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Drug discovery to treat COVID-19 two years after its outbreak

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SUMMARY Coronavirus disease 2019 (COVID-19) has had a significant impact on human health and economic development over the past two years. Therapeutics in combination with vaccines are critical measures to fight the pandemic. The three areas of drug development are blocking the entry of SARS-CoV-2 into cells, suppressing viral replication inside cells, and regulating the immune system, and important advances have recently been made in those areas. Increasing numbers of neutralizing antibodies and small molecules that show promise have been fully approved or authorized for emergency use, resulting in decreased mortality of patients with COVID-19. The use of therapeutics will have a great impact on formulating and revising public policies to control the pandemic. The pace of lifting of restrictions and economic recovery worldwide will also accelerate in the future. Here, the drugs or agents that have attracted considerable attention and that have led to remarkable progress in the fight against COVID-19 are reviewed

Keywords COVID-19, SARS-CoV-2, vaccine, drug, pandemic

1. Introduction

Coronavirus disease 2019 (COVID-19) has been prevalent worldwide for two years, and it has had a significant impact on human health and economic development. Although vaccination is an important measure for epidemic prevention and control, the effectiveness of vaccines may diminish as SARS-CoV-2 variants continue to emerge (*1-3*). As research on and development of COVID-19 vaccines is promoted, researchers and pharmaceutical companies worldwide are endeavoring to promote the research and development of therapeutics for COVID-19 (*4-6*). Drug development is mainly focused on three strategies: blocking virus entry into cells, inhibiting viral replication, and regulating the human immune system. Here, the drugs that have attracted considerable attention and that have led to remarkable progress in the fight against COVID-19 are reviewed.

2. Drugs that block the entry of SARS-CoV-2 into cells

SARS-CoV-2 entry into cells entails attaching to the angiotensin-converting enzyme 2 (ACE2) receptor by the spike glycoprotein present on the surface of the viral envelope (*7,8*). Then, the human transmembrane protease serine 2 (TMPRSS2) and other cellular proteases such as furin facilitate the virus' entry into cells through endocytosis or direct fusion of the viral envelope with the host membrane (*9*). Neutralizing monoclonal antibodies bind the spike protein of SARS-CoV-2, thus preventing the virus from adhering to the target cell receptor ACE2 and entering the cells (*10*). Vaccines effectively prevent COVID-19 by provoking the immune system into producing antibodies. For individuals who have comorbidities and who are ineligible for vaccination or who are receiving therapies that impair their immune response to vaccination, SARS-CoV-2-neutralising monoclonal antibodies may provide immediate, passive immunity and may limit disease progression and complications.

Casirivimab and imdevimab is a cocktail of two monoclonal antibodies (also known as REGN10933 and REGN10987, respectively) that are specifically directed against the spike protein of SARS-CoV-2 to block the virus' attachment and entry into human cells (*11*). The drug was first issued an emergency use authorization (EUA) by the US Food and Drug Administration (FDA) in November 2020 and received its last EUA update on November 2021 (*12*). The authorized use of this drug is to treat mild to moderate COVID-19 in adult and pediatric patients (12 years of age and older weighing at least 40 kg) with positive results of direct SARS-CoV-2 viral testing and who are at high risk for progression to severe COVID-19, including hospitalization or death

(*13*). The drug is also authorized for use as post-exposure prophylaxis for COVID-19 in individuals who are 12 years of age and older weighing at least 40 kg and are at high risk for progression to severe COVID-19, including hospitalization or death (*13*). Casirivimab and imdevimab were first approved in Japan in July 2021 to treat mild to moderate COVID-19 based on a global phase III clinical study which found that casirivimab and imdevimab reduced hospitalization or death by 70% and that the two antibodies reduced symptom duration by four days in high-risk non-hospitalized patients with COVID-19, as well as a phase I clinical study that examined the safety, tolerability, and pharmacokinetics of the antibodies in Japanese (*14,15*). Casirivimab and imdevimab have also been approved for the prophylaxis and treatment of COVID-19 in the UK, European Union, and Australia thus far (*16-18*) .

Bamlanivimab and etesevimab are neutralizing monoclonal antibodies that bind to distinct epitopes within the receptor binding domain of the spike protein of SARS-CoV-2 (*19*). This antibody combination therapy received its first EUA in the US in February 2021 and was subsequently reissued a Letter of Authorization in August, September, and December 2021 (*12*). According to the most recent EUA, emergency use of the drug combination is permitted for treatment of mild to moderate COVID-19 in adults and pediatric patients, including neonates, with positive results of direct SARS-CoV-2 viral testing and who are at high risk for progression to severe COVID-19, including hospitalization or death (*20*). In addition, bamlanivimab and etesevimab are also authorized for post-exposure prophylaxis for COVID-19 in adults and pediatric individuals, including neonates, who are at high risk of progression to severe COVID-19, including hospitalization or death (*20*). According to the results of a phase III study (BLAZE-1), patients markedly benefited from bamlanivimab plus etesevimab in terms of reducing the incidence of COVID-19–related hospitalization and death and accelerating the decline in the SARS-CoV-2 viral load (*21*). Another phase III study (BLAZE-2) evaluated bamlanivimab alone for prevention of COVID-19 in residents and staff of skilled nursing facilities following a confirmed reported case of SARS-CoV-2 infection at the facility (*22*). Results from that study revealed that bamlanivimab significantly reduced the incidence of COVID-19 in the prevention population compared to a placebo (8.5% *vs*. 15.2%) (*22*). Treatment of COVID-19 with bamlanivimab and etesevimab has been granted an EUA in about 15 countries around the world thus far (*23*).

Sotrovimab (also known as VIR-7831 and GSK4182136) is a monoclonal antibody designed to attach to a conserved epitope on the spike protein receptor binding domain of SARS-CoV-2, thus limiting the ability of the virus to enter the body's cells (*24*). Emergency use of this drug was originally authorized by the US FDA in May 2021, and this EUA was reissued in October and December 2021 (*12*). Sotrovimab is permitted for treatment of mild-to-moderate COVID-19 in adults and pediatric patients (12 years of age and older weighing at least 40 kg) with positive results of direct SARS-CoV-2 viral testing and who are at high risk for progression to severe COVID-19, including hospitalization or death (*25*). An interim analysis of a phase 3 trial (COMET-ICE) revealed that sotrovimab reduced the risk of hospitalization for more than 24 hours or death by 85% compared to a placebo (*24*). In December 2021, sotrovimab was approved to treat COVID-19 patients who do not require supplemental oxygen and who are at an increased risk of developing severe disease in the UK and European Union (*26-28*).

Tixagevimab and cilgavimab are two monoclonal antibodies that bind distinct epitopes of the viral spike protein receptor binding domain to interfere with the infection process (*29*). Tixagevimab and cilgavimab, administered together, were granted an EUA in the US to prevent COVID-19 in December 2021 (*12*). The authorized use of this antibody cocktail is for pre-exposure prophylaxis for COVID-19 in adults and pediatric individuals (12 years of age and older weighing at least 40 kg) with weakened immunity or who cannot be fully vaccinated due to a history of severe reaction to coronavirus vaccines (*30*). Results of a phase 3 clinical study (PROVENT) indicated that tixagevimab and cilgavimab reduced the risk of developing symptomatic COVID-19 by 77% in unvaccinated adults (≥ 18 years old) without a prior SARS-CoV-2 infection (*31*).

In September 2021, the South Korean Ministry of Food and Drug Safety (MFDS) approved regdanvimab (CT-P59), a neutralizing monoclonal antibody binding to the viral spike protein receptor binding domain, to treat COVID-19 in elderly patients ≥ 50 years of age with at least one underlying condition (obesity, cardiovascular disease, chronic lung disease, diabetes, chronic kidney disease, chronic liver disease, and patients receiving immunosuppressive agents) and mild symptoms of COVID-19, and in adult patients with moderate symptoms of COVID-19 (*32,33*). Preliminary results of a phase III clinical trial, released by the developer Celltrion, indicated that regdanvimab markedly decreased the risk of hospitalization or death by 72% versus a placebo in patients with mild-to-moderate COVID-19 symptoms who were considered at high risk of progressing to severe COVID-19 up to day 28 (*33*). In November 2021, regdanvimab was approved by the European Medicines Agency (EMA) to treat COVID-19 in adults who do not require supplemental oxygen and who are at increased risk of their disease becoming severe (*34*). Regdanvimab has received an EUA or conditional marketing authorization (CMA) in Indonesia, Brazil, Peru, and Australia for treatment of COVID-19 thus far (*32*).

In December 2021, China's National Medical

Products Administration (NMPA) approved amubarvimab (BRII-196) and romlusevimab (BRII-198) to treat mild to moderate COVID-19 in adult and pediatric patients (12 years of age and older weighing at least 40 kg) who are at high risk for progression to severe COVID-19 (*35*). Their indication for pediatric patients (age 12-17 weighing at least 40 kg) has been conditionally approved in China (*35*). Amubarvimab and romlusevimab are non-competing SARS-CoV-2 monoclonal neutralizing antibodies that are derived from convalesced COVID-19 patients and that have been subsequently engineered to reduce the risk of antibody-dependent enhancement and to prolong their plasma half-lives for potentially more durable efficacy (*36*). The approval of amubarvimab and romlusevimab by the NMPA is based on the results of a phase 3 clinical study (ACTIV-2) which indicated that the drugs significantly reduced the risk of hospitalization or death by 80% versus a placebo in outpatients who were considered at high risk of progressing to severe COVID-19 up to day 28 (*37*). Brii BioSciences, the developer, reported that the combination of amubarvimab and romlusevimab retains activity against major SARS-CoV-2 variants of concern, including B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.429 (Epsilon), B.1.617.2 (Delta), AY.4.2 (Delta plus), C.37 (Lambda), B.1.621 (Mu), and B.1.1.529 (Omicron) in *in vitro* studies (*38*). The US FDA is currently reviewing an application for an EUA for combination therapy with amubarvimab and romlusevimab.

The neutralizing antibodies that are currently approved or authorized for clinical use are mainly used for pre- or post-exposure prophylaxis for COVID-19 or treatment of early-stage COVID-19 in non-hospitalized patients (Table 1). Results of a recent phase III trial (ACTIV-3) indicated that neither sotrovimab nor amubarvimab plus romlusevimab displayed efficacy in improving clinical outcomes among adults hospitalized with COVID-19 (*39*), suggesting the shortcomings of neutralizing antibodies and the need for drugs with different mechanisms of action against COVID-19.

3. Drugs that suppress SARS-CoV-2 replication

SARS-CoV-2 is a spherical enveloped virus containing a single strand of positive sense RNA (*7*). Once inside the cell, the viral RNA is released into the cytoplasm and acts as messenger RNA (mRNA). Utilizing host ribosomes, two open reading frames, 1a (ORF1a) and ORF1b, of the virus genome are first translated to produce the polyproteins pp1a and pp1ab, which are then auto-proteolytically cleaved by the viral proteases PLpro and Mpro/3CLpro (encoded by ORF1a) to yield 16 nonstructural proteins (NSPs) (*4*). NSP7-16 including RNA-dependent RNA polymerase (RdRp), RNA helicase, and other proteins then form an RNA replicase-transcriptase complex (RTC) that generates new RNA genomes and mRNAs for the synthesis of structural proteins such as the spike glycoprotein (S), envelope protein (E), membrane protein (M), and the nucleocapsid phosphoprotein (N) as well as components necessary to assemble the new viral particles (*4*). The proteases Mpro/3CLpro and PLpro and components of RTC such as RdRp are potential targets for drugs to suppress viral replication (*40*).

The RdRp inhibitor remdesivir and the protease inhibitor lopinavir attracted considerable attention in early 2020 (*41-43*). A number of clinical studies have been conducted to test the efficacy and safety of these agents worldwide since the outbreak of the disease, and those studies have yielded evidence both corroborating and refuting their use to treat COVID-19 (*41*). The WHO's Solidarity clinical trial, an international collaboration to identify life-saving treatments for COVID-19, suggested that neither remdesivir nor lopinavir had obvious efficacy in hospitalized patients with COVID-19, as indicated by overall mortality, initiation of ventilation, and duration of hospitalization (*44*). Variations in baseline characteristics of study populations and therapeutic regimens may have influenced outcomes in different studies. Remdesivir (intravenous route) was approved in October 2020 in the US to treat COVID-19 in hospitalized adult and pediatric patients (aged ≥ 12 years and weighing ≥ 40 kg) (*45*). An EUA was also granted to remdesivir by the US FDA for treatment of COVID-19 in hospitalized pediatric patients weighing 3.5 kg to < 40 kg or aged < 12 years and weighing ≥ 3.5 kg (*46*). Remdesivir was approved in Japan and received a conditional marketing authorization in the European Union for treatment of COVID-19.

Molnupiravir, an orally administered form of a potent ribonucleoside analog that inhibits the replication of SARS-CoV-2, was approved in the UK on November 4, 2021 to treat patients with mild to moderate COVID-19 and at least one risk factor for developing severe illness such as obesity, older age (> 60 years), diabetes mellitus, or heart disease (*47*). On December 23, 2021, molnupiravir was authorized by the US FDA for the treatment of mild-to-moderate COVID-19 in adults with positive results of direct SARS-CoV-2 viral testing who are at high risk for progressing to severe COVID-19, including hospitalization or death (*48*). A randomized, double-blind, placebo-controlled clinical trial (MOVe-OUT) investigated the efficacy and safety of molnupiravir in non-hospitalized adult patients with mild to moderate COVID-19 at high risk for progression to severe COVID-19 and/or hospitalization. Results indicated that the risk of hospitalization or death was significantly reduced from 9.7% in the placebo group (68/699) to 6.8% (48/709) in the molnupiravir group. A fact worth noting is that nine deaths occurred in the placebo group and one occurred in the molnupiravir group (*49*). Given that molnupiravir is most effective when taken during the early stages of infection as revealed by the clinical data, its use is recommended as soon as possible following a positive COVID-19 test and

Table 1. Drugs that are approved or authorized for emergency use to treat COVID-19

Drug/agent	Mechanism	Indications/use	Status	Developer
Casirivimab/ Imdevimab	Neutralizing antibodies binding to ACE2	Post-exposure prophylaxis for COVID-19 or treatment of mild to moderate COVID-19 in adult and pediatric patients (12 years of age and older weighing at least 40 kg) who are at high risk for progression to severe COVID-19, including hospitalization or death.	Approved in Japan, the EU, the UK, and Australia; EUA in the US	Regeneron
Bamlanivimab/ Etesevimab	Neutralizing antibodies binding to ACE2	Post-exposure prophylaxis for COVID-19 or treatment of mild to moderate COVID-19 in adult and pediatric patients including neonates who are at high risk for progression to severe COVID-19, including hospitalization or death.	EUA in the US and many other countries	Eli Lilly (Eli Lilly licensed etesevimab from Junshi Biosciences)
Sotrovimab	Neutralizing antibodies binding to ACE2	Treatment of mild-to-moderate COVID-19 in adults and pediatric patients (12 years of age and older weighing at least 40 kg) who are at high risk for progression to severe COVID-19, including hospitalization or death.	Approved in the UK and EU; EUA in the US	GlaxoSmithKline and Vir Biotechnology
Tixagevimab/ cilgavimab	Neutralizing antibodies binding to ACE2	Pre-exposure prophylaxis for COVID-19 in adults and pediatric individuals (12 years of age and older weighing at least 40 kg) with weakened immunity or who cannot be fully vaccinated due to a history of severe reaction to coronavirus vaccines.	EUA in the US	AstraZeneca
Regdanvimab	Neutralizing antibodies binding to ACE2	Treatment of COVID-19 in adults who do not require supplemental oxygen and who are at increased risk of their disease becoming severe.	Approved in S. Korea and the EU; EUA in Indonesia, Brazil, Peru, and Australia	Celltrion
Amubarvimab/ romlusevimab	Neutralizing antibodies binding to ACE2	Treatment of mild to moderate COVID-19 in adult and pediatric patients (12 years of age and older weighing at least 40 kg) who are at high risk for progression to severe COVID-19.	Approved in China	Brii Bio
Remdesivir	Inhibiting viral replication by inhibiting RdRp	Treatment of COVID-19 in hospitalized adult and pediatric patients.	Approved in the US and Japan	Gilead
Molnupiravir	Inhibiting viral replication as ribonucleoside analog	Treatment of mild to moderate COVID-19 in patients who have at least one risk factor for developing severe illness.	Approved in the UK; EUA in the US	Merck
Nirmatrelvir/ ritonavir	Inhibiting viral replication by inhibiting the main protease (Mpro)	Treatment of mild to moderate COVID-19 in adults and pediatric patients (12 years of age and older weighing at least 40 kg) who are at high risk for progression to severe COVID-19.	EUA in the US	Pfizer
Dexamethasone	Immunosuppression	Treatment of severe and critical COVID-19.	Recommended by the WHO, National Health Service in the UK, and NIH in the US, and endorsed by the EMA	---
Baricitinib	Immunosuppression by inhibiting JAKs	Treatment of severe COVID-19 in adults and pediatric patients 2 years of age or older.	EUA in the US	Eli Lilly
Tocilizumab	Immunosuppression by binding the IL-6 receptor	Treatment of COVID-19 in patients two years of age and older who are receiving systemic corticosteroids and who require supplemental oxygen or mechanical ventilation.	EUA in the US; provisional approval in Australia	Genentech

Abbreviations: EUA, emergency use authorization; ACE2, angiotensin-converting enzyme 2; JAKs, Janus kinases; US, The United States; UK, The United Kingdom; EU, European Union.

within five days of the onset of symptoms (*47*).

Besides molnupiravir, the US FDA also issued an EUA for nirmatrelvir tablets and ritonavir tablets, co-packaged for oral use, for treatment of mild-to-moderate COVID-19 in adults and pediatric patients (12 years of age and older weighing at least 40 kg) with positive results of direct SARS-CoV-2 viral testing and who are at high risk for progression to severe COVID-19, including hospitalization or death (*50*). Nirmatrelvir is a SARS-CoV-2 main protease (Mpro or 3CLpro) inhibitor while ritonavir is a CYP3A inhibitor that may inhibit the CYP3A-mediated metabolism of nirmatrelvir and consequently increase nirmatrelvir plasma concentrations to levels anticipated to inhibit SARS-CoV-2 replication (*50*). Data from a phase 2/3 randomized, double blind, placebo-controlled trial (EPIC-HR) indicated that nirmatrelvir plus ritonavir reduced the proportion of patients with COVID-19-related hospitalization or death by 88% compared to a placebo in non-hospitalized adult patients who have a prespecified risk factor for progression to severe disease or who were 60 years and older, regardless of prespecified chronic conditions (*51*).

The approval or authorized emergency use of oral small molecule drugs such as molnupiravir and nirmatrelvir/ritonavir represents major progress in the fight against COVID-19. Neutralizing antibodies have several disadvantages such as costly production, stringent storage requirements, and inconvenience to use. In terms of large-scale use, small molecule oral drugs have some advantages including a high level of compliance with medication, low-cost synthesis, and less stringent storage requirements. Early intervention with oral antivirals would be more feasible for prophylaxis or treatment of COVID-19, thus preventing infection or disease progression.

4. Drugs that regulate the immune system

The immune system functions like "a double-blade sword" in the pathogenesis of COVID-19 (*52*). The innate and adaptive immune responses evoked by SARS-CoV-2 help to eliminate the virus and promote recovery (*53*). However, a hyperinflammatory syndrome induced by SARS-CoV-2 contributes to disease severity and mortality (*54*). The natural course of COVID-19 begins with an initial stage of viral replication that might be followed by a second stage in which a cytokine storm may occur, leading to severe COVID-19 (*54,55*). Thus, the optimal stage of the disease should be selected to administer immunomodulators to treat COVID-19. A dysregulated host innate immune response is regarded as a cause of the hyperinflammatory syndrome, which is characterized by elevated serum cytokines such as IL-6 and TNF-α, in patients with severe disease (*54*). Conventional anti-inflammatory drugs such as corticosteroids, novel cytokine blockades targeting specific cytokines, such as IL-6 and TNF-α or the Janus kinase (JAK) pathway, or repurposed drugs including artesunate and imatinib have been investigated in clinical studies, and some have been granted an EUA or recommended for treatment of COVID-19.

Dexamethasone is a corticosteroid used to treat a wide range of conditions because of its anti-inflammatory and immunosuppressant action (*56,57*). Dexamethasone is recommended by the WHO to treat patients with severe and critical COVID-19 but not for patients with non-severe COVID-19 (*58*). The RECOVERY study revealed that dexamethasone reduced the incidence of death for patients on invasive mechanical ventilation or receiving oxygen without invasive mechanical ventilation but not for those who received no respiratory support (*59*). Dexamethasone has also been suggested by the National Health Service in the UK and the National Institutes of Health (NIH) in the US and it has been endorsed by the European Medicines Agency (EMA) to treat severe COVID-19 (*60-62*).

Baricitinib, a drug that has been approved to treat rheumatoid arthritis by the US FDA, is an inhibitor of Janus kinases (JAKs), which transmit signals arising from cytokine or growth factor-receptor interactions on the cellular membrane to influence cellular processes of hematopoiesis and immune cell function (*63*). In December 2020, baricitinib received an EUA from the US FDA for the treatment of COVID-19, in combination with remdesivir, in hospitalized patients requiring supplemental oxygen, invasive mechanical ventilation, or extracorporeal membrane oxygenation (ECMO) (*64*). The EUA was revised based on the results of several clinical studies, and an updated EUA was issued by the US FDA in December 2021, in which baricitinib could be used to treat hospitalized adults and pediatric patients 2 years of age or older with severe COVID-19 (*65*).

Tocilizumab, an IL-6 receptor antibody approved to treat rheumatoid arthritis and cytokine release syndrome, is another drug that is widely used to clinically treat hyperinflammation in patients with COVID-19 (*66,67*). In June 2021, tocilizumab was granted an EUA in the US for the treatment of COVID-19 in hospitalized patients aged two years of age and older who are receiving systemic corticosteroids and who require supplemental oxygen, non-invasive or invasive mechanical ventilation, or ECMO based on the results of clinical studies including the RECOVERY trial, the COVACTA trial, the EMPACTA trial, and the REMDACTA trial (*68*). On December 1, 2021, tocilizumab received a provisional approval in Australia for the treatment of hospitalized patients receiving systemic corticosteroids and requiring supplemental oxygen or mechanical ventilation (*69*).

Artesunate, imatinib, and infliximab are three drugs that are already used to respectively treat malaria, certain cancers, and diseases of the immune system. These three drugs are now being tested for treatment of COVID-19 in the Solidarity PLUS trial led by the WHO (*70*). Artesunate is being evaluated for its anti-inflammatory

properties in this trial at the standard dose recommended for the treatment of severe malaria. Artesunate was reported to display anti-inflammatory action by inhibiting IL-6 and TNF-α release in animal models of acute lung injury and nephritis (*71,72*). A small-scale clinical study indicated that patients might benefit from artesunate (*73*), warranting further verification in clinical trials. Imatinib was reported to protect against capillary leakage and alveolar edema caused by inflammatory stimuli (*74*). A randomized clinical trial indicated that imatinib might confer a clinical benefit in hospitalized patients with COVID-19 (*75*), but further studies are required to validate those findings. Infliximab is a TNF-α inhibitor that has displayed efficacy and safety in restricting broad spectrum inflammation, and particularly in elderly populations who are most clinically vulnerable to COVID-19. In a small-scale clinical study, infliximab was found to abrogate pathological inflammatory signaling to facilitate clinical recovery in patients with severe or critical COVID-19 (*76*). More convincing evidence should be yielded by the WHO's Solidarity clinical trial in the future.

