dilation of main bile ducts. The etiology can be obstruction of common bile duct, for example, stone or tumor in common bile duct, or compression of common bile duct from outside to inside.

Complications of gallstones are usually considered to be acute cholecystitis, chronic cholecystitis, choledocholithiasis and MIRRIZI syndrome. To our knowledge, only MIRRIZI syndrome and

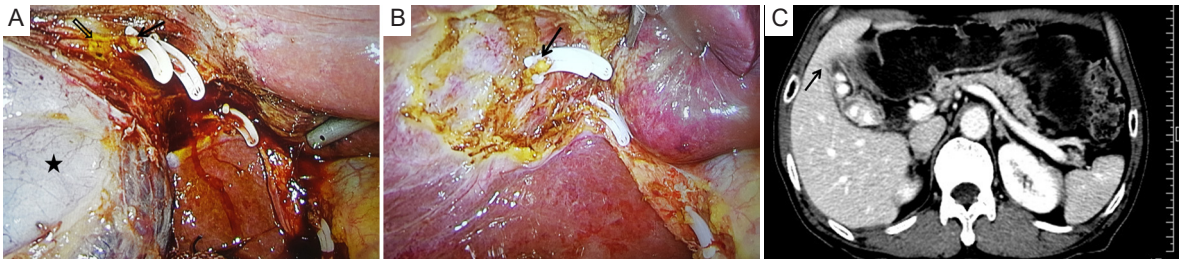

Figure 1. A. Intraoperative photo. During Laparoscopic cholecystectomy, when the detaching proceeded to the bottom of gallbladder (black star), a bile duct connecting the wall of gallbladder and cholecystic bed was encountered. The cystohepatic duct was cut off, and the photo showed the proximal cuff (black arrow) and distal cuff (hollow arrow). **B. Intraoperative photo (gallbladder removed).** The cystohepatic duct was clipped with hemolock and cut off subsequently after being identified, and the gallbladder was removed. The photo showed the proximal cuff (black arrow). **C.** Identifying the cystohepatic duct on image of computed tomography scan. Abdominal contrast-enhanced computed tomography scan showed the diameter of cystohepatic duct (black arrow) was 0.33 cm.

choledocholithiasis can cause dilation of main bile duct and extrahepatic obstructive jaundice. However, our case presented with repeated calculous cholecystitis and obstructive jaundice, whose abdominal pain were mild or even without abdominal pain, without dilation of biliary system.

As we know, intrahepatic obstructive jaundice presents with cholestasis, obstructive jaundice and without dilation of biliary system, which can be caused by drug-induced hepatitis, autoimmune hepatitis, acute viral hepatitis, acute alcoholic hepatitis, primary sclerosing cholangitis, sickle cell crisis, total parenteral nutrition and so on. Our case was with repeated calculous cholecystitis, obstructive jaundice and mild transaminitis, and without dilation of biliary system, however, when antibiotic therapy had been administrated, obstructive jaundice relieved. The patient presented with infective cholangitis and cholestasis. Most importantly cystohepatic duct was identified intraoperatively and there was no cholestasis or obstructive jaundice postoperatively. Pathological findings revealed that bile ductule was normal.

The key point to our case is that there was cystohepatic duct and repeated calculous cholecystitis. As we know, gallstones normally don't cause infective cholangitis and cholestasis, because the bile flow is normal and from intrahepatic to extrahepatic. When there is cystohepatic duct, bacteria can spread from calculous gallbladder to intrahepatic bile ducts and causes infective cholangitis and cholestasis.

Variations of the anatomy of the biliary ducts are attributed to abnormalities during embryological development of the biliary tract. It was reported that there were about 10% of humans with anomalous biliary tracts (*1-3*). The biliary ducts that communicating between the liver and the gallbladder was one kind of anomalous biliary tract, which was firstly reported by Luschka, and was named as cystohepatic duct subsequently (*4-7*).

Cystohepatic ducts are bile ducts of the right hepatic lobe and may serve a subsegment or segment, a sector, or exceptionally the whole of the right hepatic lobe, and drain into the gallbladder.

The incidence of cystohepatic duct was 0.7% of all surgical cases, and 1.5-30% of postmortem dissection according to published reports (*8*). It was recommended that magnetic resonance cholangiopancreatography would be helpful in identifying anomalous biliary tract (*9*).