5. Conclusion

As more drugs are approved or authorized on an emergency basis to fight COVID-19, patient mortality should decrease further in the future. New oral antivirals have attracted considerable attention around the world because early drug intervention, a principle when using antivirals, will benefit more people, and especially those at risk of progressing to severe COVID-19. Nevertheless, the data from current clinical trials are limited, and safety and efficacy need to be evaluated through long-term and wide-ranging use. In addition, mutations in SARS-CoV-2 may lead to drug resistance, which is a major challenge that we face. A combination of social distancing, vaccines, and therapeutics is necessary to fight a "tough battle" against the epidemic. The use of therapeutics should have a great impact on formulating and revising public policies to control the pandemic. The pace of lifting of restrictions and economic recovery worldwide will also accelerate in the future.

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Review

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Potential therapeutic effect of Shufeng Jiedu capsule and its major herbs on coronavirus disease 2019 (COVID-19): A review

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SUMMARY The outbreak and rapid spread of coronavirus disease 2019 (COVID-19) poses a huge threat to human health and social stability. Shufeng Jiedu capsule (SFJDC), a patented herbal drug composed of eight medicinal plants, is used to treat different viral respiratory tract infectious diseases. Based on its antiviral, anti-inflammatory, and immunoregulatory activities in acute lung injury, SFJDC can be effectively used as a treatment for COVID-19 patients according to the diagnosis and treatment plan issued in China and existing clinical data. SFJDC has been recommended in 15 therapeutic regimens for COVID-19 in China. This review summarizes current data on the ingredients, chemical composition, pharmacological properties, clinical efficacy, and potential therapeutic effect of SFJDC on COVID-19, to provide a theoretical basis for its anti-viral mechanism and the clinical treatment of COVID-19.

Keywords Shufeng Jiedu capsule, coronavirus disease 2019, chemical composition, pharmacological properties, antiviral, anti-inflammatory

1. Introduction

Coronavirus disease 2019 (COVID-19), characterized by a rapid spread and profound impact on public health worldwide, has led to remarkable financial investments in the research and development of new drugs and vaccines (*1*). Fever, fatigue, and dry cough are the most common clinical presentations of COVID-19. However, few patients may experience nasal congestion, runny nose, and diarrhea (*2*). The basic clinical treatment for COVID-19 includes anti-infection, anti-inflammatory cytokines, non-specific antiviral drugs, and life support therapy (*3-6*). Despite increasing understanding of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and COVID-19, no clinical trial has revealed a validated significant effect for the treatment of patients with mild and moderate symptoms.

Traditional Chinese medicine (TCM) has been used for thousands of years in China to treat human diseases. Shufeng Jiedu capsule (SFJDC), a TCM containing eight types of herbal medicines, has a history of more than 30 years as a treatment for acute lung injury (ALI) and respiratory infections in China (*7*). SFJDC has been recommended by the China Food and Drug Administration (CFDA) for the treatment of the 2009 influenza A (H1N1)-related upper respiratory tract infections since 2009. According to "Novel Coronavirus Pneumonia Diagnosis and Treatment Program (from the fourth to the eighth edition)" in China, SFJDC is recommended for use during the medical observation period when clinical manifestations of fatigue and fever are displayed.

In this review, we sought to summarize the ingredients, chemical composition, pharmacological properties, clinical efficacy, and potential therapeutic effect on COVID-19 of SFJDC to provide a theoretical basis for its anti-viral mechanism and the clinical treatment of COVID-19.

2. Ingredients of SFJDC

SFJDC is a TCM formula that is composed of eight medicinal herbs, including *Bupleurum chinense, Fallopia japonica, Forsythia suspensa, Glycyrrhiza uralensis, Isatis indigotica, Patrinia scabiosaefolia, Phragmites australis*, and *Verbena officinalis*, that exert a synergistic effect. The herbal composition of SFJDC is summarized in Figure 1.

3. Chemical composition of SFJDC

The primary analytical approach implemented in phytochemical studies includes the separation and identification of active components, which is crucial for the modernization of traditional Chinese medicine. Recently, ultra-performance chromatography (UPLC/Q-TOF) and tandem mass spectrometry (MS) methods have been established to characterize the chemical profile of extracts from SFJDC. A total of 94 compounds, including 1 carbohydrate, 7 amino acids, 1 coumarin, 11 phenylethanoid glycosides, 4 phenolic acids, 5 plant lignins, 25 flavonoids, 6 anthraquinones, 5 alkaloids, 18 triterpenoid saponins, 1 iridoid, and 7 glycosides were tentatively identified (*8*). The identification of these components may serve as a foundation for future studies on the pharmacological effects of SFJDC.

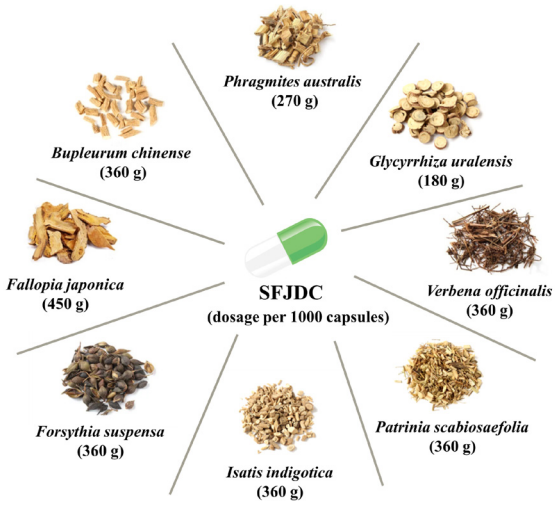

Figure 1. Herbal compositions of SFJDC. Every 1000 capsules of SFJDC comprise 360 g *Bupleurum chinense*, 450 g *Fallopia japonica*, 360 g *Forsythia suspensa*, 180 g *Glycyrrhiza uralensis*, 360 g *Isatis indigotica*, 360 g *Patrinia scabiosaefolia*, 270 g *Phragmites australis*, and 360 g *Verbena officinalis* according to data from the Chinese Pharmacopoeia.

4. Chemical composition and pharmacological activities of the constituents of SFJDC

SFJDC is mainly composed of eight Chinese traditional medicinal herbs, each of which produces its own therapeutic effect. The independent pharmacological activity of each constituent jointly and synergistically exerts antiviral, antibacterial, antitumor, and anti-inflammatory activities. The chemical composition and pharmacological activities of each constituent are summarized in Table 1.

4.1. *Bupleurum chinense*

Bupleurum chinense is a perennial herb belonging to the Umbelliferae family (*9*). *Bupleurum chinense* is widely used in TCM because of its multiple pharmacological effects. In TCM, Radix Bupleuri, the dried root of *Bupleurum chinense*, has been employed for more than two thousand years in China (*10*). According to modern pharmacological studies, Radix Bupleuri possesses a wide range of bioactive properties, including antipyretic, anti-inflammatory, hepatoprotective, antibacterial, antiviral, immune regulation, and antiplatelet agglutination functions (*11*). Among the complex constituents of Radix Bupleuri, saikosaponins have been identified as the major biologically active constituents using modern techniques (*12*).

Table 1. The family, chemical composition, and pharmacological activities of the constituents of SFJDC

Ingredient	Family	Pharmacological properties	Chemical composition
Bupleurum chinense	Umbelliferae	antipyretic, anti-inflammatory, hepatoprotective, antibacterial, anti-virus, immune regulation and antiplatelet agglutination functions	saikosaponins
Fallopia japonica	Polygonaceae	lipid regulating, anti-shock, anti-inflammatory, antioxidant, anticancer, hepatoprotective, antiviral, antibacterial and antifungal	quinones, stilbenes, flavonoids, coumarins and lignans
Forsythia suspensa	Oleaceae	anti-inflammatory, antioxidant, anti-bacterial, anti-cancer, anti-virus, anti-allergy, and neuroprotective	phenylethanoid glycosides, lignans, flavonoids, phenolic acids, terpenoids, cyclohexylethanol derivatives
Glycyrrhiza uralensis	Leguminoceae	anti-inflammatory, anti-allergic, antioxidant, antiulcer, hepatoprogenic, and neuroprotective	flavonoids and triterpenoid saponins
Isatis indigotica	Cruciferae	antiviral, anti-inflammatory and anticancer	alkaloids, phenolic compounds, polysaccharides, glucosinolates, carotenoids, volatile constituents, and fatty acids
Patrinia scabiosaefolia	Valerianaceae	anti-cancer, anti-inflammation, anti-pathogenic microorganisms, anti-oxidation, sedation, and hypnosis	triterpenes, iridoids, saponins, sesquiterpenes, flavonoids, coumarins, and lignans
Phragmites australis	Poaceae	antiasthmatic, antiemetic, antipyretic, antitussive, depurative, diuretic, febrifuge, lithontriptic, sedative, sialogogue, and stomachic	terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, sterols, and amylase
Verbena officinalis	Verbenaceae	antioxidant, antimicrobial, anti-inflammatory, neuroprotective anticancer, analgesic, or anticonvulsant	iridoids, phenylpropanoid glycosides, phenolic acids, flavonoids, terpenoids, and essential oil

4.2. *Fallopia japonica*

Fallopia japonica is a perennial herb belonging to the Polygonaceae family (*13*). *Fallopia japonica*, a traditional Chinese medicinal herb, is widely distributed in southern China and Japan. According to numerous studies, the root of *Fallopia japonica* has a wide range of pharmacological activities, including lipid-regulating, anti-shock, anti-inflammatory, antioxidant, anticancer, hepatoprotective, antiviral, antibacterial, and antifungal effects (*14-16*). More than 67 chemical compounds have been isolated from *Fallopia japonica*, and its major components have been determined to be quinones, stilbenes, flavonoids, counmarins, and ligans (*13*). Among the chemical compounds, resveratrol, piceid, and emodin have been found to exhibit various biological activities. Resveratrol and piceid have been shown to possess antioxidant, anti-inflammatory, anti-cancer, anti-aging, and cardioprotective properties (*17*). Emodin has been shown to exert anti-inflammatory, antibacterial, and antineoplastic activities (*18-20*).

4.3. *Forsythia suspensa*

Forsythia suspensa is a flowering plant belonging to the Oleaceae family (*21*). *Forsythia suspensa* is widely distributed in China, Southeast Asia, and many European countries (*22*). Fructus Forsythiae, the seeds of *Forsythia suspensa*, exhibits high pharmacological activity and is documented in every edition of the Chinese Pharmacopoeia. In fact, a total of 114 Chinese medicinal preparations containing Fructus Forsythiae are listed in the 2015 edition of the Chinese Pharmacopoeia. Based on modern pharmacological studies, Fructus Forsythiae exerts anti-inflammatory, antioxidant, anti-bacterial, anti-cancer, anti-viral, anti-allergy, and neuroprotective effects (*23*). To date, approximately 210 compounds have been identified from *Forsythia suspensa*, including phenylethanoid glycosides, lignans, flavonoids, phenolic acids, terpenoids, cyclohexylethanol derivatives, and others (*23*). Among them, lignans and phenylethanoid glycosides, such as forsythiaside, phillyrin, rutin, and phillygenin, are considered the characteristic and active constituents of this herb (*23*).

4.4. *Glycyrrhiza uralensis*

Glycyrrhiza uralensis is a medicinal herb that belongs to the Leguminaceae family (*24*). *Glycyrrhiza uralensis* is found in southern Europe (*Glycyrrhiza glabra*) and East Asia (*Glycyrrhiza uralensis*), and has been used for traditional medicinal purposes for almost two thousand years (*24*). According to phytochemical studies, the main bioactive constituents of *Glycyrrhiza uralensis* are flavonoids and triterpenoid saponins, including licochalcone A, glycyrrhizic acid, isoliquiritigenin, liquiritigenin, and liquiritin, which exhibit a variety of pharmacological activities, such as anti-inflammatory, anti-allergic, antioxidant, antiulcer, hepatoprogenic, and neuroprotective activities (*25-27*).

4.5. *Isatis indigotica*

Isatis indigotica is a biennial herbaceous plant belonging to the Cruciferae family (*28*). *Isatis indigotica* is distributed across China, and Radix Isatidis, the dried roots of *Isatis indigotica*, are widely employed in the prevention and treatment of a wide range of viral infections, including fever, influenza, epidemic hepatitis, and bacterial infection for thousands of years (*29*). Based on recent clinical data, Radix Isatidis has clinical effects on severe acute respiratory syndrome (SARS) and H1N1-influenza (*30,31*). Numerous phytochemical studies have led to the isolation of valuable bioactive compounds, such as alkaloids, phenolic compounds, polysaccharides, glucosinolates, carotenoids, volatile constituents, and fatty acids, among which alkaloids are the dominant compounds (*29*). Owing to numerous studies, these ingredients have been identified to have antiviral, anti-inflammatory, and anticancer effects (*32-34*).

4.6. *Patrinia scabiosaefolia*

Patrinia scabiosaefolia is a herbaceous perennial plant belonging to the Valerianaceae family. *Patrinia scabiosaefolia* is a Chinese herbal medicine with high nutritional and medicinal value, and is mainly distributed in mainland China (*35*). Modern pharmacological studies have shown that *Patrinia scabiosaefolia* has various effects, including anti-cancer, anti-inflammatory, anti-pathogenic, anti-oxidation, sedation, and hypnosis (*36*). According to previous phytochemical investigations, this genus contains a variety of components, including triterpenes, iridoids, saponins, sesquiterpenes, flavonoids, coumarins, and lignans (*37*). Among them, triterpenoid aglycones and triterpenoid saponins are considered the main active constituents of *Patrinia scabiosaefolia* (*36*). Typical representatives of triterpenoid aglycones in *Patrinia scabiosaefolia* include ursolic acid, hederagenin, and oleanolic acid.

4.7. *Phragmites australis*

Phragmites australis is a species belonging to the family, Poaceae (*38*). *Phragmites australis* found in wetlands throughout the temperate and tropical regions of the world. The root of *Phragmites australis* is used as a perennial Chinese herbal medicine (*39*) and the rhizoma of *Phragmites australis* has been used clinically for patients with pulmonary diseases throughout the long history of TCM use (*40*). The roots of *Phragmites australis* have been reported to have a

wide range of pharmacological activities, including antiasthmatic, antiemetic, antipyretic, antitussive, depurative, diuretic, febrifuge, lithontriptic, sedative, sialogogue, and stomachic (*39*). Phytochemical investigations have proven that this genus is rich in terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, sterols, and amylase (*40*).

4.8. *Verbena officinalis*

Verbena officinalis is a herbal species of the family, Verbenaceae. Although *Verbena officinalis* is a perennial herb native to Europe, it is now growing worldwide (*41*). *Verbena officinalis* has traditionally been used to treat melancholia, hysteria, seizures, jaundice, fever, cholecystaliga, anxiety, depression, insomnia, menstrual disorders, abdominal problems, malaria, pharyngitis, edema, cough, asthma, rheumatic, and thyroid problems (*42-44*). *Verbena officinalis* has been reported to consist of several compounds, including iridoids, phenylpropanoid glycosides, phenolic acids, flavonoids, terpenoids, and essential oils (*45,46*). Numerous modern pharmacological studies have confirmed the antioxidant, antimicrobial, anti-inflammatory, neuroprotective, anticancer, analgesic, and anticonvulsant effects of *Verbena officinalis* herb extracts (*47*).

5. Clinical efficacy of SFJDC

SFJDC is mainly used to treat fever, parotitis, amygdalitis, plague, and other diseases (*48*). Recent studies have shown that SFJDC has been widely used in the clinical treatment of viral diseases, such as Middle East Respiratory Syndrome (MERS), influenza, human infection with H7N9 avian influenza, and respiratory diseases (such as acute upper respiratory illness, acute exacerbation of chronic obstructive pulmonary disease, and pneumonia) (*7*). Based on clinical data, SFJDC might be a promising candidate for the treatment of COVID-19. The combination of SFJDC with conventional antiviral drugs for the treatment of COVID-19 patients can effectively improve clinical symptoms, including dry cough, fever, and systemic fatigue (*48,49*). In particular, the combination of arbidol, a synthetic broad-spectrum antiviral drug, and SFJDC to treat common-type COVID-19 reduces the duration of symptoms and increases the clinical effectiveness without causing significant adverse reactions (*50*). Similarly, another clinical study showed that SFJDC, added to standard antiviral therapy, significantly reduced the clinical recovery time of COVID-19, fatigue, and cough days compared to AVD alone (*51*). SFJDC therapy was also found to be significantly more effective when administered within the first 8 days after symptom onset (*51*). Based on case reports, four patients with mild or severe 2019-nCoV pneumonia were cured or had significant improvement in their respiratory symptoms after treatment with combined lopinavir/ritonavir, arbidol, and SFJDC on the basis of supportive care (*52*). The recommended diagnosis and treatment schemes for SFJDC are summarized in Figure 2.

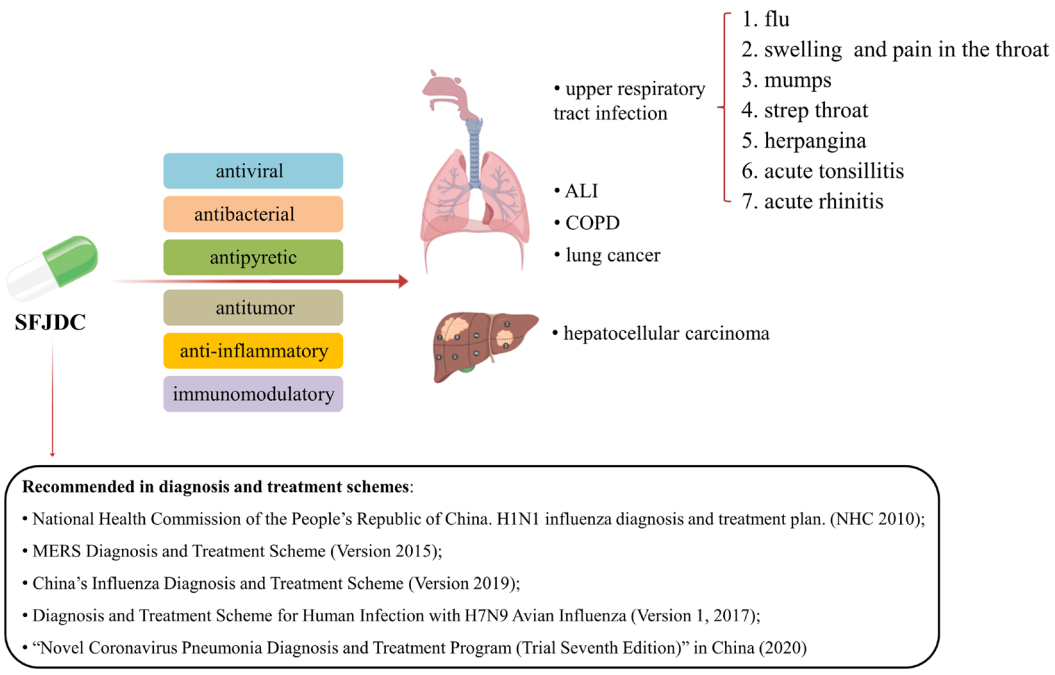

Figure 2. The pharmacological properties and clinical efficacy of SFJDC. SFJDC exerts antiviral, antibacterial, antitumor, and anti-inflammatory activities and is used to treat different upper respiratory tract infections, ALI, COPD, lung cancer, and hepatocellular carcinoma. SFJDC has been recommended in several diagnosis and treatment schemes.

6. Potential therapeutic effect of SFJDC on COVID-19

Currently, SARS-CoV-2 infection and immune dysfunction are believed to be the two main factors driving the pathogenesis of COVID-19 (*53*). In the early course of infection, the manifestation of the disease is primarily driven by the replication cycle of SARS-CoV-2. In the late course of infection, the severity of the disease is driven by a remarkable inflammatory/immune response to the virus. Thus, the anti-viral and anti-inflammatory/anti-oxidative capabilities and properties of SFJDC might act in tandem to improve the outcomes of infected patients. The potential therapeutic effects of SFJDC on SARS-CoV-2 are summarized in Figure 3.

6.1. Potential inhibitory effect of SFJDC on the replication cycle of COVID-19

6.1.1. Antiviral activity of SFJDC

Based on clinical investigations and basic research, SFJDC alone or in combination with other chemotherapeutic drugs exhibits antiviral effects. Modern clinical studies have shown that SFJDC has therapeutic effects on viral diseases, including MERS, influenza, and human infection with H7N9 avian influenza (*48*). Moreover, pre-clinical studies have shown that treatment with SFJDC and/or oseltamivir could decrease the elevated levels of NLRP3-inflammasome-associated components in human bronchial epithelial cells inoculated with the influenza A virus (IAV) (*54*). The combination of SFJDC and oseltamivir improved survival rates, alleviated lung damage, and reduced viral titers in lung homogenates from IAV-infected chronic obstructive pulmonary disease (COPD) rats (*54*). Furthermore, SFJDC significantly reduced the viral load in the lungs of HCoV-229E mice (*51*). Clinical data have shown that the addition of SFJDC to standard antiviral therapy significantly reduces the clinical recovery time of COVID-19 (*50,51*).

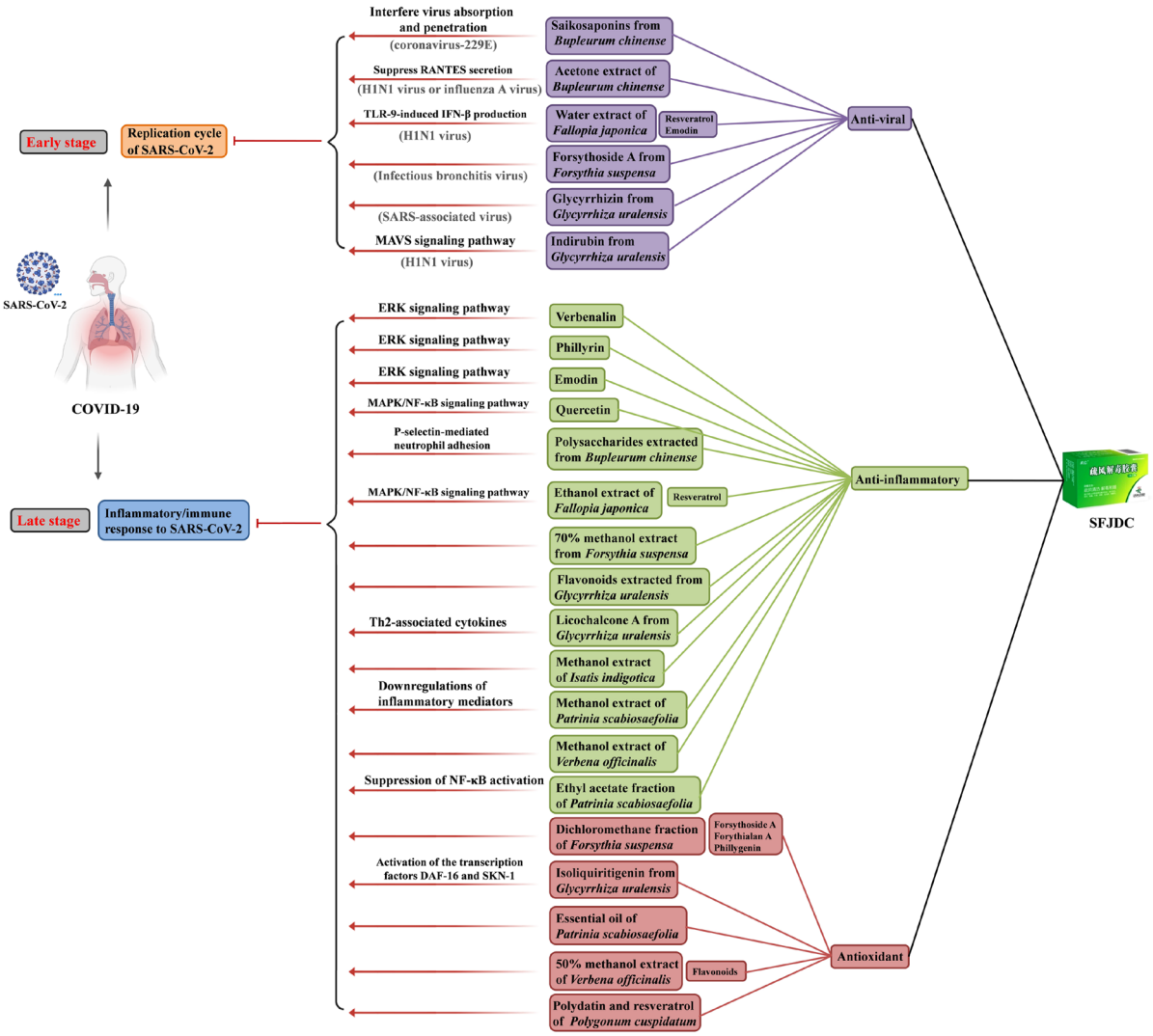

Figure 3. The potential therapeutic effect of SFJD on SARS-CoV-2. The replication cycle of SARS-CoV-2 in the early course and immunity dysfunction in the late course of infection are the two main factors driving the pathogenesis of COVID-19; the anti-viral and anti-inflammatory/anti-oxidative capabilities and properties of SFJD and its bioactive components might act in tandem to improve the outcomes of infected patients.

6.1.2. Active ingredients of SFJDC with antiviral activity

The acetone extract of Radix Bupleuri (the dried roots of *Bupleurum chinense*) has been reported to exhibit a significant antiviral effect on acute respiratory tract infections with H1N1 virus infection, the mechanism of which may be related to its suppression of influenza A virus-induced regulation of activation normal T cell expressed and secreted (RANTES) secretion (*55*). Moreover, saikosaponins (a, b_2, c, and d), the main active ingredient of Radix Bupleuri, exerted definitive antiviral activity against human coronavirus-229E by interfering with the early stage of viral replication, including absorption and penetration of the virus (*56*). These results suggest that Radix Bupleuri may have therapeutic benefits for the treatment of viral infection-associated diseases.

Fallopia japonica and its active components, resveratrol and emodin, have been shown to suppress influenza virus replication in A549 cells (*57*). Moreover, they preferentially inhibit the replication of multiple subtypes of influenza A virus. Mechanistically, *Fallopia japonica*, emodin, and resveratrol could upregulate the expression of interferon beta (IFN-β) through Toll-like receptor 9 (TLR9) and downregulate the expression of hemagglutinin and neuraminidase (*57*). Additionally, the anti-viral activity of resveratrol was abolished when supplemented with neutralizing anti-IFN-β antibodies or a TLR9 inhibitor in A549 cells, indicating that resveratrol may inhibit influenza virus replication by acting synergistically with IFN-β.

Forsythoside A is a major active constituent of *Forsythia suspensa* fruits. The antiviral activity of forsythoside A was confirmed by infecting primary chicken embryo kidney cells with infectious bronchitis virus (IBV) infection. The results indicate that forsythoside A inhibited the replication of avian IBV infection *in vitro* in a dose-dependent manner (*58*).

Glycyrrhizin is an active component of *Glycyrrhiza uralensis*. High concentrations of glycyrrhizin (4,000 mg/L) have been demonstrated to completely block SARS-CoV-2 replication (*59,60*). Glycyrrhizin can also inhibit the adsorption and penetration of the virus (*59*).

Indirubin, a bisindole alkaloid, is the main active ingredient in *Isatis indigotica*. A previous study demonstrated that indirubin significantly decreased the susceptibility of restrained mice to influenza H1N1 virus owing to the lowered mortality and reduced viral replication in the lungs (*31*). Mechanistically, indirubin maintained the morphology and function of mitochondria following influenza A virus infection and enhanced IFN-β production by promoting the mitochondrial antiviral signaling pathway (*31*).

6.2. Potential anti-inflammatory and anti-oxidative activities of SFJDC on the inflammatory/immune response to SARS-CoV-2

6.2.1. Anti-inflammatory and anti-oxidative activities of SFJDC

The anti-inflammatory and immunomodulatory properties of SFJDC have been demonstrated in several studies. Some active compounds of SFJDC, including forsythiaside (*61*), vitexin (*62*), and emodin (*63*), have been reported to possess anti-inflammatory effects. Tao *et al.* used an LPS-induced ALI rat model to investigate the anti-inflammatory effect of SFJDC (*64*). Based on the results, SFJDC can alleviate LPS-induced stress injury and inhibit inflammation in lung tissue by suppressing the mitogen-activated protein kinase (MAPK)/nuclear factor kappa-B (NF-κB) signaling pathway (*64*). Recently, target prediction and RNA sequencing (RNA-Seq) based on transcriptome analysis have been used to clarify the inflammation-eliminating mechanism of SFJDC (*65*). According to the results, various ingredients of SFJDC, especially verbenalin, phillyrin, and emodin, could ameliorate *Pseudomonas aeruginosa-induced* acute lung injury, among which the extracellular regulated protein kinases (ERK) pathway was identified as a key pathway related to its anti-inflammatory effect (*65*). Similarly, airway inflammation and lung injury in IAV-infected rats could be controlled by the combination of SFJDC and oseltamivir by modulating the nucleotide-binding oligomerization domain (NOD)-like receptors containing pyrin domain 3 (NLRP3) inflammasome and subsequently downregulating interleukin-1β (IL-1β) and IL-18 levels (*54*).

6.2.2. Active ingredients of SFJD with anti-inflammatory and anti-oxidative activities

Recently, the anti-inflammatory effects and possible mechanisms of water-soluble polysaccharides (BCPs) extracted from *Bupleurum chinense* were investigated. According to the results, BCPs could significantly ameliorate lung injury in an LPS-induced acute pneumonia model by inhibiting P-selectin-mediated recruitment of neutrophils (*66*). As P-selectin, which mediates adhesion between endothelium and neutrophils, is a promising target for inflammation-related diseases, it provides a new therapeutic strategy for improving inflammation-related disease processes with polysaccharides.

Another study investigated the effects of licorice flavonoids (LF) extracted from the roots of *Glycyrrhiza uralensis* on LPS-induced acute pulmonary inflammation in mice. Treatment with LF significantly decreased LPS-induced inflammatory cells and tumor necrosis factor-α (TNF-α) and IL-1β mRNA expression in lung tissues, suggesting that LF effectively attenuates LPS-induced pulmonary inflammation (*67*). Licochalcone A, isolated from *Glycyrrhiza uralensis*, has been reported to have anti-inflammatory effects. According to *in vitro* studies, licochalcone A significantly inhibited reactive

oxygen species, eotaxin, and proinflammatory cytokines in inflammatory human tracheal epithelial (BEAS-2B) cells (*68*). Consistently, *in vivo* studies revealed that licochalcone A significantly decreased oxidative responses, reduced malondialdehyde levels, and increased glutathione levels in the lungs of ovalbumin (OVA)-sensitized mice (*68*). These findings suggest that licochalcone A has excellent potential to ameliorate asthmatic inflammation and oxidative stress.

The antinociceptive, anti-inflammatory, and antipyretic effects of *Isatis indigotica* were previously demonstrated. The root extract of *Isatis indigotica* was revealed to significantly inhibit the writhing responses of mice and decrease the licking time in the early and late phases of the formalin test (*69*). Moreover, carrageenan-induced paw edema in rats and pyrexia induced by LPS were attenuated by treatment with the root extract of *Isatis indigotica* (*69*).

Patrinia scabiosaefolia is usually used to treat anti-inflammatory diseases, especially colonic inflammation, viral infections, hepatitis, and uteritis in Asia. Numerous studies have shown that oleanonic acid and ursolic acid from *Patrinia scabiosaefolia* have good anti-inflammatory effects (*70-72*). More recently, the anti-inflammatory effect of the methanol extract of the roots of *Patrinia scabiosaefolia* in a dextransulfate sodium-induced colitis mouse model was investigated. According to the results, *Patrinia scabiosaefolia* can not only significantly attenuate tissue myeloperoxidase accumulation, but also inhibit the abnormal secretion and mRNA expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (*73*). Similarly, the ethyl acetate fraction of *Patrinia scabiosaefolia* suppressed LPS-induced nitric oxide (NO) and IL-6 production in RAW 264.7 cells, and inhibited the production of IL-6 and TNF-α in LPS-stimulated splenocytes from BALB/c mice. Mechanistically, the ethyl acetate fraction of *Patrinia scabiosaefolia* could downregulate the LPS-induced increase in NF-κB activity. Therefore, *Patrinia scabiosaefolia* may be a potential therapeutic candidate for the treatment of inflammatory diseases.

Verbena officinalis has traditionally been used for the treatment of topical inflammation. Recently, the anti-inflammatory and gastroprotective activities of *Verbena officinalis* were evaluated in an acute gastric ulcer model induced by ethanol in rats. All extracts obtained with different solvents (methanol, enriched flavonoids, and supercritical CO_2) of *Verbena officinalis* exerted anti-inflammatory activity and decreased the area of ethanol-induced gastric damage in rats (*74*).

In recent years, several studies have shown that extracts from *Forsythia suspensa* exhibit remarkable antioxidant activity. Previously, phillyrin and forsythoside were found to be the major components of *Forsythia suspensa*, which are responsible for its antioxidant activities (*75*). Subsequently, forsythialan A, forsythialan B, phillygenin, and 8-hydroxypinoresinol, extracted from the fruits of *Forsythia suspensa*, also exerted potent protective effects against peroxynitrite-induced oxidative stress in LLC-PK1 cells (*76*). Additionally, the dichloromethane (CH_2Cl_2) fraction of *Forsythia suspensa* exerted the strongest scavenging activity in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging experiment (*77*). These results indicate that *Forsythia suspensa* exerts protective effects against oxidative stress.

7. Network pharmacology tools to analyze the mechanism of SFJDC prevention and treatment of COVID-19

More recently, to identify new candidates with potential activity against SARS-CoV-2 viral targets, several studies employed computer modeling to explore the mechanism of SFJDC using network pharmacology and molecular docking technology. In this section, the active ingredients of SFJDC for the treatment of COVID-19 were predicted by network pharmacology methods, including quercetin, resveratrol, emodin, and phillyrin.

Among the viral proteins of SARS-CoV-2, 3C-like protease (3CLpro), a protease highly conserved among coronaviruses, is an attractive target for antiviral inhibitors owing to its indispensable role in viral replication and gene expression of viral proteins (*78*). Thus, molecules that can inhibit SARS-CoV-2 3CLpro would hinder viral replication and represent appropriate candidates for the development of low-toxicity drugs against this devastating pathogen. Quercetin has been reported to interact with 3CLpro using biophysical techniques and bind to the active site in molecular simulations (*79*). In particular, the study revealed a significant inhibition by quercetin of 3CLpro with a docking binding energy corresponding to -6.25 kcal/mol (*79*). Consistently, protein-chemical interactions suggest quercetin is a promising drug candidate against COVID-19 and other SARS-like viral infections (*80*).

Recently, network pharmacology and bioinformatics analysis were conducted to uncover the pharmacological mechanisms of resveratrol against COVID-19 (*81*). A significant overlap in geneontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was found between SFJDC targets and SARS-CoV-2 differentially expressed genes (DEGs) (*81*). The shared targets were highly enriched in inflammation-related pathways, including the IL-17 signaling pathway, NF-κB signaling pathway, and TNF signaling pathway (*81*). Resveratrol has also been suggested to be a promising drug candidate against COVID-19 through protein-chemical interactions (*80*). Collectively, these studies revealed that resveratrol is a promising therapeutic candidate for COVID-19 and highlighted the probable key targets and pathways involved.

Another study screened and harvested the candidate genes or targets of emodin and COVID-19 using

bioinformatics databases (*82*). According to the results, the core targets of emodin for the treatment of COVID-19 include MAPK1, tumor protein (TP53), and TNF. GO analysis of emodin against COVID-19 mainly highlighted the cytokine-mediated signaling pathway, response to LPS, and response to molecules of bacterial origin. KEGG analysis revealed that the molecular pathways mainly included the IL-17 signaling pathway, advanced glycationend-product (AGE)-advanced glycation end-product receptor (RAGE) signaling pathway in diabetic complications, and TNF signaling pathway (*82*). Molecular docking results revealed the docking capability of emodin and COVID-19 (*82*). Taken together, the current bioinformatic findings revealed the targets and pharmacological mechanisms of emodin in the treatment of COVID-19.

A research strategy combining network pharmacological analysis, protein docking, and molecular docking virtual computation was adopted to identify potential inhibitors of COVID-19 from active compounds in Mongolian medicine (*83*). Phillyrin was found to block the combination of SARS-CoV-2 S-protein and angiotensin-converting enzyme 2 (ACE2) at the molecular level (*83*). ACE2 is a functional receptor on the cell surface through which SARS-CoV-2 enters host cells (*84*). Therefore, phillyrin can be used as a potential inhibitor of the ACE2 receptor of SARS-CoV-2 in further research and development.

8. Conclusions

The global outbreak of COVID-19 has had a catastrophic impact on the global economy and human health. However, no specific therapeutic drugs are available to treat COVID-19. SFJDC is a commonly used Chinese medical preparation for the treatment of viral influenza due to its good clinical efficacy and few side effects. According to modern pharmacological studies, SFJDC, composed of eight traditional Chinese medicines, contains a variety of active ingredients. These ingredients exhibit a wide range of biological activities and pharmacological effects, including antiviral, antibacterial, antitumor, and anti-inflammatory properties, making SFJDC an adjuvant therapy for COVID-19. Nevertheless, further studies are required to elucidate the unconfirmed effects, regulatory mechanisms, and adverse reactions of SFJDC in the treatment of COVID-19.

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Review

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Dual targeting, a new strategy for novel PARP inhibitor discovery

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SUMMARY As a hallmark for cancer treatment, PARP inhibitors can effectively kill tumor cells with a mechanism termed as synthetic lethality, and are used to treat various cancers including ovarian, breast, prostate, pancreatic and others with DNA repair defects. However, along with the clinical trials progressing, the limitations of PARP-1 inhibitors became apparent such as limited activity and indications. Studies have shown that a molecule that is able to simultaneously restrict two or more targets involving in tumors is more effective in preventing and treating cancers due to the enhancing synergies. In order to make up for the shortcomings of PARP inhibitors, reduce the development cost and overcome the pharmacokinetic defects, multiple works were carried out to construct dual targeting PARP inhibitors for cancer therapy. Herein, they were summarized briefly.

Keywords PARP, BRCA, dual targeting, inhibitor, antitumor

1. Introduction

Poly (ADP-ribose) polymerase (PARP), a family of at least 17 members, catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) to itself and substrate proteins (*1*) and plays a role in recruiting DNA repair proteins to the site of DNA injury and triggering the DNA damage repair *via* homologous recombination (HR) pathway (*2*). PARP is also involved in chromatin remodeling, gene transcription, signal transduction, cell cycle regulation, cell death control and aging (*3*). Abnormal expression of PARP is associated with many human disorders including cancers, oxidative stress, metabolic diseases and inflammation (*4*). Since its discovery, PARP has become an important drug targets.

Among all PARPs, PARP-1 is the founding member and the most widely investigated one. As a 116-kDa protein, PARP-1 is one of the most abundant proteins in the nucleus, and plays a role of more than 90% in DNA damage repair (*5*). PARP-1 can be activated up to 500-fold by DNA strand breaks (*6*). PARP-1 contains three functional domains: The N-terminal DNA-binding domain contains two zinc fingers which are important for the PARP-1 binding to single-strand break (SSB) and double-strand break (DSB). The central auto-modification domain, specific glutamate and lysine residues serve as acceptors of ADP-ribose moieties, thereby allowing the enzyme to poly(ADP-ribosyl)ate (PARylate) itself. Finally, the C-terminal catalytic domain transfers ADP-ribose subunits from NAD^+ to protein acceptors, and forming poly (ADP-ribose) polymer. C-terminal catalytic domain is also the binding site of PARP inhibitors (*7*).

PARP can directly participate in the base resection repair pathway and repair single-stranded DNA gaps by activating ataxia telangiectasia mutated (ATM) to promote homologous recombination (HR) (*8*). When DNA damage occurs, PARP binds to DNA gap as a dimer through DNA binding domain which causes a conformational change that activates PARP-1 to cleave NAD^+ into nicotinamide and an ADP-ribose moiety. ADP ribose is covalently transferred to PARP-1 itself or other receptor proteins and forms poly ADP ribose (PAR) chains, whose high negative charge alters the function of the nuclear receptor protein. The high steric hindrance generated causes chromosome relaxation and sends signal to recruit DNA repair proteins and guide them to bind to the gap and repair the damaged site (*9*).

1.1. Mechanism of action of PARP inhibitors

Studies have shown that targeting more than two DNA repair pathways in tumor cells can induce "synthetic lethality" mechanism and PARP inhibitors are the first anticancer drugs approved for clinical use under the concept of synthetic lethality (*10*). BRCA1 and BRCA2 are two tumor suppressors responsible for DSB repair by HR and humans with mutations in these two genes are inclined to suffer from breast and other cancers (*11*). Inhibition of PARP-1 bring about the blockage of base excision repair and the persistent SSB of DNA chain,

finally resulting in DSBs. In normal cells, these DSBs can be repaired through HR but not in BRCA1- and/or BRCA2-deficient tumor cells (*12,13*). Accumulation of DSBs becomes highly toxic to tumor cells and results in synthetic lethality. So, cancer patients with disabled BRCA1/2 are susceptible to the treatment with PARP inhibitors, and due to this feature, the efficacy of PARP inhibitors is expected to be extended to cancers with the same DNA repair dysfunction, such as breast cancer, ovarian cancer (*14*), prostate cancer (*15*), pancreatic cancer (*16*) and lung cancer (*17*). Besides the synthetic lethality mechanism, PARP inhibitors also exert cytotoxic effects through trapping PARP-DNA complexes, thereby preventing DNA replication and transcription (*18*).

The development of PARP inhibitors based on synthetic lethality is a hallmark for cancer therapy. Accordingly, the research and development of PARP inhibitors has become a hot topic in the anticancer field.

1.2. The binding mode of PARP inhibitors

The NAD^+ binding domain of PARP-1 is divided into three subdomains, including the nicotinamide-ribose binding domain, the phosphate binding domain and the adenine-ribose binding domain. Most of the reported PARP inhibitors were designed to mimic the nicotinamide structure and bind competitively with NAD^+ at the nicotinamide-ribose binding domain (*19*).

So far, the most important pharmacophore in PARP inhibitors is a amide group free or in an cycle system which is capable of competing with the natural substrate NAD^+ at the catalytic site of PARP (*20*). The amide groups can form two critical hydrogen bonds (HBs) with Ser904 and Gly863 residues. Apart from the amide group, the aromatic ring to which the amide group attaches can form a π-π stacking with Tyr907 residue, which is another key interaction between inhibitors and PARP (*21*). The other part of the PARP inhibitors go through another two domains. Some conserved water molecules can form extra HBs and contribute to the design of PARP-1 selective inhibitors.

After the failure of iniparib, currently, a total of six PARP inhibitors are approved on the market, including olaparib (1), rucaparib (2), niraparib (3), talazoparib (4), fluzoparib (5), and pamiparib (6) (*22*) as showed in Figure 1 below.

1.3. Existing problems of PARP inhibitors

As clinical trials were published, the limitations of PARP inhibitors became apparent. On the one hand, PARP inhibitors as monotherapy are only effective against BRCA1/2 defective cancers (*23*). To cancers with normal expression of BRAC, the effectiveness of PARP inhibitors is pale. On the other hand, long-term use of PARP inhibitors also faces drug resistance problems induced by different mechanisms (*24*).

The pathogenesis of cancer is complex, and a single antitumor drug usually cannot provide effective and lasting inhibition. There are several strategies to address this problem (*25*), one of which is the design of dual targeting PARP inhibitors which can inhibit other cancer related targets other than PARPs.

2. Dual targeting PARP inhibitors

2.1. PARP/HDAC dual targeting inhibitor

Acetylation as the best studied epigenetic modification plays important roles in the regulation of a host of normal cellular processes such as cell differentiation, proliferation, angiogenesis, and apoptosis. Dysregulation of acetylation is implicated in diverse cellular events in pathologies of cancer.

The level of acetylation of histones and non-histone proteins is governed by two antagonistic families of enzymes: histone deacetylases (HDACs) and histone acetyl transferases (HATs) (*26*). HDACs are a family of ubiquitous enzymes capable of removing acetyl groups from the ε-amino groups of lysine residues present within core histones and many non-histone substrates (*27*). Consequently, the positive charge on the N-terminal of core histones increases and strengthens interactions with negatively charged DNA while blocking access of transcriptional machinery to the DNA template, leading to gene silencing.

There are 18 known members in HDAC family which is further subdivided into four classes based on their sequence homology: Class I HDACs include HDAC1, 2, 3, and 8; Class II HDACs include Classes IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10); Class III HDACs, known as sirtuins (sirt1-7); and Class IV HDAC (HDAC11) (*28*). Silencing or

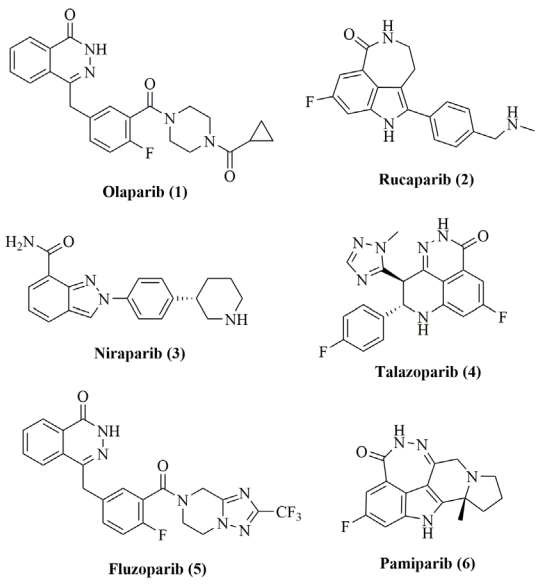

Figure 1. Six approved PARP inhibitors.

inhibiting HDACs can impair cell cycle, cell growth, chromatin decondensation, and angiogenesis and induce cell differentiation, apoptosis in several cancer cell types. HDACs have therefore emerged as important therapeutic targets for cancers. Accompanied by the extensive elucidation of mechanisms and functions of HDAC in tumorigenesis, the development of HDAC inhibitors represents a powerful weapon fighting against for cancers.