For the first time, we report here that calculous cholecystitis can affect intrahepatic biliary tract *via* cystohepatic duct, and cause obstructed jaundice and cholangitis without bile duct dilation. This entity sometimes could be misdiagnosed, according to our experience, the appropriate treatment for this entity is laparoscopic cholecystectomy.

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Conflicts of interest: The authors have no conflicts of interest to disclose.

Ethics: This study was approved by Ethics Committee of the third affiliated hospital of Sun Yat-sen university. Written informed consent was taken from the patient for publication of case details and photographs.

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Letter to the Editor

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Relaxation of all-case reporting of COVID-19 cases in Japan

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SUMMARY Japan is facing the largest outbreak of COVID-19 in history in 2022. The number of new infections per day surpassed 200,000 for the first time in July and peaked in August. Japan has required the reporting of information on all infected persons, but maintaining this system is difficult. Starting in September 2, 2022, four prefectures have implemented a trial policy to limit the infected that must be reported in order to reduce the burden on medical personnel. The policy obliges medical facilities to report only people with a high-risk infection, but the number of the infected will continue to be counted regardless of whether they have a high-risk or low-risk infection. More prefectures are expected to adopt this policy in the future.

Keywords COVID-19, all-case reporting, Japan

To the Editor,

In July, the number of new cases of COVID-19 exceeded 200,000 per day for the first time in Japan, and the total number of new cases in August reached 5,964,088, representing an unprecedented outbreak (*1*). In response to the outbreak, Japan began simplifying the reporting of all COVID-19 cases starting on September 2 in four prefectures (Miyagi, Ibaraki, Tottori, and Saga) (*2,3*). In Japan, COVID-19 is treated as a designated Category 2 infectious disease; all examination and treatment costs are publicly funded, and medical facilities are obliged to report information on every patient (*4*). Therefore, each medical facility must submit a detailed report on each new case to national authorities. Originally, 19 items such as "symptoms", "the method of diagnosis", and "the cause of infection, route of infection, and site of the infection" had to be reported (*5*). In response to the rapid increase in the number of new cases compared to 2020 and 2021, the items to report were revised on June 30, 2022. The items were simplified by deleting 10 items such as "symptoms", " the method of diagnosis", and "the cause of infection, route of infection, and site of the infection" (*6*). This simplification was expected to reduce the administrative work of medical staff, but simplified reporting of COVID-19 was introduced as an emergency measure in response to the largest increase in the number of new cases in August.

This emergency measure allows prefectures to apply to the national government to limit the persons infected with COVID-19 whom they must report. Patients must be reported only when one of the following four criteria is met: Criterion 1: People over the age of 65, Criterion 2: People who require hospitalization, Criterion 3, If a doctor determines that there is a risk of aggravation or if a COVID-19 treatment or oxygen is required, Criterion 4: Pregnant women. The criteria indicate people with COVID-19 that is likely to be aggravated. The intention of limiting reporting to at-risk patients is to reduce the number of reports and to reduce the administrative burden on medical personnel. However, this emergency measure does not mean that Japan's policy of ascertaining the total number of cases will cease. If the emergency measure is taken, the number of COVID-19 cases diagnosed by doctors themselves will be counted, and the total number of new infections by day and the age group of the infected must be published. If the four criteria are not met, a report need not be submitted, but the total number of new infections by prefecture and the total number of the infected by age group will be counted, so the number of new cases can be ascertained. That said, there are issues with this measure. New cases will only be counted based on a diagnosis by a doctor, so some people who test positive according to a simple test kit will not be seen at a medical facility unless they meet the criteria. This hampers determination of the exact number of new cases. In addition, if COVID-19 is not reported, then voluntary isolation of the infected person and voluntary isolation of his or her close contacts may not be encouraged by public health centers, leading to the further spread of the infection. Moreover, symptoms may worsen even in cases with a low risk of aggravation; in the worst-case scenario, death could occur due to delayed treatment.

At present, this emergency measure has been urgently implemented on a trial basis. If the infection spreads further in the future, more prefectures will adopt this emergency measure. However, Japan needs to respond flexibly to problems arising in the future.

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Conflict of Interest: The author has no conflicts of interest to disclose.

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DD&T
Drug Discoveries & Therapeutics

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