In 2006, the FDA approved SAHA used in treating the rare cutaneous T-cell lymphoma (*29*). Following SAHA, romidepsin, belinostat and panobinostat were approved by the FDA for treatment of cancers including cutaneous T-cell lymphoma, peripheral T-cell lymphoma (PTCL), and multiple myeloma. The benzamide-based Class I HDAC-selective inhibitor chidamide was approved by NMPA for the treatment of relapsed or refractory PTCL. Apart from these five drugs, several HDAC inhibitors are undergoing different stages of clinical trials against human disorders.

The canonical pharmacophore of the HDAC inhibitors is composed of three parts: a cap structure that can interact with the rim at the entrance of the active pocket of HDACs; a zinc ion (Zn^{2+}) binding group (ZBG); and a linker responsible for the connection of cap and ZBG and for interaction with the hydrophobic tunnel of the active site (*30*). Of these three constitutive parts of the HDAC inhibitor, the cap can accept extensive structural derivatization which make it possible to design huge amounts of HDAC inhibitors.

It was reported that acetylation blocks DNA damage-induced chromatin ADP-ribosylation (*31*) and HDAC inhibitors can decrease expression of proteins involved in DNA repair (*32,33*), which support the combinatorial application of PARP and HDAC inhibitors for the treatment of PARP-dependent cancers. As a fact, many works have proved that HDAC inhibitors can synergize with PARP inhibitors in treating cancers (*34,35*), which validate the design of dual PARP/HDAC inhibitors.

Yuan *et al.* firstly designed four hydroxamic acid containing derivatives of compound 1 as dual PARP and HDAC inhibitors to induce cancer cell death (*36*). All four compounds displayed potent inhibitory activities against PARP-1/2 and HDAC1/6. Compounds 7 and 8 (Figure 2) showed the best HDAC6 inhibitory activities with the IC_{50} values of 8.21 and 10.18 nM. These two hybrids also potently inhibited the activity of PARP-2 with IC_{50} value of 5.02 and 2.53 nM.

In vitro, 7 and 8 possessed excellent antiproliferative activities, comparable to compound SAHA and much better than 1, suggesting that the HDAC inhibitory activities of 7, 8 should play a predominant role in tumor cell response. The significant antiproliferative activities of 7 and 8 were maintained even to the Raji and HCC1937 tumor cell lines that have been reported to be resistant to SAHA treatment. The inhibitory activities of 7 (IC_{50} = 1.29 μM) and 8 (IC_{50} = 0.81 μM) against Raji were 7- and 12-fold more potent than that of SAHA, respectively, while the IC_{50} value of 1 was more than 50 μM. In HCC1937 tumor cells, both 7 (IC_{50} = 2.02 μM) and 8 (IC_{50} = 0.45 μM) exhibited more potent antiproliferative activities than 1 (IC_{50} = 8.65 μM) and SAHA (IC_{50} = 4.23 μM). So, compounds 7 and 8 may represent especially bona fide leads for further optimization in the development of novel antitumor agents against both PARP and HDAC.

Besides hydroxamic acid, *o*-amine aniline is another frequently used ZBG. Reported by Liao, *et al.*, a series of PARP/HDAC inhibitors was synthesized still with 1 as the core skeleton and *o*-amine aniline as the ZBG (*37*). Compound 9 (Figure 2) came up as the most promising candidate possessing balanced

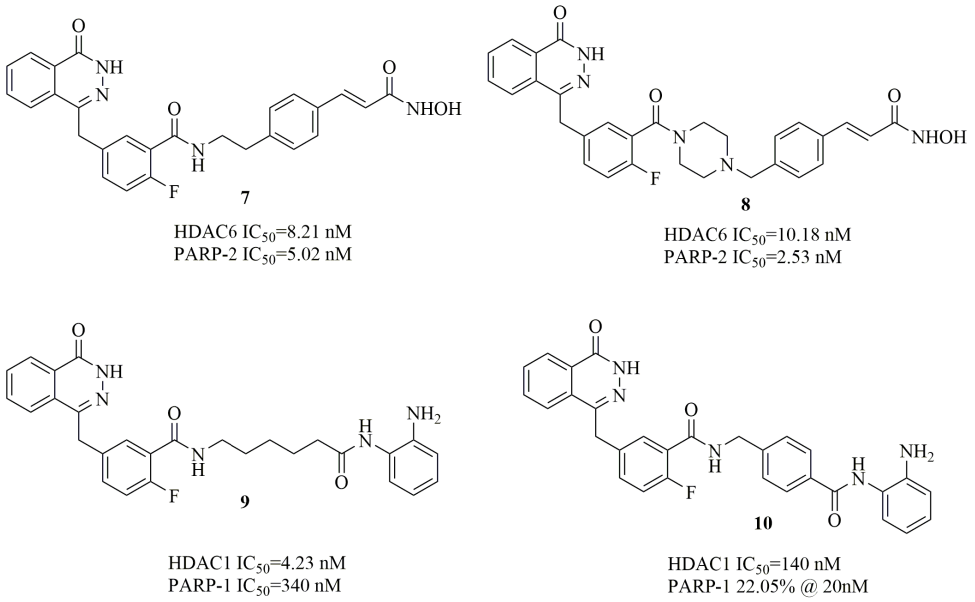

Figure 2. PARP/HDAC dual targeting inhibitors.

inhibitory activities toward PARP-1 and HDAC1 with the IC_{50} values of 4.23 and 340 nM, respectively. 9 showed potent inhibition against growth of K562 and MDA-MB-231 cells with GI_{50} values of 5.6 and 4.3 μM, respectively. However, in this series, compound 10 (Figure 2) with benzyl as linker showed the most potent antiproliferative activity against K562 and MDA-MB-231 cells with GI_{50} values of 0.4 and 1.9 μM, respectively, due to its strong inhibitory activity to HDAC1 (IC_{50} = 140 nM), however, the PARP-1 inhibition activity was much weaker (inhibition rate = 22.06 % at 20 nM) than 9.

2.2. PARP/PI3K dual target inhibitor

Phosphatidylinositol 3-kinases (PI3Ks) are a family of intracellular signal transducer enzymes possessing regulatory roles in critical cellular processes including cell growth, proliferation, differentiation, motility, and intracellular trafficking (*38*). These lipid kinases specifically phosphorylate the 3-position hydroxyl group upon the inositol ring of phosphatidylinositol. The generation PIP3 resulting from PI3K activation activates Akt which is also known as protein kinase B through phosphorylation, Akt targets many downstream proteins that results in many cancer-related consequences such as tumor cell survival, cell cycle progression, cell proliferation and growth (*39-41*).

PI3K-Akt-mTOR is one of the most frequently activated signaling pathways controlling a number of essential cellular processes including cell survival, proliferation, motility, and differentiation in tumors, and mediated nearly 50% of malignant tumors. This pathway has been one of the most extensive studied pathways for cancer therapeutics and the development of its inhibitor is attractive. The first antitumor drugs targeting the PI3K/mTOR signaling pathway are the rapamycin derivatives temsirolimus and everolimus which are inhibitors of mTORC1. The former is used for the treatment of advanced renal cell carcinoma (*42*), and the latter is used for the treatment of advanced breast cancer (*43*), renal cell carcinoma, and pancreatic neuroendocrine tumors (*44*). Besides that, more than 40 PI3K inhibitors with different isoform selectivity have advanced to clinical trials, four of those, idelalisib, copanlisib, duvelisib, and alpelisib have been approved by the FDA (*40*).

It has been proved that the PI3K signaling pathway maintains the stability of HR repair pathway and controls the repair process of DNA double-strand damage (*45*). Inhibition of the PI3K signaling pathway activates ERK, enhances ETS1 activity, and thus inhibits the expression of BRCA1/2, resulting in HR defects that sensitize tumor cells to PARP inhibitors (*46*). Inhibition of the PI3K related pathway has displayed synergistic effects with PARP inhibitors for the treatment of cancers (*47*).

Triple negative breast cancer (TNBC) as an invasive breast cancer with poor prognosis and high recurrence rate, currently, the only treatment option for it is still highly toxic and incurable chemotherapy, so more effective and safety therapeutics are urgently needed. Ibrahim and Juvekar both found that PI3K inhibition attenuate BRCA1/2 expression which making TNBC cells much more sensitive to PARP inhibitors (*46,48*). In another work, Yang, *et al.* reported that combinatory treatment with PI3K inhibitor BKM120 and PARP inhibitor 1 is effective in inhibiting the gastric cancer cells with ARID1A deficiency (*49*). The combination of BKM120 and 1 also showed promising efficacy for the treatment of ovarian cancer due to the low expression of BRCA (*50*). Considering the synergistic effect of dual inhibition of PI3K and PARP, some works have been reported for the PI3K and PARP dual targeting inhibitors.

In the work of Wu *et al.*, the authors discovered highly effective dual PARP/PI3K inhibitors through pharmacophores combination and scaffold hopping strategy, demonstrating the practicability of targeting PARP and PI3K together with a single chemical entity (*51*) . Compound 1 was firstly selected as the starting point to design the hybrid inhibitors, and the imperative structure of a PI3K inhibitor GDC-0980 was merged. In this serial, compounds 11 (Figure 3) exhibited excellent inhibitory activities against PARP-1 (IC_{50} = 1.57 nM) and PI3Kα (IC_{50} = 2.0 nM). In another serial, the benzofuran carboxamide structure was utilized to design the hybrid inhibitors in place of compound 1. Compound 12 (Figure 3) exhibited balanced and more potent inhibitory activities against two targets (PARP-1: IC_{50} = 0.91 nM, PI3Kα: IC_{50} = 1.5 nM). Compound 11 and 12 showed promising antiproliferative activities against both BRCA-deficient (HCT-116, HCC-1937) and BRCA-proficient (SW620, MDA-MB-231/468) tumor cells with IC_{50} values in μM or sub-μM ranges. 11 and 12 also exhibited considerable *in vivo* antitumor efficacy in an MDA-MB-468 xenograft mouse model, with tumor growth inhibition values of 56.39% and 48.77%, respectively. Excitingly, 12 possessed promising profiles including high kinase selectivity and low cardiotoxicity. Overall, this work indicates two compounds 11 and 12 might be potential PARP/PI3K dual inhibitors for cancer therapy and deserve further research.

In another work, Wang, *et al.* also reported the design and synthesis of novel PARP/PI3K dual inhibitors (*52*). By taking the enzyme inhibitory activity, solubility and pharmacokinetic parameters all into account, compound 13 (Figure 3) was obtained whose structure encompassed a benzofuran carboxamide moiety for PARP inhibition and 1,3,5-triazine scaffold for PI3K inhibition. 13 potently inhibited the activities of PARP-1 and PI3Kα with IC_{50} values of 13.8 and 64 nM, respectively. 12 also showed excellent antiproliferative activity against MDA-MB-468 cells with the IC_{50} value 1.40 μM, much stronger than compound 1 (IC_{50} = 13.72

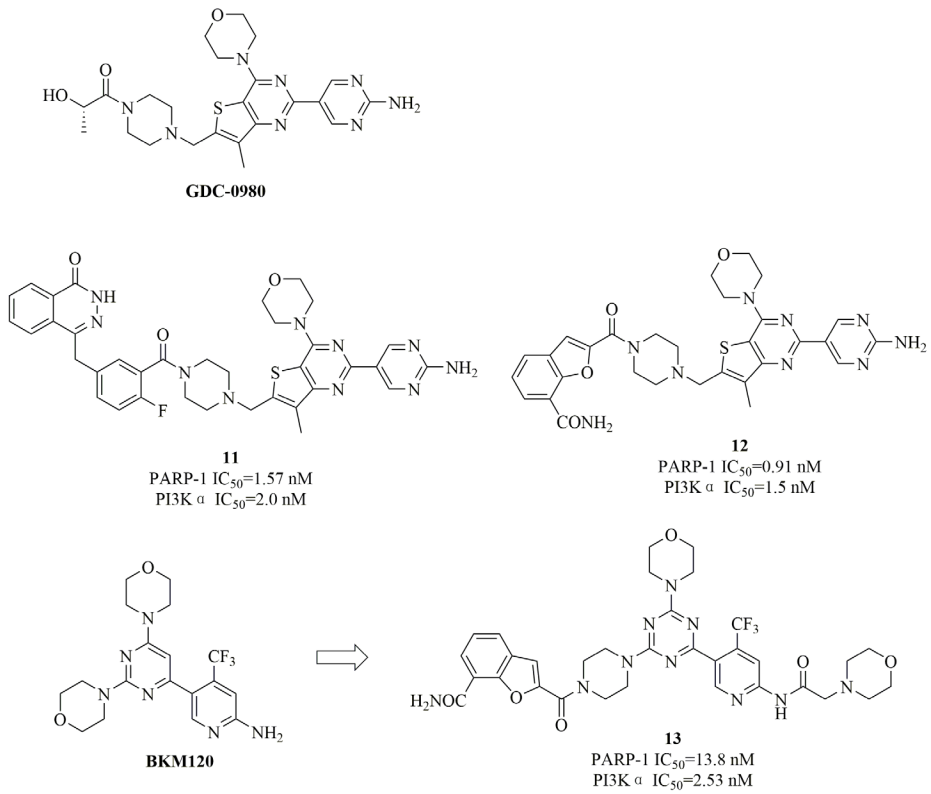

Figure 3. PARP/PI3K dual targeting inhibitors.

μM). Compound 13 displayed a stronger capability to downregulate the expression of BRCA1/2 at the mRNA level than 1 and BKM120, a PI3K inhibitor, suggesting that compound 13 was likely to induce HR deficiency through the downregulation of BRCA1/2. In MDA-MB-468 cell derived xenograft model, compound 13 displayed excellent antitumor efficacy at a dose of 50 mg/kg, more efficacious than the single administration of 1 or BKM120.

2.3. PARP/topoisomerase dual targeting inhibitors

Topoisomerase, including type I and II (Topo I/II), are well-studied enzymes that participate in the cleavage and religation of DNA. They can dissolve topological problems caused by supercoiling of DNA and play important roles in cell replication and gene transcription. Topo I transiently unknits a single strand of DNA, while Topo II can cleave double strands of DNA powered by ATP. Both of these two processes can change the topological structure of DNA and relax it, ultimately facilitating the process of DNA replication during cell division (*53*).

Topo I/II also plays key roles in cancer cell proliferation. Since cancer cells divide much more rapidly than normal cells, cancer cells can be killed disproportionately by inhibition of Topo inhibition, which makes Topo I/II important targets for anticancer drug development. Compounds targeting Topo can be divided into poisons and catalytic inhibitors, with the majority of Topo I/II inhibitors belonging to the former class that firmly bind to cleaved DNA-Topo complex to prevent DNA relinking and lead to tumor cell death (*54*).

The overexpression of PARP-1 and other various DNA damage repair proteins may contribute to the repair of DNA lesions induced by Topo I/II inhibitors, and then enable tumor cells to resist to Topo I/II inhibitors treatment. While, the combination of a Topo inhibitor and PARP inhibitor is considered to be an option to obtain a synergistic effect against tumors. Topo I poisons such as camptothecin (CPT) stabilize the complex in the broken conformation leading to persistent SSB. While PARP-1 plays a major role in the sensing and repair of DNA SSB and contributes to the restart of stalled replication forks during HRR. PARP-1 is activated by CPT-induced DNA breaks and promotes the separation of Topo I from the DNA and the subsequent DNA repair (*55*). So, PARP reduces CPT-induced replication fork reversal and limits DNA strand breakage. While, inhibition of PARP-1 will sensitize cells to Topo I poisons and PARP inhibitors could enhance the cytotoxicity of Topo I poisons by the inhibition of DNA repair (*56*). It was also reported that inhibition of PARP was able to potentiate the antitumor activity of Topo II inhibitors (*57,58*). So, it is reasonable to design Topo/PARP dual targeting inhibitors.

Acridine and its derivatives which possess tricyclic planar structures have been widely explored as Topo I or II inhibitors (*59,60*). In a work accomplished by Yuan, *et al.*, an acridine derivative 14 (Figure 4) was

selected as the skeleton to design Topo I/PARP dual targeting inhibitors by retaining the key pharmacophore of veliparib, a potent PARP inhibitor under clinical trials (61). A serial of fourteen compounds was finally obtained. Out of them, compound 15 (Figure 4) displayed the most potent PARP-1 inhibition activity with IC_{50} value of 90 nM. While another compound 16 (Figure 4) with moderate PARP-1 inhibitory activity (IC_{50} = 450 nM) possessed the highest antiproliferative activity toward MCF-7 cells with GI_{50} value of 2.14 μM. On the other hand, 16 exhibited comparable Topo I inhibitory potency with that of reference compound CPT at the concentration of 100 μM which proved that 16 was a Topo I/PARP dual targeting compounds. As the candidate, *in vivo* antitumor activity was also assessed with 16 against the xenografts tumor models of MCF-7. At two dose, 20 and 40 mg/kg, 16 significantly reduce the tumor growth compared to the blank control group.

2.4. PARP/EZH2 dual targeting inhibitors

Like acetylation mentioned above, lysine methylation is another important protein covalent modification in the field of epigenetics. Many enzymes function lonely or as a subunit in a complex act as lysine methyltransferase such as DOT1L (*62*), SMYD (*63*), G9a (*64*) and enhancer of zeste homolog 2 (EZH2). EZH2 an enzymatic subunit of PRC2 complex, catalyzes histone H3 lysine 27 trimethylation, which results in multiple gene silencing (*65*). EZH2 is frequently overexpressed or mutated in many kinds of cancers (*66*). Many studies have shown that EZH2 promotes cancer cell proliferation, tumor growth, cancer stem cell (CSC) expansion and metastasis (*67*). Thus, EZH2 is considered as a promising anticancer drug target. Tazemetostat as an EZH2 inhibitor was approved in 2020 for treating epithelioid sarcoma (*68*). Following the HDAC inhibitors, one more drug in epigenetics was approved.

EZH2 is subjected to multiple posttranslational modifications including phosphorylation (*69*), ubiquitination (*70*) and O-GlcNAcylation (*71*), all of which participate in the regulation of EZH2 activity. Yamaguchi, *et al.* demonstrated that PARylation of EZH2 mediated by PARP-1 negatively regulates EZH2 activity, leads to its dissociation from the PRC2 complex and subsequent degradation. Conversely, PARP inhibitor could induce EZH2 activity and increased cancer stem cell population which could attenuate the therapeutic efficacy of the PARP inhibitor. Inhibition of EZH2 could sensitize BRCA-mutant cancers to PARP inhibition. So, concurrent inhibition EZH2 and PAPR-1 should be a promising therapeutic strategy for BRCA-mutated breast and ovarian cancers (*72*). Accordingly, it is reasonable to design dual EZH2/PARP inhibitors.

In the work reported by Wang, *et al.*, they used compound 1 and tazemetostat as the starting point to design the dual PARP and EZH2 inhibitors (*73*). Analysis of the complex of tazemetostat-EZH2 revealed that the benzylmorpholine moiety oriented to the solvent and can tolerate structural modification. Thus, the authors replace the benzylmorpholine with a linker in order to incorporate the key pharmacophore of compound 1. In addition, different substitution groups were installed on the benzene ring of the tazemetostat to investigate their influence on the activity towards two targets. Finally, compound 17 (Figure 5) was selected as the candidate. 17 potently inhibited the PARP-1 and EZH2 with IC_{50} values of 6.87 and 36.51

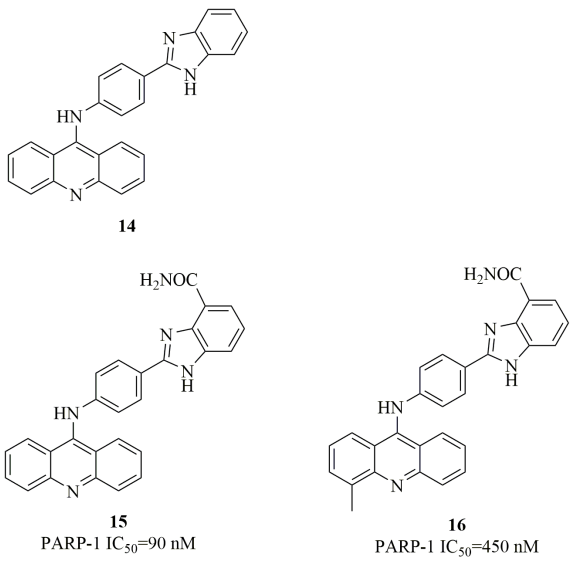

Figure 4. PARP/Topo I dual targeting inhibitors.

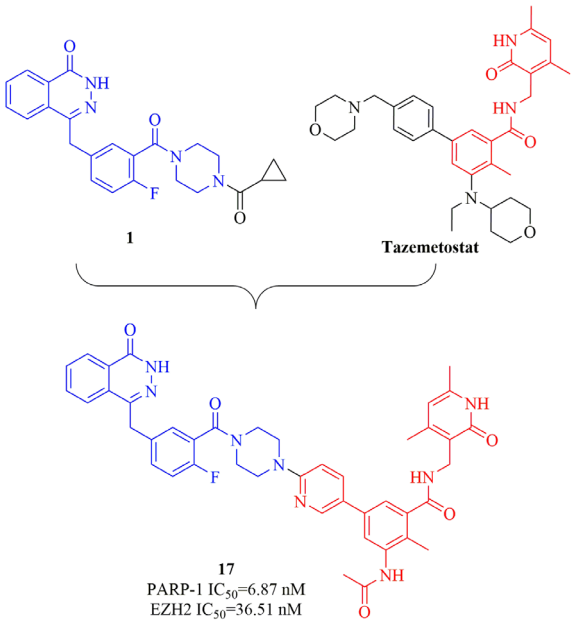

Figure 5. PARP/EZH2 dual targeting inhibitors.

nM, respectively, comparable to two positive controls (IC_{50} of compound 1 against PARP-1 = 7.09 nM, IC_{50} of tazemetostat against EZH2 = 13.05 nM) In cellular assay, 17 could suppress the proliferation of MDA-MB-231 and MDA-MB-468 breast tumor cells with IC_{50} values of 2.63 and 0.41 μM, respectively, much more potent than 1 (36.92 and 35.57 μM) and tazemetostat (44.45 and 46.76 μM). 17 could induce autophagy death of tumor cells and cause less damage to normal cells. Therefore, compound 17 as a first-in-class dual PARP and EZH2 inhibitor should be a potential anticancer drug candidate for breast cancer treatment.

2.5. PROTAC for PARP

Targeted protein degradation (TPD) which can eliminate a protein of interest (POI) have been drawing immense attentions and holds great promise for the development of novel drugs for human diseases (*74*). One focus of TPD is the development of hetero-bifunctional small-molecule degraders, such as Proteolysis Targeting Chimera (PROTAC). A PROTAC molecule is composed of two different ligands, one is for binding to corresponding POI and another binding to an E3 ligase. These two ligands are tethered *via* a linker. PROTACs are able to tag the POI with ubiquitination and then hijack the ubiquitin-proteasome system (UPS) to bring about the degradation of the POI (*75*).

Comparing to the traditional small molecular inhibitors (SMIs) of POI, PROTAC possess several advantages. PROTACs can function in a low concentration, just like a catalysis in the field of organic chemistry, which enable it to degrade multiple POIs with single PROTAC molecule (*76*). Accordingly, PROTAC can confine the toxicities induced by SMIs. Another advantage of PROTAC over SMIs is that PROTAC can degrade undruggable proteins such as transcription factors (TFs), of which no suitable binding pocket exist on the surface, such as STAT3 and Ras. Additionally, PROTACs can overcome the drug resistance resulted from the mutations of one or more amino acids.

So far, the most frequently used in literature including CRBN with immunomodulatory imide drugs (IMiD) as the ligand (*e.g.* thalidomide, lenalidomide and pomalidomide), VHL with the peptoid as the ligand, MDM2 with nutlin-3 derivatives as ligand and IAPs with bestatin, a CD13 inhibitor, as the ligand.

Following the upsurge of the PROTAC technology and considering the promising therapeutic value of harnessing PARP-1, Zhao, *et al.* was encouraged to develop potential PROTAC for PARP-1 degradation (*77*). Niraparib (3) was selected as the PARP-1 ligand. The analysis of the crystal structure of 3 in the complex with PARP-1 (PDB# 4R6E, Figure 6) suggested that the piperidine ring of 3 experiences the opening of the ligand binding pocket and thus may represent a suitable site for tethering the linker and E3 ligase ligand. Five molecules were synthesized coupling with the ligands of three different E3 ligases: MDM2, CRBN and VHL. Finally, compound 18 (Figure 6) equipped with nutlin-3, a MDM2 ligase, could effectively induce PARP-1 degradation in a concentration dependent manner, but not 3, nutlin-3 or their combination. In cellular level, 18 could induce the apoptosis of MDA-MB-231 cells and suppress the proliferation of MDA-MB-231 cells with the IC_{50} of 8.45 μM and 6.12 μM for 24 h and 48 h treatment, respectively. In contrast, only marginal or no inhibitory effects were observed upon treatment with 3, nutlin-3 or in their combination. All these results demonstrated that compound 18 was

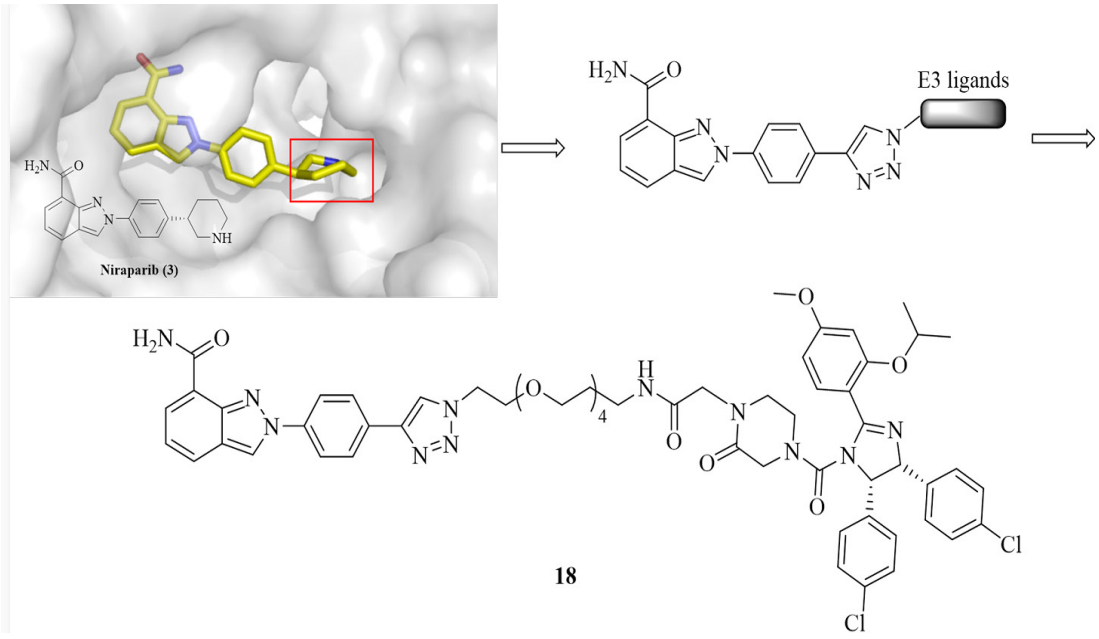

Figure 6. The PROTAC for PARP degradation.

a promising PROTAC for PARP-1 of value for further biological activity tests. In our opinion, this work also shed light on the work for design of more PARP PROTACs.

3. Conclusion

Since 2010, many important advances in the field of PARP inhibitors for cancer therapy have been achieved. As the most successful tumor targeting agents in the field of synthetic lethality, six PARP inhibitors have been approved for clinical use mainly for breast and ovarian cancers, which verified the theory that PARP inhibition can block the single-chain repair pathway and kill tumor cells with BRCA mutations. Although the use of PARP inhibitors is a hallmark event, some bottlenecks are still present. PARP inhibitors have limited indications in cancer therapy. And it is apparent that patients with or without BRCA-mutant cancers will eventually become resistant to PARP inhibitors. Therefore, PARP inhibitors are usually used in combination with other anticancer drugs in order to extend the therapeutic spectrum, enhance the efficacy and conquer the drug resistance. But drug combinations meet some drawbacks including unpredictable metabolism, complex drug-drug interaction that induces undesirable side effects and poor patients' compliance. The design of multi targeting drugs that are capable of repressing more than one pathway can promisingly address these problem including the PARP inhibitors. But till now, only a few of dual targeting PARP inhibitors are reported. There is still a large space for the development of dual targeting PARP inhibitors. In theory, inhibition of drug targets that are associated with PARP or involved in DNA breaks repair have the potential to cooperate with PARP inhibitors and design dual targeting PARP inhibitors. The key point is to pinpoint the structural moiety that can tolerate modification without effecting the inhibitory activities towards two targets. There are many PARP inhibitors with abundant structure features available which make great opportunity to design more dual targeting PARP inhibitors possessing druggable characteristics.

But, the design of dual targeting PARP inhibitors has its dark side which is that the toxicity maybe increased along with the inhibition of two targets which should be paid attention. More works need to be accomplished to assess the toxicity of the dual targeting PARP inhibitors.

Moreover, PROTACs as a sophisticated technology for drug discovery have drawing more and more attentions. But to our best knowledge, only one work reported the PROTACs for PARP-1. Along with the increasing number of PARP inhibitors and E3 ligase ligands, more PROTACs for PARP can be designed. Overall, design of dual targeting PARP inhibitor is newly emerging filed and hold great promise for novel anticancer drug discovery, so, more attentions should be paid.

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Original Article

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Pneumothorax and pneumomediastinum in patients with COVID-19: A retrospective study from tertiary care institute in India

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SUMMARY COVID-19 is associated with rarer extra-parenchymal manifestations, namely pneumothorax (PTX) and pneumomediastinum (PM) leading to complications and increased mortality. The study aims to describe the prevalence, risk factors for mortality, radiological characteristics and outcome of PTX/PM in patients admitted with COVID-19. This was a retrospective, single-centre, observational study in patients with confirmed COVID-19 presenting with non-iatrogenic PTX/PM from April 2020 to May 2021. Details pertaining to demographics, presentation, radiological characteristics, management and outcome were collected. Cases were classified into spontaneous and barotraumatic PTX/PM and a between-group comparison was performed using Chi-square and *t*-test. A total of 45 cases (mean age: 53.2 years, 82% males) out of 8,294 confirmed COVID-19 patients developed PTX/PM, the calculated incidence being 0.54%. 29 cases had spontaneous PTX/PM and the remaining 17 cases were attributed to barotrauma. The most common comorbidities were diabetes-mellitus (65.3%) and hypertension (42.3%). The majority of the cases had large PTX (62.1%) with tension in 8 cases (27.5%). There were predominant right-sided pneumothoraces and five were diagnosed with bronchopleural fistula. 37.7% of cases had associated subcutaneous emphysema. The median duration of PTX/PM from symptom onset was delayed at 22.5 and 17.6 days respectively. The mean CT severity score (CTSS) was 20.5 (± 4.9) with fibrosis (53.8%), bronchiectatic changes (50%) and cystic-cavitary changes (23%). There was no statistically significant difference between the spontaneous and barotrauma cohort. 71% of cases died and the majority belonged to the barotrauma cohort. It is imperative to consider the possibility of PTX/PM in patients having COVID-19, especially in those with deterioration in the disease course, both in spontaneously breathing and mechanically ventilated patients. These patients may also have a high incidence of death, reflecting the gravity of COVID-19.

Keywords Pneumothorax, pneumomediastinum, COVID-19

1. Introduction

The COVID-19 pandemic has resulted in significant mortality and morbidity around the globe. A varied number of manifestations and complications have been reported to be associated with the disease, however, the frequency of such manifestations is difficult to quantify (*1*). SARS-CoV-2 primarily affects the lung parenchyma with features ranging from mild self-limiting upper respiratory infection to critical acute respiratory distress syndrome (ARDS). A myriad of extra-parenchymal manifestations has been documented in the backdrop of COVID-19 such as pulmonary embolism, pleural effusion, empyema, cavitation and cyst formation (*2-5*). Pneumothorax (PTX) and pneumomediastinum (PM), defined as gas in the pleural cavity and the mediastinum respectively, has also been reported in association with COVID-19, although not as frequently as lung parenchymal involvement.

PTX and PM can be either arise spontaneously or due to barotrauma. Necrotizing pneumonia or lung parenchymal involvement, due to various aetiologies,

can result in PTX/PM. The causes include commonly *Pneumocystis jirovecii* pneumonia (PCP), bacterial pneumonia caused by *Staphylococci, Mycoplasma pneumoniae, Klebsiella* and *Pseudomonas* spp., bronchiolitis obliterans organising pneumonia (BOOP) and sometimes by rarer organisms such as *Herpes simplex*, *cytomegalovirus* pneumoniae, *Aspergillus* and even influenzae virus (*6-12*). The previous Middle East respiratory syndrome (MERS) and severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) outbreaks have been associated with an increased incidence of PTX and PM (16.4% and 1.7-11.6%, respectively) associated with increased mortality (*13-15*). Although several studies of PTX/PM in COVID-19 exist, there is sparse data on the incidence or frequency of occurrence, especially from the Indian subcontinent. In one of the earliest studies reporting pneumothoraces by Chen *et al.*, only ~1% (1/99 patients) had radiological evidence of PTX (*16*). In another study by Yang *et al.*, out of 92 deceased COVID-19 patients, one (1.1%) had a PTX (*17*). A recent systematic review by Chong *et al.* found the incidence of PTX to be ~0.3% of hospitalised COVID-19 patients with rates increasing to 12.8-23.8% with 100% mortality in patients on invasive mechanical ventilation (*18*). The incidence of barotrauma has varied from 0-49% in traditional ARDS patients (*19*). Miro *et al.* reported a higher incidence of spontaneous PTX in patients with COVID-19 disease than among patients without COVID-19 disease (*20*). Similarly, McGuinness *et al.* reported a higher incidence of barotrauma among patients with COVID-19 acute respiratory distress syndrome in comparison to the historical controls with non-COVID-19 acute respiratory distress syndrome patients (*21*). Lemmers *et al.* found that the incidence of PTX was significantly greater in patients with COVID-19 ARDS despite the use of lung-protective ventilation, indicating that the frailty of the lung due to lung damage was contributing to the higher incidence of PTX in this patient population (*22*).

We present here a large cohort of PTX/PM from India, developing in admitted COVID-19 patients, and describe the demographic details, clinical features, management, risk factor for mortality and outcome of COVID-19 patients presenting with these entities.

2. Materials and Methods

This retrospective observational study was approved by the institutional ethics committee of the All India Institute of Medical Sciences (Institute Reference number IECPG/373/6/2021) and the need for patient consent was waived given the retrospective nature of the study. The authors used the electronic medical record (EMR) to attain clinical variables and admission details. All the relevant imaging studies were obtained by using the Picture Archiving and Communication System (PACS) and were reviewed by a pulmonologist and a radiologist independently (each with experience in the respective field of more than 10 years each). Only the cases where there was an agreement on diagnosis were included in this study.

2.1. Subject population

Case files of all patients aged > 18 years with COVID-19 confirmed by RT-PCR or SARS-CoV-2 Antigen-RDT assay between 1st April 2020 to 31st May 2021 at our tertiary care facility were reviewed. Only those cases meeting the World Health Organisation (WHO) case definition of confirmed SARS-CoV-2 infection were included (*23*). Medical records reporting PTX and/or PM were reviewed. Cases with a history of iatrogenic PTX as a result of central venous catheter insertion or tracheal injury post-intubation were excluded.

2.2. Clinical data collection

To describe the characteristics of the cohort, demographic, clinical course and management details were obtained for each case and recorded in a predesigned proforma. Demographic parameters included age, sex, smoking status and underlying comorbidities. Details pertaining to COVID-19 included the initial COVID-19 severity classification at the time of presentation to our facility, and the severity at the time of developing PTX/PM, initial symptomatology of COVID-19 chiefly fever, cough, expectoration, shortness of breath, fatigue, myalgia, sore-throat, anosmia, ageusia and diarrhoea. Details about PTX/PM include the size of PTX, site of PTX (unilateral *vs.* bilateral and right *vs.* left-sided), associated PM and subcutaneous emphysema, day of onset of PTX/PM from COVID-19 symptom onset and the mode of oxygenation at the time of diagnosis of PTX/PM. Patients were subsequently divided into two groups for comparison – spontaneous and barotraumatic PTX/PM. Any patient who developed PTX/PM at room air, facemask, nasal prong or high-frequency nasal cannula (HFNC) were considered to have secondary spontaneous PTX/PM, while patients on positive pressure ventilation viz. non-invasive ventilation mask (NIV) and invasive mechanical ventilation (IMV) resulting in PTX/PM were considered to have barotraumatic PTX/PM. Size of PTX was based on the 2003 British Thoracic Society (BTS) guideline (*24*) for classification of PTX based on the radiographic film. Small PTX: distance < 2 cm between the lung margin and the chest wall at the level of the hilum. Large PTX: distance ≥ 2 cm between the lung margin and the chest wall at the level of the hilum. Tension PTX: Presence of mid-line shift or presence of hemodynamic compromise due to the PTX. In cases of broncho-pleural fistula, Cerfolio's classification was used for grading (*25*). Details about the diagnosis, management were collected including modality of

imaging used for diagnosis of PTX/PM, conservative versus pigtail insertion/water seal intercostal tube drainage for management of PTX/PM and final outcome of the patient, *i.e.*, death or discharge.

2.3. Imaging characteristics

Chest radiograph imaging feature of all patients was analysed and was scored based on the 18-point Brixia scoring developed by Borghesi *et al.* (*26*) exclusively for COVID-19 patients. This was done to quantify the degree of lung involvement and its correlation to developing PTX/PM. HRCT chest was also evaluated whenever available and the findings were recorded into the following categories: CTSS, ground-glass opacification, consolidation, bronchiectasis, interlobular septal thickening, fibrosis, cysts and others.

2.4. Statistical analysis

STATA version 12.1 (StataCorp) was used for the statistical analyses in this study. Categorical variables were represented as frequency and percentages while continuous variables were represented as mean (± standard deviation). Chi-square test and *t*-test/ Wilcoxon rank-sum test were applied to calculate statistical differences between categorical variables and continuous variables respectively. Survival data were used to generate Kaplan-Meier curves with STATA version 12.1 (StataCorp). Survival was compared using the log-rank test. A p-value of < 0.05 was considered to assume statistical significance.

3. Results

From 1st April 2020 to 31st May 2021, a total of 45 patients were identified to have PTX/PM after reviewing the electronic database. During this study period, a total of 8,294 patients diagnosed with COVID-19 were admitted to our tertiary care centre which is dedicated to COVID-19 services. The calculated frequency was 0.54% (95% CI: 0.4-0.73%). A total of 16 patients had isolated PTX, 16 patients had isolated PM and 13 patients developed concurrent PTX with PM as shown in Table 1. The mean (± SD) age of the cohort was 53.2 ± 14.9 years and the male to female ratio was 4.6:1.

Pre-existing comorbidities were present in 57.7% (n = 26) of the population with 12 cases (46%) having more than one comorbidity. The most common comorbidity was diabetes mellitus (65.3%) followed by hypertension (42.3%), coronary artery disease and malignancy (19.2% each), hypothyroidism and chronic kidney disease (7.6% each) and hypertriglyceridemia (3.8%). Three cases had a prior history of pulmonary tuberculosis. A total of 9 cases (31%) were current or ex-smokers in this cohort. There was no significant statistical difference between the spontaneous and barotrauma group concerning comorbidities and smoking status.

Table 1. Baseline characteristics of COVID-19 patients with PTX and/or PM and comparison between patients developing spontaneous versus barotraumatic PTX/PM

Variables	Total, *n* (%)	Spontaneous *n* (%)	Barotrauma *n* (%)	*p*-value
Age in years[a]	53.28 ± 14.9	51.8 ± 13.9	55.5 ± 16.5	
Sex				0.98
Male	37 (82.2%)	23 (62.1%)	14 (37.8%)	
Female	8 (17.7%)	5 (62.5%)	3 (37.5%)	
Comorbidities (n = 26)				
Diabetes Mellitus	17 (65.3%)	11 (64.7%)	6 (35.2%)	0.77
Hypertension	11 (42.3%)	8 (72.7%)	3 (27.2%)	0.49
Coronary artery disease	5 (19.2%)	3 (60%)	2 (40%)	1.00
Hypothyroidism	2 (7.6%)	0 (0%)	2 (100%)	0.13
Malignancy	5 (19.2%)	5 (100%)	0 (0%)	0.14
Chronic kidney disease	2 (7.6%)	2 (100%)	0 (0%)	0.51
Hypertriglyceridemia	1 (3.8%)	1 (100%)	0 (0%)	0.37
COPD/Asthma	1 (3.8%)	0 (0%)	1 (100%)	0.37
Smoking status (n = 29)				
Smoker	9 (31%)	7 (77.7%)	2 (22.2%)	0.43
Symptoms (n = 45)				
Fever	32 (71.1%)	20 (62.5%)	12 (37.5%)	0.95
Dry cough	21 (46.6%)	14 (66.6%)	7 (33.3%)	0.56
Expectoration	4 (8.8%)	1 (25%)	3 (75%)	0.14
Shortness of breath	40 (88.8%)	26 (65%)	14 (35%)	0.35
Fatigue	3 (6.6%)	3 (100%)	0 (0%)	0.27
Myalgia	3 (6.6)	2 (66.6%)	1 (33.3%)	1.00
Sore-throat	7 (15.5%)	6 (85.7%)	1 (14.2%)	0.22
Diarrhoea	2 (4.4%)	1 (50%)	1 (50%)	1.00
Nausea	1 (2.2%)	1 (100%)	0 (0%)	1.00
Anosmia/Ageusia	2 (4.4%)	1 (50%)	1 (50%)	1.00

a: Mean ± Standard Deviation.

The most common presenting symptom at admission was shortness of breath (88%) followed by fever (71.1%), cough without expectoration (46.6%), sore throat (15.5%), cough with expectoration (8.8%), fatigue and myalgia (both 6.6%), diarrhoea and anosmia/ageusia (4.4%) and nausea (2.2%). There was no significant statistical difference between the two groups (spontaneous and barotrauma induced) with regards to cough. Of the 29 cases with pneumothoraces, based on the BTS 2003 classification of size of PTX, 37.9% (*n* = 11) had a small PTX and 62.1% (*n* = 18) had large pneumothoraces of which 27.5% (*n* = 8) had tension pneumothoraces. Five cases were diagnosed with bronchopleural fistula with three cases having grade 3 air leak based on Cerfolio classification. Majority of the cases had unilateral PTX (86.2%, *n* = 25), however 68% (*n* = 16) had right-sided involvement. Associated subcutaneous emphysema was present in 37.7% (*n* = 17) cases with similar distribution between the two groups. The mean (± SD) duration from symptom onset to developing PTX/PM was 22.5 (± 11.5) days and 17.6 (± 8.8) days respectively. The most common mode of oxygenation at the time of developing PTX/PM in the spontaneous group was nasal prong/face-mask (57%, *n* = 16) followed by HFNC (28.5%, *n* = 8) and room air (14.2%, *n* = 4). In the barotrauma patients, NIV (58.8%, *n* = 7) was the most common modality of respiratory support while the rest (41%, *n* = 7) were on invasive mechanical ventilation. The mean PEEP given in the barotrauma cohort was 8.9 (± 1.4) cm of H_2O. The radiological modality of PTX/PM diagnosis was chest radiograph in 75.5 (*n* = 34) and the rest were diagnosed based on chest computed tomography. Immediate intercostal tube drainage or pigtail insertion was done in 57.7% (*n* = 26) cases while the rest were managed conservatively. A conservative approach was taken in all of the cases of isolated PM (with or without subcutaneous emphysema) and three cases of small PTX without much symptomatology. In our cohort, 71% (*n* = 32) of the cases died. The proportion of patients discharged was higher in the spontaneous group although it was not statistically significant (*p* value 0.08). The median duration of hospitalisation was 17.5 (± 12.9) days. The details are enumerated in Table 2. Kaplan-Meier curve was plotted to determine the probability of survival between the PM cohort and the PTX with/without PM cohort. Even though the overall survival duration was not significant between the two groups concerning days from the event (PM and/or PTX) to death, there was a trend towards significance (*p* value = 0.07) showing an increased survival in patients with PM *vs.* PTX as shown in Figure 1A. On the other hand, overall survival was non-significant with regards to the time of COVID-19 symptom onset to developing PTX/PM (*p* value = 0.27) as shown in Figure 1B.

Table 3 demonstrates the radiological characterization of the cohort. The most common chest CT feature was ground-glass opacification (82%) characteristic of COVID-19 pneumonia. Other principal findings were consolidation (50%), fibrosis (53.8%) with interlobular septal thickening (42.3%) and bronchiectatic changes (50%). A significant number of cases had cystic changes (23%) and lung cavitation, which is an unusual feature of COVID-19 infection and was present in 3 cases (11.5%). The mean CT severity score was 20.5 (± 4.9) which indicated that the majority of cases had severe

Table 2. Characteristics, management and outcome of PTX/PM in COVID-19 and comparison between patients developing spontaneous versus barotraumatic PTX/PM

Parameters	Total, *n* (%)	Spontaneous, *n* (%)	Barotrauma, *n* (%)	*P*-value
Isolated pneumothorax	16 (35.5%)	11 (68.7%)	5 (31.2%)	-
Isolated pneumomediastinum	16 (35.5%)	10 (62.5%)	6 (37.5%)	
Pneumothorax and pneumomediastinum	13 (28.9%)	7 (53.8%)	6 (46.1%)	
Pneumothorax size (*n* = 29)				0.51
Small	11 (37.9)	6 (54.5)	5 (45.4)	
Large/Tension	18 (62.1)	12 (66.6)	6 (33.3)	
Site of pneumothorax (*n* = 29)				0.13
Unilateral	25 (86.2)	17 (68)	8 (32)	
Right side	17	12	5	
Bilateral	4 (13.7)	1 (25)	3 (75)	
Associated Subcutaneous emphysema (*n* = 45)	17 (37.7)	9 (52.9)	8 (47.0)	0.31
Mode of oxygenation (*n* = 45)	45			
Room air		4 (14.2)	0	
Nasal prong/face mask		16 (57.1)	0	
HFNC		8 (28.5)	0	
NIV		0	10 (58.8)	
Invasive mechanical ventilation		0	7 (41.1)	
Management				0.91
Intercostal tube drain/pigtail	26 (57.7)	16 (61.5)	10 (38.4)	
Conservative	19 (42.2)	12 (63.1)	7 (36.8)	
Outcome				0.08
Death	32 (71)	17 (53.1)	15 (46.8)	
Discharge	13 (28.9)	11 (84.6)	2 (15.3)	

COVID-19. For the evaluation of the chest radiograph, the Brixia scoring developed by Borghesi *et al.* (26) was utilised. The mean (± SD) Brixia score for the patients was 3.275 (± 2.59) and there was no statistical difference between patients who survived and patients who died.

4. Discussion

In our cohort of 8,294 hospitalized COVID-19 patients, the prevalence of PTX and/or PM was 0.54% (95% CI: 0.4-0.73%), with prevalence of PTX and PM being both 0.2% (95% CI: 0.1-0.3%) and that of combined PTX/PM being 0.16% (95% CI: 0.08-0.3%). This study pegs the prevalence of PTX and/or PM in COVID-19 patients reflecting on the frequency of occurrence of this serious complication against the backdrop of lung involvement by SARS-CoV-2. A systematic review, which did not include any Indian studies, had found the prevalence of PTX of 0.3% and 12.8-23.8% in hospitalized COVID-19 patients and those requiring invasive mechanical ventilation respectively (*18*). The incidence of spontaneous PTX/PM was 0.48% in our cohort, which was similar to the incidence of spontaneous PTX (0.66%) reported from over 3,000 patients by Zantah *et al.* in their single centre study from the USA (*27*). However smaller studies had reported higher percentages (≥ 1%) (*17,28*). The incidence of PTX associated with ARDS or mechanical ventilation in COVID-19 patients seems to be higher ranging from 10-56% (*18,29*). This study also provides a prevalence estimate of 0.2% of PM with or without PTX in COVID-19 patients, which is lower than that observed by Tacconi *et al.* (2.3%) in a select group of patients (*29*). Spontaneous and barotrauma-related PTX and/or PM constituted 62% and 38% of the total 45 cases in our cohort. There were no significant differences between the spontaneous and barotrauma-related groups in terms of clinical parameters, radiological features or outcome.

The postulated mechanism for the development of PTX/PM is linked to the development of diffuse alveolar damage, cystic lesions due to fibro-inflammatory changes and fibrosis. Alveolar rupture, with or without the contribution of increased intrathoracic pressure during coughing or positive pressure ventilation, typically results in PTX or PM. PM and resulting subcutaneous emphysema, without concomitant significant PTX, can ensue due to the phenomenon of the Macklin effect (*30*). The pathologic features in the lungs of patients with COVID-19 pneumonia closely resemble those of SARS and MERS outbreaks. Histologic examinations have reported diffuse alveolar damage with fibromyxoid inflammatory exudates along with cystic lesions (*31*). In our cohort where CT imaging was available (26 cases), 53.8% of cases had evidence of fibrosis while 23% had cystic-cavitation changes, both of which are known contributing factors for the development of PTX/PM. The mean CTSS score for the cases was 20.5, indicating

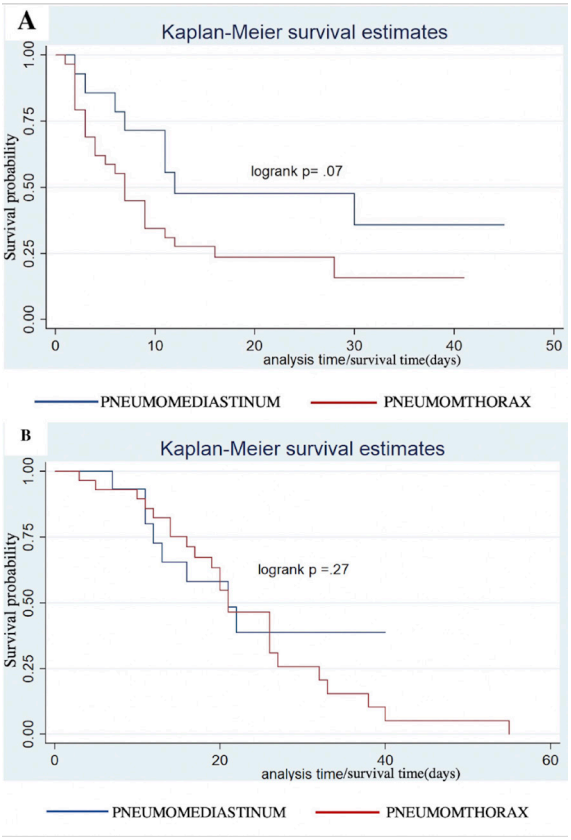

Figure 1. (A) Kaplan-Meier survival curve from the time of the event (PTX/PM) to time of death in days between PM (blue) and PTX (red). Log-rank test, p = 0.07. **(B)** Kaplan-Meier survival curve from the time of the COVID-19 symptom onset to time of the event (PTX/PM) in days between PM (blue) and PTX (red). Log-rank test, p = 0.27.

Table 3. Radiological characteristics in COVID-19 patients with PTX/PM

	Total, *n* (%)	Spontaneous, *n* (%)	Barotrauma, *n* (%)	*P*-value
Radiological features (*n* = 26)				
Ground glass opacities	22 (84.0)	17 (77.2)	5 (22.7)	0.28
Consolidation	13 (50.0)	7 (53.8)	6 (46.1)	0.07
Bronchiectasis	14 (53.8)	10 (71.4)	4 (28.5)	1.00
Fibrosis	13 (50)	9 (69.2)	4 (30.7)	1.00
Interlobular septal thickening	11 (42.3)	8 (72.7)	3 (27.2)	1.00
Cysts	6 (23.0)	5 (83.3)	1 (16.6)	1.00
Cavitation	3 (11.5)	1 (33.3)	2 (60.6)	0.16

that the development of PTX/PM was related to the severity of lung involvement by COVID-19. The mean age of the cases was 53 years and the complication was more common in the male gender. Incidentally, men, as compared to women, are more likely to be afflicted by severe form of the COVID-19 infection as shown by a recent meta-analysis by Peckham *et al.* (32). Only one patient had underlying respiratory comorbidity (3.8%) while significant smoking history was present in 31% indicating that COVID-19 was responsible for PTX/PM in the vast majority. Brixia Scoring for the cases showed a mean value of 3.275 and it did not predict mortality.

An interesting observation made in our study was that right-sided PTX was more common than the left side (Right: left = 2:1). A similar observation was found by Miro *et al.* who reported that COVID-19-related PTX was 3.85-fold more likely to occur on the right side and at higher frequency (81.1% versus 52.7%; $p < 0.001$) as compared to non-COVID-19 patients (*20*). The likely explanation for such observation may be a heightened degree of involvement of the right lung vis-à-vis the left lung, as reported by previous research (*33-35*). There could be several reasons for this, including the larger size and number of segments of the right lung, as well as the larger diameter of the right main bronchus leading to greater delivery of viral particles to the right lung - causing relatively more damage (*34*).

Another interesting finding in this study was the median duration of PTX/PM from symptom onset which was 22.5 and 17.6 days respectively. Similarly, delayed onset of PTX/PM has been seen in most of the previous larger studies. The late onset of this complication suggests a sustained period of lung inflammation with extensive parenchymal injury followed by fibrosis and cyst formation in the late phase of COVID-19 pneumonia. The late finding is also supported by the typical radiologic findings in COVID-19 patients (*36*) comprising of inflammation of the lung parenchyma that predominantly affects the peripheries progressing to consolidation and eventually involves the overlying pleura leading to various pleural manifestations such as PTX and pleural effusion. The majority of the cases in our study died (71%) and most of the cases who survived belonged to the spontaneous group as opposed to the barotrauma group even though there was no statistical significance. Such high mortality is comparable to a recent systematic review by Chong *et al.* reporting an overall mortality rate of 74.2% (*18*). The mortality rate of severe COVID-19 is known to be very high and our study demonstrates that severe COVID-19 patients with PTX/PM have a very prognosis. With regards to isolated PM, the mortality rate was 0.56% which was considerable drawing to the conclusion that not only PTX but also PM is a significant predictor of poor outcome in patients with COVID-19. Kaplan-Meier survival curve depicted in Figures 1A and 1B shows that the PM cohort of cases had better chances of survival as compared to cases with PTX. The possible explanation lies in the fact that PTX results in decreased lung volume leading to further compromise of lung function in the already poor compliant lung parenchyma culminating in a poor outcome. On the other hand, pneumomeiastinum doesn't result in compromise of lung function unless the size is massive with all the cases undergoing conservative management possibly explaining the improved survival.

This study involves the largest cohort of patients from the Indian subcontinent concerning the prevalence, clinico-radiological characteristics and outcome of PTX and/or PM in COVID-19 patients. However, this study is not without limitations. The most significant one was that it was a retrospective study from a single tertiary centre, with potential risks of information and selection biases.

5. Conclusion

It is thus imperative to consider the possibility of PTX and/or PM in patients having COVID-19, especially in those with deterioration in the disease course, both in spontaneously breathing as well as mechanically ventilated patients. These patients may also have a high incidence of death, apparently reflecting the gravity of COVID-19 disease.

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Original Article

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Quantification of antipsychotic biotransformation in brain microvascular endothelial cells by using untargeted metabolomics

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SUMMARY Most studies of antipsychotic-therapies have highlighted the discrepancy between plasma and brain pharmacokinetics of antipsychotics, but how the drug changes through the blood brain barrier (BBB) has not been investigated. Cell-based metabolomics using liquid chromatography-mass spectrometry (LC-MS) combined with multivariate data analysis were applied for screening of antipsychotic metabolites in the BBB. We applied this approach to analyze the antipsychotic biotransformation in brain microvascular endothelia cells (BMVECs), the main component of the BBB. From this study, five, four, three, and one metabolite of chlorpromazine, clozapine, haloperidol and risperidone, respectively, were locally metabolized on the BMVECs. These results confirm that there is a drug biotransformation process within the BBB and show that drug metabolite screening employed cell-based metabolomics using LC-MS, combined with multivariate analysis in the study of BMVECs exposed to antipsychotics can provide a way to screen drug metabolites in the BBB.

Keywords Biotransformation, antipsychotics, BBB, BMVECs, cell-based metabolomics, LC-MS, multivariate data analysis

1. Introduction

Antipsychotics have been widely used in treating mental health illnesses such as schizophrenia and bipolar disorder (*1*). Antipsychotics are normally classified as typical (such as chlorpromazine and haloperidol) or atypical (such as risperidone and clozapine). Clinical research on how to best predict the therapeutic effects and side effects of antipsychotics has been ongoing for several decades (*2-4*). Most studies have highlighted the studies on the pharmacokinetics and pharmacodynamics in brain tissue, cerebrospinal fluid and interstitial fluid for drugs used in the treatment of antipsychotics (*5,6*), but how the drug biotransformation through the blood brain barrier (BBB) has not been studied.

The BBB is a complex vascular structure that physically and physiologically separates the peripheral blood circulation from the central nervous system (CNS). It acts very effectively in maintaining brain homeostasis, regulating the influx and efflux transport of nutrients, and protecting the CNS from pathogens and toxins (*7*). The basic anatomy of the BBB consists of brain microvascular endothelial cells (BMVECs), pericytes, astrocyte foot processes and nerve endings. Although, this structure contributes to the function of the microvasculature in the brain, the permeability of the BBB is controlled only by the BMVECs (*7*).

The combined surface area of BMVECs constitutes by far the largest surface area for blood-brain exchange. This surface area, depending on the anatomical region, is between 150 and 200 cm^2/g of tissue giving a total area for exchange in the brain of 12-18 m^2 for the average human adult (*8*). BMVECs are the major site of blood-central nervous system (CNS) exchange and shield the brain against drugs, toxins and immune cells *via* paracellular, transcellular, transporter, and extracellular matrix proteins (*7,8*). While evidence for drug biotransformation exists in the BBB, has not investigated, and whether BMVECs themselves are functionally compromised metabolism and lead to the clinical response to drugs is unclear.

Cell-based metabolomics is the comprehensive analysis of small molecule metabolites in cell cultures by the integration of state-of-art analytical tools and bioinformatics (*9,10*). At present, liquid chromatography-mass spectrometry (LC-MS) as an analytical platform is quite commonly used in cell-based metabolomics. This method offers advantages over other analytical platforms; these include speed, sensitivity, relative ease of sample preparation and large dynamic range (*11*). Multivariate

data analysis, such as principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), is an essential component in cell-based metabolomics analysis, to assist in the extraction of valuable information from large LC-MS datasets (*12*). PCA statistical analysis is commonly employed to analyze multivariate data, due to its rapid provision of an overview of the information hidden in the LC -MS data (*13*). The OPLS-DA model is intended for the modeling of two classes of LC-MS data in order to improve class separation, simplify interpretation and identify potential biomarkers (*14*). The advantages of LC-MS, coupled with multivariate data analysis, mean they have been widely used in various fields, such as toxicology, pharmacology and medicine (*10,11,15*). Cell-based metabolomics using LC-MS coupled with multivariate data analysis has also been adopted in drug metabolism and is used for the screening of stable metabolites and reactive metabolites (*16*).

In this study, the implications of the cell-based metabolomics using LC-MS, coupled with multivariate data analysis, the in profiling of antipsychotic metabolism and bioactivation were firstly provided in BMVECs.

2. Materials and Methods

2.1. Materials

Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=87*) provides the details of the chemicals used in this study.

2.2. Cell lines and culture

For the current study, the brain microvascular endothelial cells, a fundamental of the BBB, were provided by Paul A. Smith (School of Life Science, University of Nottingham Medical School, Nottingham, UK). The BMVECs were used from passage 21-23 and were cultured and maintained as previously described in Elmorsy *et al.* (2004) (*17*).

2.3. Cytotoxicity assay

For this assay, the cell proliferation kit I (Merck, Bangkok, Thailand) was used to analyze the number of viable BMVECs. This kit is based on a colorimetric assay that analyzes the number of cell viable cells by the cleavage of tetrazolium salts (MTT) added to the culture medium. Briefly, BMVECs were seeded at 1 × 10^4 cells per well in 96-well plastic plates (Gibthai, Bangkok, Thailand) and incubated overnight at 37°C under humidified 5% CO_2 conditions. To assess the cytotoxicity of antipsychotics used were then incubated for 24 h. in the presence of drug or its vehicle (ethanol). The antipsychotics concentrations used in this study were 0.2 µM chlorpromazine (*18*), 1 µM clozapine (*19*), 0.7 µM haloperidol (*20*), and 0.5 µM risperidone (*21*); these concentrations are similar to those measurement in patients. The original drug and vehicle were corrected *via* subtraction of a blank (media with the kit reagents but without BMVECs).

2.4. Antipsychotic metabolite study

BMVECs were used at a density of 1 × 10^8 cell/mL and were cultured and maintained as described in the previous protocol (*17*). When 80% confluence had been achieved, unattached BMVECs and cell culture media were discarded and attached BMVECs were washed with 5 mL of phosphate buffer solution (PBS) (Merck, Bangkok, Thailand). Then, 10 mL of fresh cell culture media (Gibthai, Bangkok, Thailand) was added, followed by antipsychotics. The concentration of each drug was described in the previous section. This solution was then incubated for 24 h, then the cell culture media was removed, and attached BMVECs were washed with 5.0 mL of PBS followed by 0.5 mL of cold methanol (4oC). Then a cell scraper was used to detach cells from a flask and the cell suspension was transferred to an Eppendorf tube and kept at -80°C for further processes.

2.5. Drug metabolite extraction

The cell extraction was based on the method described by the previous paper (*22*). Briefly, 0.50 mL of 4°C chloroform and 0.50 mL of 4°C water were added to the suspension and then vortexed vigorously for 10 min at 4°C. Then, the suspension was centrifuged at 15,000 g for 10 min at 4°C. The hydrophilic fraction and hydrophobic fraction were collected separately and transferred into fresh Eppendorf tubes and evaporated to dryness at room temperature. The dried hydrophobic layer was reconstituted in 50 µL of chloroform and methanol (1:2, v/v), and the dried hydrophilic layer was reconstituted in 50 µL of water. The reconstituted samples were centrifuged at 15,000 g for 10 min at 4°C to remove any cell debris. Finally, the supernatant was transferred into an HPLC vial and stored at -80°C for LC-MS analysis.

2.6. LC-MS analysis

Metabolic profiling was performed on an LC Accela™ system (Thermo Scientific Ltd., Loughborough, UK) coupled with high resolution mass spectrometry (Exactive®, Thermo Scientific Ltd., Loughborough, UK). Hydrophobic chromatographic separations were performed on an Agilent SB C8 column (1.8 µm particle size, 2.1 × 100 mm, Crawford Scientific Ltd., Lanarkshire, UK) and hydrophilic chromatographic separations were performed on a C18 (2) column (2.5 µm particle size, 3 × 100 mm, Phenomenex Ltd, Cheshire, UK). The details of LC and MS conditions are

summarized in Supplementary Tables S2 and S3 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=87*), respectively. In both hydrophobic and hydrophilic metabolic profiling, samples were performed in six replicates, to account for any biological variability. The retention time consistency and mass accuracy were confirmed though the pooled samples.

2.7. Data analysis

The feasibility of the method was first performed using a high mass resolution mass spectrometer. Six replicates of each drug-treated sample were analyzed by LC-MS. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were employed to process the acquired LC-MS data. Samples were grouped together for OPLS-DA modelling. The PCA and OPLS-DA results were displayed as score plots to visualize sample clustering and to indicate sample similarity. Discriminatory metabolites between the treated and the control for each antipsychotic drug were first screened with the variable importance in the projection (VIP) ranks > 1.00 and then validated using ANOVA statistical analysis of false discovery rate (FDR) with a significance level of 0.05 for antipsychotic treatments. According to the identity check, based on raw data and the features of peaks, the target masses of candidate metabolites identified in the profiling process were searched over a narrow ± 5 ppm mass window in the HMDB database and confirmed by available standards. The possible drug metabolite analysis combined results from the publications to help our studies identify the most relevant drug metabolites involved in the conditions under study. A results report was then presented graphically as well as in a detailed table.

3. Results

3.1. BMVEC viability

The viability results of cytotoxicity assay after incubation for 24 h with chlorpromazine, clozapine, haloperidol, risperidone, and vehicle (ethanol) were 99.7 ± 1.2%, 101.2 ± 0.7%, 100.5 ± 2.0%, 99.5 ± 1.3%, and 100.2 ± 0.3%, respectively. These results confirmed that the drug concentration used did not affect the cell growth.

3.2. LC-MS data quality

Figures 1 and 2 illustrate the LC-MS chromatograms

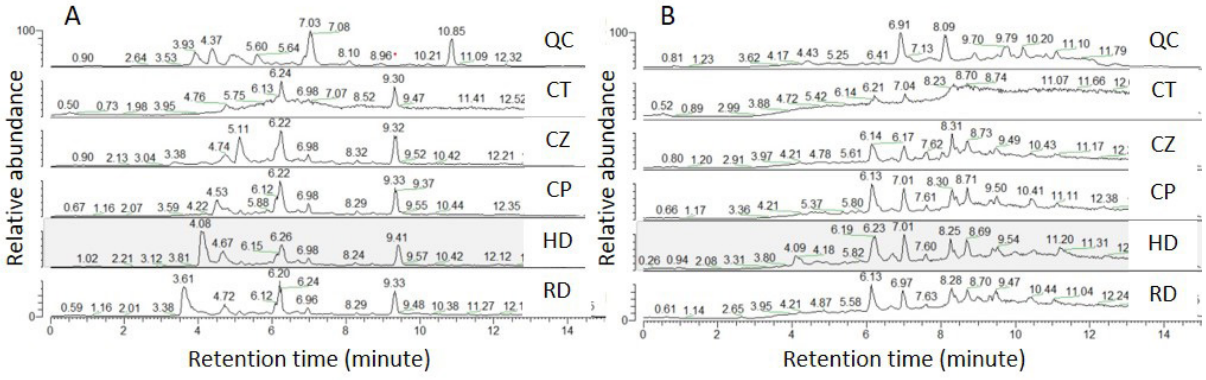

Figure 1. LC-MS chromatogram of hydrophobic fraction in ESI positive (A) and ESI negative (B) of pooled sample (QC), control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

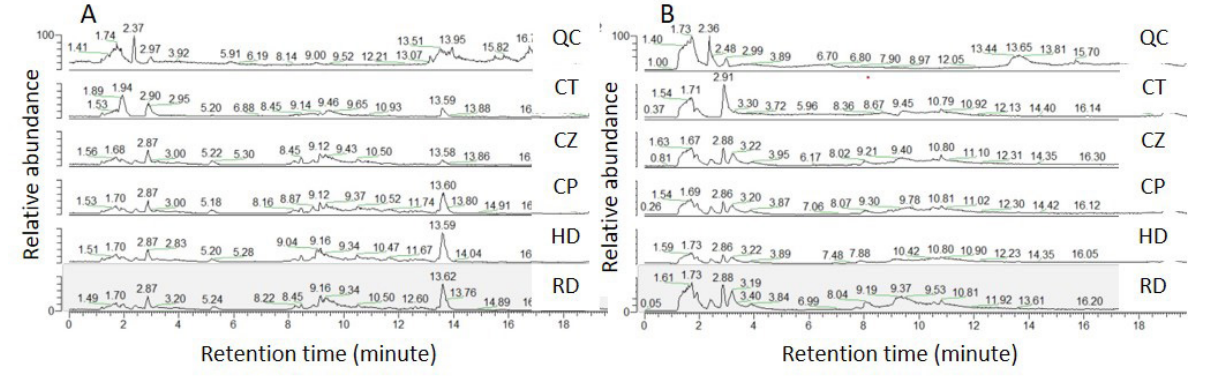

Figure 2. LC-MS chromatogram of hydrophilic fraction in ESI positive (A) and ESI negative (B) of pooled sample (QC), control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

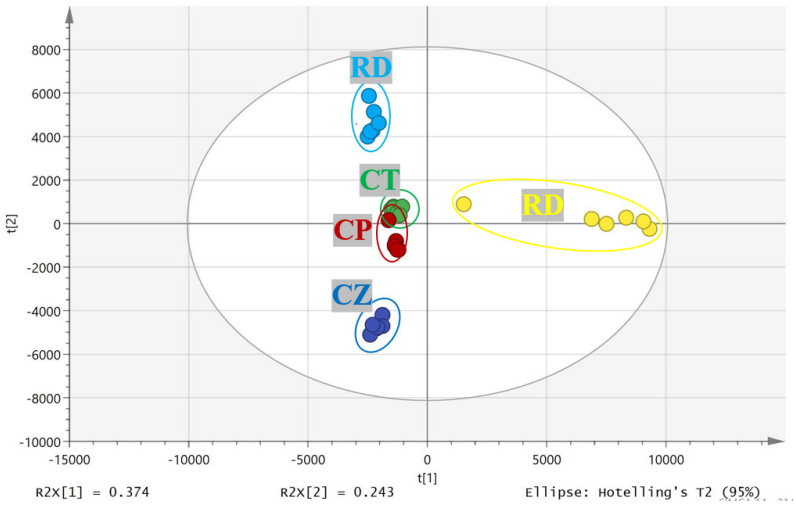

Figure 3. PCA scores plots of metabolites of brain microvascular endothelia cells treated with control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

of hydrophobic and hydrophilic fractions extracted from BMVECs treated with antipsychotics in ESI positive and negative, respectively. To check the LC-MS instrument performance in the current study, 10.0 µL of cellular extraction from each BMVEC sample were pooled to get a quality control (QC) sample in both hydrophobic and hydrophilic fractions. Several consecutive injections of the QC sample were made to obtain a stable LC-MS system. One in five QC samples was analyzed throughout the whole analysis procedure for both hydrophobic and hydrophilic fractions.

According to the optimized conditions, principal component analysis (PCA) of all samples from BMVECs is shown in Figure 3. The QC samples were gathered together for analysis during the data collection. The data from the QC samples were then analyzed to determine the number of ions present in the samples, their intensity and their % RSD values. The average RSDs of peak abundance for the QC samples was 18.0% with a standard deviation of 7.8% for the hydrophobic part, and 22.9% with a standard deviation of 10.2% for the hydrophilic part. Since the recommendation of the FDA is that biochemical analysis ions should show RSDs of less than 30%, this recommendation was used in the subsequent analysis of the test and control sample data (*23*). The results demonstrated that the system employed in this study had excellent stability during the analysis procedure for both hydrophobic and hydrophilic part.

For data analysis, the aligned data array was filtered using the QC samples. In line with the recommendations of the FDA for biomarker analysis ions (*23*), those showing RSDs less than 30% were used in the subsequent analysis of the BMVECs treated with four antipsychotics and control cell sample data.

3.3. Cell metabolic profiling of BMVEC treated with four antipsychotics

From our data, PCA score plots showed clear separation between control and antipsychotic treatment of BMVECs (Figure 3). Figure 3 shows that separate clusters from each model are revealed, which indicates metabolic differences in terms of level and compositional changes of cellular metabolites among control, chlorpromazine treatment, clozapine treatment, haloperidol treatment and risperidone treatment. Then, OPLS-DA was applied to visualize samples in an attempt to distinguish between control and each antipsychotic treatment.

A very clear separation was revealed in Figures 4A-4D. In order to identify drug metabolites, VIP statistics (VIP > 1.0) were initially used to pre-select detected mass ions. Then, from those detected mass ions with FDR (ANOVA) < 0.05 selection was made of those which were most correlated highly with the OPLS-DA discriminant scores in order to decrease the risk of false positives in the selection of significantly altered mass ions.

3.4. Antipsychotic drug metabolism in BMVECs

For the LC-MS analysis of the four antipsychotic drugs mentioned above, samples obtained from incubation with BMVECs with chlorpromazine, clozapine, haloperidol, or risperidone were extracted by liquid extraction and concentrated by speed vacuum. The MS spectrum of the chlorpromazine $[M+H]^+$, haloperidol $[M+H]^+$, clozapine $[M+H]^+$, and risperidone $[M+H]^+$ revealed ions at m/z = 319.1028, 376.1473, 327.1327, and 411.2190, respectively. Of interest from the current perspective was the detection of each drug metabolism as shown in Table 1, while Figures 5-8 show possible chemical structures.

4. Discussion

Antipsychotic metabolite identification in BMVECs is challenging, since thousands of cellular metabolites

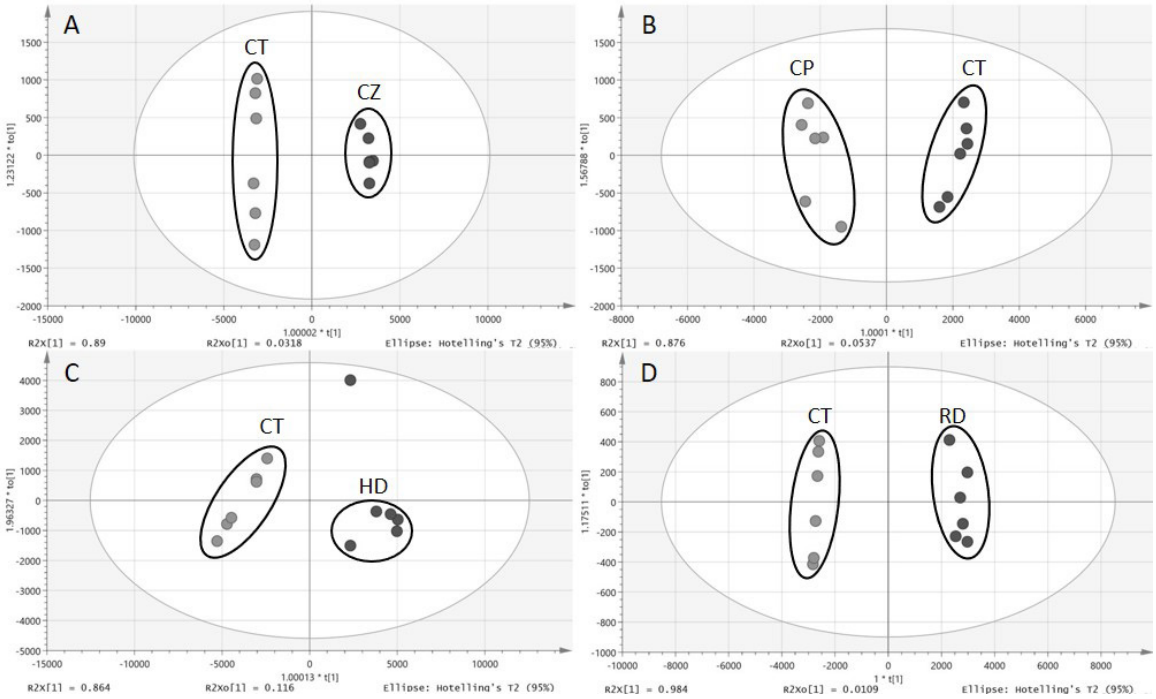

Figure 4. OPLS-DA of untargeted metabolomics data from BMVECs treated with antipsychotics. Two-dimensional OPLS-DA scores plots reveal separation between **(A)** the control group (CT) and the chlorpromazine-treated group (CZ), **(B)** the control group (CT) and the clozapine-treated group, **(C)** the control group (CT) and haloperidol-treated group (HD), **(D)** and the control group (CT) and the risperidone-treated group (RD).

Table 1. Possible biotransformation of chlorpromazine, haloperidol, clozapine, and risperidone by brain microvascular endothelial cells

Name	m/z	Adduct	RT (min.)	MW	Formula	VIP	FDR	Confirmation
Chlorpromazine	319.1028	$[M+H]^+$	5.08	318.0957	$C_{17}H_{19}ClN_2S$	1.85	3.53×10^{-5}	Standard
7-Hydroxy-chlorpromazine	355.0978	$[M+H]^+$	6.72	334.8636	$C_{17}H_{19}ClN_2OS$	2.01	9.12×10^{-6}	Standard
3-Hydroxy-chlorpromazine	355.0978	$[M+H]^+$	6.72	334.8636	$C_{17}H_{19}ClN_2OS$	2.02	4.28×10^{-6}	Standard
Norchlorpromazine	305.0872	$[M+H]^+$	4.69	304.8376	$C16H_{17}ClN_2S$	2.03	3.32×10^{-8}	Standard
Chlorpromazine-N-oxide	335.0978	$[M+H]^+$	3.37	334.0907	$C_{17}H_{19}ClN_2OS$	2.02	3.16×10^{-7}	Standard
Promazine	285.1419	$[M+H]^+$	4.53	284.4191	$C_{17}H_20N_2S$	2.02	2.84×10^{-6}	Standard
Haloperidol	376.1473	$[M+H]^+$	4.06	375.8642	$C_{21}H_{23}CLFNO_2$	1.98	1.62×10^{-6}	Standard
Haloperidol-N-oxide	392.1421	$[M+H]^+$	3.27	391.8636	$C_{21}H_{23}CLFNO_3$	2.03	3.43×10^{-7}	Standard
Reduced haloperidol	378.1631	$[M+H]^+$	4.34	377.8801	$C_{21}H_{25}CLFNO_2$	2.04	8.91×10^{-7}	Standard
Dechloro haloperidol	342.1863	$[M+H]^+$	3.61	341.1492	$C_{21}H_{24}FNO_2$	2.03	6.00×10^{-6}	Standard
Clozapine	327.1327	$[M+H]^+$	4.46	326.8233	$C18H_{19}ClN_4$	1.97	1.71×10^{-4}	Standard
Hydroxy-clozapine	343.1319	$[M+H]^+$	3.40	342.8227	$C18H_{19}ClN_4O$	2.00	4.86×10^{-6}	Mass error < 5 ppm
Clozapine-N-oxide	343.1318	$[M+H]^+$	6.41	342.8227	$C18H_{19}ClN_4O$	2.02	3.79×10^{-3}	Standard
N-Desmethylclozapine	313.1213	$[M+H]^+$	3.78	312.7967	$C_{17}H_{17}ClN_4$	2.03	1.80×10^{-3}	Standard
Hydroxyl-desmethyl-clozapine	307.1553	$[M+H]^+$	3.32	294.3510	$C_{17}H18N_4O$	2.04	5.48×10^{-4}	Mass error < 5 ppm
Risperidone	411.2190	$[M+H]^+$	3.61	410.4845	$C_{23}H_27FN_4O_2$	1.99	1.69×10^{-13}	Standard
Hydroxy-risperidone	427.2139	$[M+H]^+$	3.67	426.4839	$C_{23}H_27FN_4O_3$	2.04	6.77×10^{-6}	Standard

m/z: mass per charge ratio, RT: retention time, min: minute, MW = monoisotopic molecular weight, VIP: variable importance in the projection, FDR: false discovery rate and ppm: part per million.

exist in cells. Radiotracking is commonly utilized as a method for identifying the drug metabolites *in vivo* and *in vitro*. However, this method greatly depends on the availability of the radiolabeled molecules that are sometimes difficult and expensive to synthesize and require containment facilities. Moreover, radiolabeled molecules can be metabolized at different rates by enzymes. Accompanying the development of technology associated with metabolomics, several metabolomics-based methods have been developed, such as cell-based metabolomics using LC-MS, combined with multivariate data analysis for screening and characterizing drug metabolites. Moreover, this method is an unbiased approach for metabolite

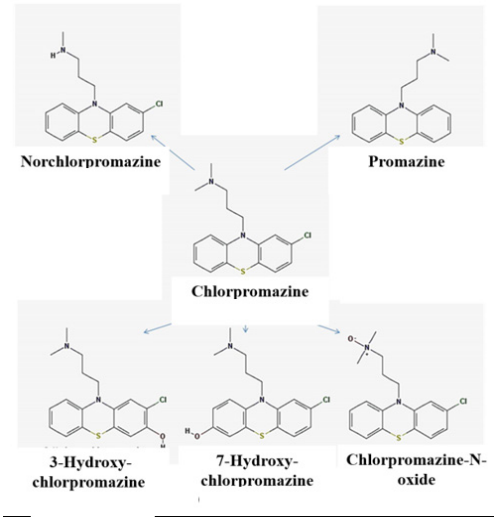

Figure 5. Schematic representation of the possible metabolism of chlorpromazine by brain microvascular endothelial cells.

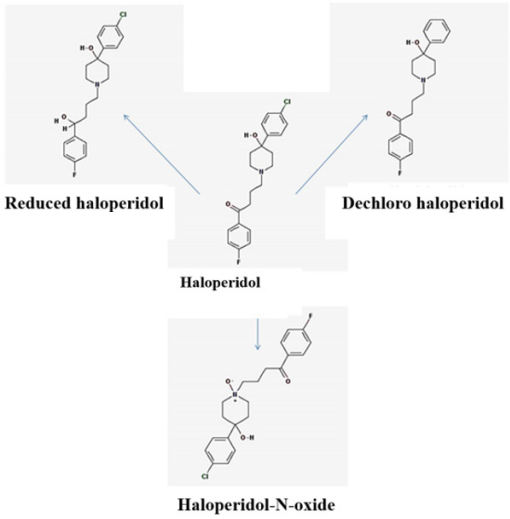

Figure 6. Schematic representation of the possible metabolism of haloperidol by brain microvascular endothelial cells.

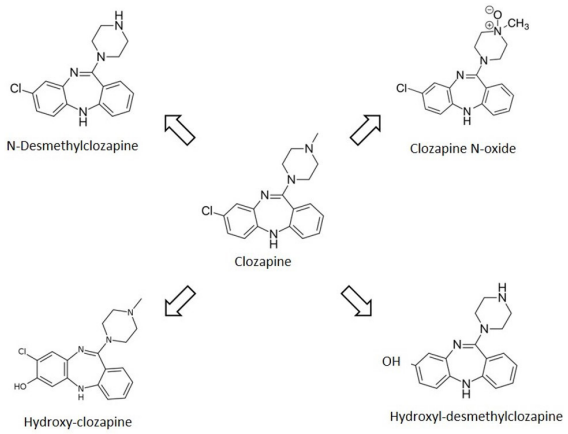

Figure 7. Schematic representation of the possible metabolism of clozapine by brain microvascular endothelial cells.

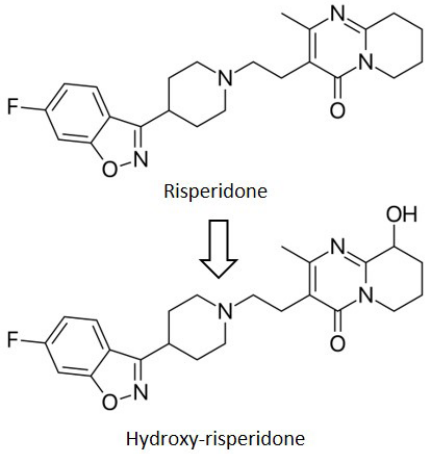

Figure 8. Schematic representation of the possible metabolism of risperidone by brain microvascular endothelial cells.

identification. The purpose of this study was to firstly describe the outcomes of cell-based metabolomics using LC-MS combined with multivariate data analysis in profiling of antipsychotic metabolism and bioactivation in BMVECs.

Although only five chlorpromazine metabolites were identified in this study, these metabolites were identified in human serum (*24,25*) and *in vitro* study (*26,27*). Previous studies had suggested that chlorpromazine could be transformed into other metabolites through hydroxylation, N-oxidation, demethylation and dechlorination. To achieve chlorpromazine metabolites, LC-MS analysis was applied to screen for these compounds. The ions were identified as the putative chlorpromazine metabolites, based on the exact m/z as shown in Table 1 and confirmed by standards. This *in vitro* study detected the two hydroxylation chlorpromazine metabolites (8-hydroxychlorpromazine and 3-hydroxychlorpromazine), one demethylation chlorpromazine metabolite (N-desmethyl chlorpromazine), one N-oxidation chlorpromazine metabolite (chlorpromazine-N-oxide), and one chlorination chlorpromazine metabolite (promazine).

Moreover, using LC-MS, Khelfi *et al.* (2018) (*28*) reported 14 haloperidol metabolites formed from an *in vitro* study of human liver microsomal incubation. In this study they found only 3 drug metabolites in BMVECs as shown in Table 1 and Figure 7. This illustrates the possible chemical structures of the detected metabolites: N-oxidation, hydroxylation, and dechlorination forms. These drug metabolites were similarly found in the previous study (*28*).

In addition, there were 12 clozapine metabolite forms of clozapine which were reported in previous studies (*29*). In our study, four clozapine metabolite forms were detected in the BMVECs treatment group: one oxidation, one hydroxylation, one dechlorination, and one oxidative dechlorination (as shown in Table

1 and Figure 7). 9-Hydroxy risperidone was detected for risperidone metabolism in the BMVECs, as shown in Table 1 and Figure 9, according to the previous experiment (*30*).

These results confirm that there is a drug biotransformation process at the BBB and show that drug metabolite screening employed cell-based metabolomics using LC-MS combined with multivariate analysis in the study of BMVECs exposed to antipsychotics could provide a way to use for screening of drug metabolites in the BBB.

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

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Brief Report

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Effects of consultation for voiding behavior on nocturnal urination status of older adults living alone: A preliminary study

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SUMMARY Nocturia and its related arousal may impair the quality of life and increase the risk of falls in older adults. This study aimed to clarify the change in urination status during the main sleeping period within 1 year. We also aimed to examine the effects of a consultation for voiding behavior in addition to the traditional behavioral therapy on urination status during sleep in a group of independent community-dwelling older adults. A single-arm intervention study was conducted in 10 older adults, with a mean age of 80.1 years and nocturia frequency of 1-4 times/day. Natural changes in urination status were observed between 2016 and 2017. Participants received traditional behavioral therapy and a consultation related to voiding behavior four times from summer 2017 to spring 2018. Urination status was monitored using sensing devices placed in the participant's home. The average time staying in the toilet significantly increased after 1 year. Although this parameter significantly decreased after the first consultation in 2017, this change was not observed with the subsequent consultation. A combination of traditional behavioral therapy and consultation for voiding behavior may be effective in improving urination status during the main sleeping period.

Keywords community-dwelling older adults, living alone, nocturia, arousal time, sensing device

1. Introduction

In 2018, the International Continence Society defined nocturia as waking to pass urine during the main sleeping period (*1*). Clinically, nocturia is diagnosed when a person awakens during sleep more than once to urinate. In Japan, approximately 38% of individuals aged > 40 years experienced nocturia, which increases in older adults (*2*). However, most older adults assume that nocturia is a sign of aging; therefore, they do not seek consult for appropriate treatment.

In community-dwelling older adults, nocturia can cause falls, which are associated with bone fractures, increased mortality, and impaired quality of life (*3-5*); therefore, detecting nocturia is important in this population. The Japanese Continence Society guideline recommends that patients with nocturia caused by storage dysfunction undergo pharmaceutical and behavioral therapy, such as water and salt restriction, regardless of nocturnal polyuria (*6*). Additionally, the guideline recommends a multidisciplinary team to manage the voiding behavior including removing underwear, sitting on the toilet seat, cleaning after voiding, wearing clothes, and toilet environment to improve the cognitive and motor dysfunctions of older adults in need of long-term care. However, the recommendations remain lacking for older adults who live alone.

Our previous study (*7*) revealed that older adults with sufficient mental and behavioral function to live alone awakened several times for several minutes during night sleep to urinate. However, being awake for certain duration increases the difficulty in subsequent sleeping attempts, which may lead to sleeping disorders. In addition to medication and water and salt restriction, consulting nurses for voiding behavior may resolve problems and shorten the awake time during nocturia. However, the deterioration of voiding behavior in older adults and the effect of consultations on improving voiding behavior and shortening awakened time remain unclear. Therefore, this longitudinal study aimed to clarify urination status changes during the main sleeping period in 1 year. Additionally, we aimed to examine the effects of a consultation and traditional behavioral therapy by a nurse on the voiding behavior during the main sleeping period of independent community-dwelling older adults.

2. Methods

2.1. Study design and participants

This single-arm interventional study was conducted from July 2016 to March 2018. Data regarding urination status during the main sleeping period were collected during five research periods: 1 week during summer 2016, 2 weeks each during summer, autumn, and winter 2017, and 2 weeks during spring 2018. Consultations were provided on the intermediate day in the four research periods, except during the summer of 2016.

The study was approved by the Medical Ethics Review Committee of Kanazawa University (No. 555-1) and complied with the provisions of the Declaration of Helsinki (revised 2013).

Flyers containing the research introduction were distributed by staff to older adults who had support from one of three regional comprehensive support centers in Kanazawa City. Older adults who were interested in the study attended a briefing session at a university. At the session, they received a written explanation of the study from a researcher and provided their informed consent for participation.

This study included older adults aged ≥ 65 years who lived alone, with the following characteristics: 1) experiencing nocturia, 2) evaluated as at risk of falls by the staff of the centers, 3) allowed setting of sensing devices in their homes, and 4) their families agreed with the participation. The exclusion criteria were as follows: 1) diagnosis of dementia or higher brain dysfunction and 2) lives with animals, which may cause noise for sensing data.

2.2. Intervention

An expert nurse in continence care provided consultations and traditional behavioral therapy regarding nocturia and voiding behavior. Prior to the consultations, participants were asked to complete a 3-day bladder diary. At the beginning of the intervention, the nurse measured the residual urine volume and interviewed participants regarding lower urinary tract symptoms, bed environment, sleeping clothes, and toilet environment. A portable ultrasound device, Lilliam® α-200 (Lilliam Otsuka Co., Ltd., Tokyo, Japan), was used to measure residual urine volume, with a measurement accuracy of ± 15% or ± 20 mL.

Regarding traditional behavioral therapy, the nurse provided explanations to all participants regarding appropriate volume and timing of fluid intake to improve nocturia and nocturnal polyuria. The main lower urinary tract symptoms and their causes were identified. Subsequently, for participants with urinary incontinence, the nurse instructed them to perform pelvic floor muscle training using a pamphlet, recommended an appropriate pad based on the amount of urinary leakage, and taught them methods to easily change pads. In participants without residual urine, but had voiding difficulty or fear of residual urine, the nurse reassured them that no problematic residual urine was observed and instructed them to leave the toilet after urination.

Regarding voiding behavior care, the nurse recommended the use of handrails and other devices to allow easier getting up from a bed in participants who complained of physical symptoms including back and knee pain. For participants with weak grip strength or those who wore clothing with buttons around the waist, the nurse suggested wearing clothes with an elastic waistband that is easily worn and removed. Participants at risk of falls were instructed regarding footwear and environment safety (electricity, removing luggage from the hallway) to prevent falls.

2.3. Measurements

2.3.1. Urination status during the main sleeping period

To monitor the urination status during the main sleeping time, we used an electrical device that utilizes an infrared sensor to detect nearby motions. The device was set at four places: two were placed 20 cm and 1.5 m above the floor on the wall adjacent to the bed, one on the outside wall of the toilet, and one on the inside wall of the toilet. Sensing data were continuously stored *via* the Internet (Wireless Smart Utility Network) in the cloud using Nippon Electric Company Solution Innovators, Ltd. (Tokyo, Japan) throughout the research period. Using these data, we detected three timings: leaving the bed, opening the door of the toilet, and lying on the bed. A urination-related arousal included the three timings. These arousals were quantified as the number of nocturnal urinations during the main sleeping period.

We measured three times: the time from leaving the bed to the first opening of the toilet door (time going to the toilet), the time from the first to the second opening of the toilet door (time staying in the toilet), and the time from the second opening of toilet door to sitting on bed (time going back to the toilet). Nocturia was represented as the sum of the time going to the toilet and staying in the toilet because some older adults loitered and stayed in other rooms before returning to bed from the toilet. The arousal time related to nocturia was calculated as the sum of the three times (from leaving to sitting on the bed).

The 1-week data in the summer of 2016 and data from 2 weeks before (control period) and after (intervention period) the consultation in the summer, autumn, and winter of 2017 and the spring of 2018 were extracted.

2.3.2. Basic characteristics

Age, sex, walking ability, assistive devices used, and major diseases were collected through interviews during the recruitment period.

2.4. Statistical analysis

Variables related to nocturnal urination status including the cumulative number of nocturnal urinations, cumulative nocturia-related time, and cumulative arousal time were calculated using the 7-day data. The average nocturia-related time and the average arousal time were calculated by dividing each variable by the cumulative number of nocturnal urinations.

A paired t-test was performed to analyze the changes in nocturnal urination between the summers of 2016 and 2017. Similarly, a paired *t*-test was performed to compare the control and intervention periods to evaluate the effect of the intervention. Differences in nocturnal urination among the four seasons were examined by multivariate analysis of variance with repeated measures using the values of the four control periods. JMP ver. 16 (SAS Institute Japan Ltd.) was used for data analysis. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

Initially, 12 participants provided their consent; however, one was unable to attend consultation due to conflicting schedules, and another was unable to provide sensing data for > 50% of the day in both summers of 2016 and 2017. Therefore, this study included 10 older adults with a mean age of 80.1 ± 5.8 years. Among them, eight (80%) were female, and four (40%) walked with an assistive device occasionally. The prevalence of back and knee pain was 40%. All participants can urinate independently. All participants experienced nocturia, four experienced urinary incontinence, and three experienced voiding difficulty (Table 1).

Table 1. Participant characteristics

Items	
Age (years)	80.1 ± 5.8
> 75 years	8 (80%)
65 to < 75 years	2 (20%)
Sex	
Female	8 (80%)
Male	2 (20%)
Walking condition	
Independent	6 (60%)
Occasional use of assistive device	4 (40%)
Back pain	4 (40%)
Knee pain	4 (40%)
Lower urinary tract symptoms	
Nocturia	10 (100%)
Urinary incontinence	4 (40%)
Voiding difficulty	3 (30%)

Data are mean ± standard deviation, or n (%).

One participant had missing sensing data in 2016. The remaining nine participants had an 11.2 cumulative number of nocturnal urinations in 2017, which was not significantly different from 2016. Additionally, the average nocturia-related time and the average time staying in the toilet in 2017 were significantly increased compared with 2016 data ($p = 0.030$ and $p = 0.035$, respectively) (Table 2).

Seasonal changes in nocturnal urination status were examined in five participants who had complete data for all four seasons from summer 2017 to spring 2018; however, no significant difference was observed between the cumulative numbers of nocturnal urinations among the four seasons (Table 3). A significant difference was observed in the average time staying in the toilet among the four seasons; among them, the 2017 winter obtained the shortest time (208.0 ± 43.5 s). The average arousal time in summer and autumn were 428.2 and 333 s, respectively, which were approximately 7 and 5 min, respectively (Table 3).

In the summer of 2017, the intervention was initiated in 10 participants. Based on the bladder diary, all 10 participants had nocturia, with a frequency of 1-4 times/day and a residual urine volume of < 100 mL. After the behavioral therapy and consultation for voiding behavior, the average nocturia-related time significantly decreased ($p = 0.024$) (Table 4). Similarly, the average time staying in the toilet was significantly decreased ($p = 0.027$). After consultation at autumn 2017, a significant decrease was observed in the cumulative time going to the toilet in eight participants ($p = 0.027$). However, no changes were observed in nocturnal urination status after the intervention in five participants who underwent consultation during winter

Table 2. Change in nocturnal urination status after 1 year ($n = 9$)

Items	Summer 2016	Control period in 2017 summer	*p*-value
Cumulative number of nocturnal urinations	10.2 ± 3.0	11.2 ± 3.0	0.382
Cumulative nocturia-related time	2596.2 ± 1679.1	3696.7 ± 2756.4	0.161
Cumulative time going to the toilet	274.5 ± 132.1	284.4 ± 141.8	0.866
Cumulative time staying in the toilet	2321.8 ± 1675.5	3412.3 ± 2768.1	0.170
Cumulative arousal time	4402.8 ± 2059.0	6660.4 ± 4123.0	0.189
Average nocturia-related time	254.1 ± 160.8	320.4 ± 173.9	0.030
Average time going to the toilet	27.5 ± 12.5	25.6 ± 11.3	0.751
Average time staying in the toilet	226.6 ± 158.7	294.8 ± 175.1	0.035
Average arousal time	438.2 ± 204.7	576.0 ± 269.6	0.213

Unit: Seconds. Data are mean ± standard deviation. Paired *t*-test.

Table 3. Seasonal changes in nocturnal urination status (n = 5)

Items	Control period in summer 2017	Control period in autumn 2017	Control period in winter 2017	Control period in spring 2018	p-value
Cumulative number of nocturnal urinations	9.4 ± 2.2	10.2 ± 2.7	10.2 ± 2.2	10.0 ± 2.3	0.365
Cumulative nocturia-related time	2526.8 ± 785.5	2672.9 ± 1293.9	2410.0 ± 681.3	2508.0 ± 929.8	0.609
Cumulative time going to the toilet	238.6 ± 121.6	266.5 ± 118.5	239.6 ± 87.8	243.2 ± 115.9	0.946
Cumulative time staying in the toilet	2288.2 ± 696.4	2406.4 ± 1190.6	2170.4 ± 633.7	2264.8 ± 923.5	0.717
Cumulative arousal time	3988.5 ± 1057.8	3540.8 ± 1631.9	3843.3 ± 1303.5	3869.0 ± 2054.6	0.829
Average nocturia-related time	268.6 ± 60.5	249.0 ± 65.4	230.8 ± 41.6	254.8 ± 102.0	0.062
Average time going to the toilet	24.3 ± 7.3	25.2 ± 8.6	22.8 ± 4.8	23.4 ± 7.7	0.802
Average time staying in the toilet	244.3 ± 57.5	223.8 ± 59.6	208.0 ± 43.5	231.4 ± 107.6	0.046
Average arousal time	428.2 ± 90.7	333.0 ± 80.8	371.5 ± 129.0	393.4 ± 229.9	0.428

Unit: Seconds. Data are mean ± standard deviation. Multivariate analysis of variance with repeated measures.

and spring.

Daily monitoring of nocturnal urination status is difficult; therefore, the actual nocturia-related behaviors in community-dwelling older adults remain unclear. Sensing devices allow continuous monitoring of the daily urination status without changing voiding behavior. Despite the relatively small sample, this study revealed a significant increase in average time staying in the toilet in 1 year. Annual age-induced motor function decline may be observed (*8-10*); therefore, the time staying in the toilet may be prolonged by the deterioration of voiding behaviors. When motor function deteriorates due to paralysis or muscle weakness, older adults easily lose truncal balance when wearing and removing underwear and clothes, cleaning the buttocks, and standing from the toilet seat (*11-13)*. This functional deterioration may gradually manifest as difficulties in performing repeated joint movements, such as dressing and standing. However, this study found no decrease in the time going to the toilet. This might may be attributed to the presence of back and knee pain in most participants; additionally, their walking ability may have already deteriorated in 2016, since their average age at time was already at > 80 years.

The first consultation in summer 2017 significantly decreased the average nocturia-related time, especially the time staying in the toilet; however, no significant change in average nocturia-related time was observed in the following three sessions. This may be due to the ease of application of the recommendations for voiding behaviors that were provided during the first consultation, such as avoiding clothing that interferes with urination. Moreover, the older adults seemed to continue to follow this advice, which may explain the non-effectiveness of the consultation. Another possible reason was the non-problematic nature of residual urine volume; therefore, older adults may completely urinate without uncertainty. The average time staying in the toilet decreased by approximately 1 min before and after the consultation in every season; despite the non-significant difference, this may cause improvements in the quality of life in older adults. Therefore, the results of this study suggest that consultation related to voiding behavior is effective for older adults living alone.

In autumn 2017, the cumulative time going to the toilet significantly decreased after consultation, which may be related to the 30-s increase during the control period. Many people tend to use socks or slippers in colder climates, including autumn and winter, while they prefer walking barefoot during summer. Indoor footwear influences balance and gait patterns (*14*); therefore, wearing inappropriate footwear could prolong the cumulative time going to the toilet in the autumn, which may be reduced by a consultation regarding appropriate footwear. The cumulative time going to the toilet was similar between the control and the intervention periods of winter 2017. This suggests that older adults with cognitive function sufficient to live independently in the community can maintain behaviors with a single instruction at summer 2017.

No change in the cumulative number of nocturnal urinations was observed before and after the consultation at any time. The habit of going to the toilet during sleeping time has been associated with anxiety related to urinary incontinence (*15,16*). For older adults with this habit and fear of urinary leakage, it is psychologically difficult to avoid urination during the sleeping period, despite healthcare professionals explaining that voiding is not needed. This study found a large discrepancy between the urination-related time and the arousal time, indicating that older adults did not directly return to the toilet after urination. Previous studies have found that polyuria is caused by lifestyle habits, such as drinking excessive amounts of water at night because of dehydration-induced stroke (*6,17,18*). It is presumed that the older adults did not return to their bedrooms immediately after voiding but stayed in other rooms performing other activities. Staying in a bright place for long periods promotes arousal and interferes with subsequent falling asleep (*19-21*); thus, providing advice to sleep promptly and to immediately return from the toilet is more beneficial than to ignore going to the toilet considering their lower urinary function.

This study has two limitations. First, the sample size is relatively small due to difficulties in monitoring behaviors in homes using sensors; therefore, this study

Table 4. Changes in nocturnal urination status with consultation

Items	Summer 2017 (n = 10)			Autumn 2017 (n = 8)			Winter 2017 (n = 5)			Spring 2018 (n = 5)		
	Control period	Intervention period	p-value	Control period	Intervention period	p-value	Control period	Intervention period	p-value	Control period	Intervention period	p-value
Cumulative number of nocturnal urinations	11.0 ± 2.9	11.4 ± 3.4	0.591	11.1 ± 3.1	10.0 ± 2.6	0.268	10.2 ± 2.2	10.0 ± 2.5	0.749	10.0 ± 2.3	10.6 ± 2.3	0.208
Cumulative nocturia-related time	3416.9 ± 2745.2	3120.0 ± 2415.1	0.386	4118.9 ± 2435.4	3026.2 ± 1592.1	0.282	2410.0 ± 681.3	1796.4 ± 836.2	0.100	2508.0 ± 929.8	1975.7 ± 849.6	0.279
Cumulative time going to the toilet	273.7 ± 137.8	263.1 ± 126.7	0.642	298.8 ± 116.4	235.5 ± 91.7	0.027	239.6 ± 87.8	222.6 ± 50.2	0.430	243.2 ± 115.9	249.3 ± 104.3	0.782
Cumulative time staying in the toilet	3143.2 ± 2745.0	2856.9 ± 2365.2	0.394	3820.1 ± 2345.8	2790.8 ± 1598.8	0.302	2170.4 ± 633.7	1573.7 ± 791.5	0.109	2264.8 ± 923.5	1726.4 ± 767.2	0.275
Cumulative arousal time	6114.3 ± 4253.6	4607.7 ± 2654.9	0.223	4989.1 ± 2541.3	4065.8 ± 1492.6	0.390	3843.3 ± 1303.5	3046.4 ± 847.5	0.166	3869.0 ± 2054.6	3443.3 ± 2157.1	0.693
Average nocturia-related time	298.4 ± 178.2	250.4 ± 143.7	0.024	350.2 ± 151.5	316.7 ± 175.1	0.622	230.8 ± 41.6	173.2 ± 50.2	0.163	254.8 ± 102.0	180.8 ± 53.2	0.195
Average time going to the toilet	25.0 ± 10.8	22.8 ± 8.0	0.208	26.3 ± 7.7	24.0 ± 9.0	0.306	22.8 ± 4.8	22.8 ± 4.3	0.977	23.4 ± 7.7	22.7 ± 4.9	0.747
Average time staying in the toilet	273.3 ± 178.5	227.6 ± 144.4	0.027	323.8 ± 147.9	292.7 ± 172.7	0.644	208.0 ± 43.5	150.4 ± 50.0	0.168	231.4 ± 107.6	158.1 ± 51.8	0.206
Average arousal time	531.7 ± 290.2	388.2 ± 156.1	0.088	432.7 ± 169.4	429.8 ± 191.6	0.967	371.5 ± 129.0	310.6 ± 69.4	0.303	393.4 ± 229.9	306.4 ± 144.2	0.483

Unit: Seconds. Data are mean ± standard deviation. Paired t-test.

was unable to detect any significant seasonal variation in nocturnal urination status. Second, the content of the consultation covered individual's symptoms, lifestyle, and house conditions. However, to maintain the quality of the intervention, a nurse conducted the consultation. After identifying the components of the consultation that lead to beneficial effects on voiding behavior during sleeping, protocols should be standardized and further studies with larger sample sizes are needed.

In conclusion, sensors were installed at the participant's homes to continuously detect nocturnal urination status in this study, and we found a 1-year increase in the average nocturia-related time among community-dwelling older adults. This study also showed that consultation for voiding behavior effectively decreased the average time staying in the toilet and the cumulative time going to the toilet. Future interventional studies with a larger sample size are warranted to validate the results of this study.

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Brief Report

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CASC5 is a potential cancer-testis gene in human urinary bladder transitional cell carcinoma

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SUMMARY Urinary Bladder cancer (UBC) is a diversified disease with an array of clinicopathological attributes. Several studies have shown that cancer susceptibility candidate 5 (CASC5) plays important roles in various types of malignancies; however its expression and clinical significance in human UBC remain largely unknown. This research study was intended to explore mRNA/protein expression pattern of CASC5 as a member of the cancer-testis (CT) gene family and assess its clinical utility in diagnostic management of patients with UBC. Quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC) was employed to appraise the detailed expression profile of CASC5 in patients with UBC. The mRNA over expression of CASC5 was detected in testis tissue and relatively high frequency 59.2% (45 of 76) of CASC5 mRNA was detected in UBC tissues. CASC5 mRNA relative mean fold expression was also significantly ($p < 0.01$) higher in the muscle-invasive tumor tissues compared to non-muscle-invasive tumor tissues (12.26 ± 9.53 *vs.* 4.64 ± 2.50, $p = 0.005$). Heterogeneous staining pattern of CASC5 protein was exclusively detected using IHC. The frequency of CASC5 protein over expression was detected in 67.7% (44 of 65) UBC patients and negative in benign prostatic hyperplasia (BPH). Further, CASC5 protein expression was significantly ($p < 0.001$) associated with cigarette smoking habit in UBC patients. Our study findings testified that CASC5 over expression among patients with UBC as compared to controls and concludes that CASC5 is a potential CT gene in UBC.

Keywords Cancer susceptibility candidate 5, urinary bladder cancer, cancer-testis, immunotherapy, peptide vaccines

1. Introduction

Urinary Bladder cancer (UBC) is a diversified disease with an array of clinicopathological attributes and natural histories, which are characterized by complex networks of molecular alterations and gene expressions. With the high recurrence rate among solid tumors, UBC remains most commonly diagnosed malignancy of the urinary tract as well as second major cause of death associated with genitourinary cancer (*1*). Transitional cell carcinoma (TCC), which is most natural form of UBC, constitutes approximately 95% of all urothelial tumors (*2*). The nature of TCC is immensely diverse and characterized by two different, but associated processes: tumor recurrence and progression (*3*). In the developed world, the occurrence of TCC has the fourth highest incidence of all cancers and thus UBC patients is also affected from substantial morbidity and mortality (*4*). UBC include highly differentiated, non-invasive tumors on one side to high-grade lamina propria invasive malignant lesions on the other side. More than 60% of the early staged bladder tumors recur at least once and progress to invasive neoplasms with poor prognosis in a significant proportion of patients (*5,6*). Although radical cystectomy (RC) has been the mainstay treatment for muscle-invasive bladder cancer (MIBC), many patients with several comorbidities are unfit for RC (*7*). Thus, the 5-year survival rate for MIBC patients is approximately 50% (*8,9*). Therefore, to upgrade the clinical management of UBC, it is crucial to identify additional potential candidates to be used in diagnostic, prognostic and therapeutic of patients.

UBC is the only malignancy, for which immunotherapy is usually included as part of standard care (*10*). In non-muscle-invasive bladder cancer (NMIBC) intravesical use of the Bacillus Calmette Guerin (BCG) immunotherapy reduces the risk of local recurrence by approximately 60% by unknown mechanism, which can achieve 5-year survival rates of approximately 90% in UBC patients having unifocal disease (*11,12*). This suggested that cancer immunotherapy targeting specific cancer testis antigens (CTAs) in UBC might be a potent and associated with less morbidity than BCG. Moreover, innovative therapy such as immunotherapy have appeared as encouraging therapeutic modality to increase overall patient survival and effective cure, which has revived enthusiasm towards characterization of tumor-specific targeted antigens (*13*). Thus identification of cancer-testis (CT) genes in UBC towards the development of effective and specific immunotherapy is need of the hour.

Cancer susceptibility candidate 5 (CASC5) was initially characterized as a gene involved in chromosomal translocation in leukemia and as a member of the CT gene family. It is predominantly expressed in testis, widely expressed in various human tumor cell lines, primary tumors from various tissues and organs (*14-19*). Till date, CASC5 expression has been documented in various diseases such as infertility, spermatogenesis and lung cancer, however its expression profiles in UBC have not been explored to date. Thus in the present study, we have evaluated quantitative mRNA expression of CASC5 in UBC. Protein expression of CASC5 was also assessed using immunohistochemistry (IHC). The expression pattern was correlated with clinical characteristics of patients to to determine clinical utility of CASC5.

2. Materials and Methods

2.1. Clinical specimens

To quantify CASC5 mRNA expression, 76 bladder tumor tissues ((male: 67; female: 9) were collected for qRT-PCR analysis. Tissue specimens were immediately immersed in RNAlater buffer (Ambion-Applied Biosystems, Milan, Italy), and stored at −80°C to extract RNA. Further, to characterize CASC5 immunohistochemical expression, 75 archival, formalin-fixed, paraffin-embedded (FFPE) tissues (65 of bladder tumor tissues and 10 benign prostatic hyperplasia (BPH) tissues) were obtained from Pathology Department, King George's Medical University (KGMU), Lucknow, India. BPH tissues were also used as negative control in IHC analysis of CASC5. The study protocol was approved by our institutional ethics committee (Approval number: XXXIECM-II B/P13). All the bladder tumors were TCCs, which were diagnosed histologically and clinically by the two independent pathologists.

Demographic data and medical history of each patient was recorded, which is summarized in Table 1. All patients underwent cystoscopy as a reference standard for detection of UBC. All bladder tumors or suspicious lesions found were either resected or biopsied. The diagnosis of UBC was interpreted via histopathological observation. The 2004 World Health Organization (WHO) bladder tumor classification criteria were used for grading (*20*) and pathologic staging of bladder tumors was performed according to the 2002 tumor-lymph node-metastasis (TNM) classification system (*21*).

2.2. RNA extraction and quantitative reverse transcription-PCR

Total RNA extraction was performed from the bladder tumor tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Sixteen different normal tissues derived RNA was also purchased (Clonetech, Palo Alto, CA, USA). The RNA solution was treated with RNase-free DNase set (Qiagen, Valencia, CA, USA) as per manufacturer's instruction. RNA samples were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quality of RNA was also analyzed using the 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and stored at –80°C. cDNA was synthesized from ≥ 1µg RNA using Quantitect® Reverse Transcription Reagent (QIAGEN GmbH, Hilden, Germany) as per manufacturer instructions. Quantitative mRNA expression of CASC5 was analyzed using LightCycler 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. The PCR primer sequences were 5'-GCGCTCGTCGTCGACAA-3' and 5'-CGCCCACATAGGAATCCTTCT-3' for β-actin; and 5'-CGTGTGGACCCCAAACAAGT-3' and

Table 1. Patient clinciopathological characteristics

Clinicopathological characteristics	Real-time-PCR Assay, *n* = 76, (%)	Immunohistochemistry Assay, *n* = 65, (%)
Age (years, %)		
≤ 45	24 (31.6%)	17 (26.2%)
> 45	52 (68.4%)	48 (73.8%)
Sex		
Male	67 (88.2%)	63 (96.9%)
Female	9 (11.8%)	2 (3.1%)
Grade		
Low	33 (43.4%)	23 (35.4%)
High	43 (56.6%)	42 (64.6%)
Stage		
Ta	3 (3.9%)	6 (9.2%)
T1	33 (43.4%)	23 (35.4%)
T2-T4	40 (52.6%)	36 (55.4%)
Smoking		
No	34 (44.7%)	29 (44.6%)
Yes	42 (55.3%)	36 (55.4%)
Tobacco chewers		
No	36 (47.4%)	33 (50.8%)
Yes	40 (52.6%)	32 (49.2%)

5'-CACCCCATCCATTTT TGAAGA-3' for CASC5 (*19*). Quantitative real-time PCR thermal cycling consisted of an initial 40 cycles of denaturation at 95°C for 15s followed by annealing at 55°C for 1 min and extension at 72°C for 45 s. Each experiment was performed in triplicate, with normalization to the β-actin gene as an internal control. Human Bladder Total RNA (Clontech) was used as a reference for evaluation of CASC5 mRNA levels in bladder tumor tissues. Among normal tissues, CASC5 mRNA levels were expressed as n-fold differences relative to β-actin (internal control) and the levels in the normal testis (calibrator).

2.3. Immunohistochemistry

IHC was performed on FFPE sections of bladder tumor and BPH tissues with classical protocol as described previously (*22*). The EnVision FLEX mini kit High pH (K802321; Dako, Glostrup, Denmark) was used to perform all steps of IHC according to manufacturer's protocol. Except primary antibody, all reagents and buffers used for IHC were from the EnVision FLEX mini kit High pH (K802321; Dako). The CASC5 protein was detected using a rabbit polyclonal antibody against CASC5 (ab95127; Abcam plc, Cambridge, UK). The tissue sections were incubated with anti-CASC5 antibody (1:100) overnight at 4 ℃.

2.4. Statistical analysis

Continual data were illustrated as mean ± SD while discrete (categorical) data were illustrated in percentages (%). Qualitative variables were represented as numbers and percentages. Independent Student's *t*-test was performed to evaluate comparison between two independent groups. ANOVA was used to evaluate comparison between more than two groups and significance of mean difference was analyzed by Tukey's post hoc test after adjusting the multiple contrasts for significance. Associations between categorical groups (*i.e.*, CASC5 mRNA/protein expression and clinicopathological parameters) were assessed applying the chi-square (χ^2) test. Two-tailed $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS (Windows version 18.0) statistical software packages.

3. Results and Discussion

3.1. Quantitative mRNA expression of CASC5

The frequency of CASC5 mRNA expression was recognized in 38.9% (14 of 36) non-muscle-invasive and 77.5% (31 of 40) muscle-invasive patients. Thus, overall frequency of CASC5 mRNA expression was detected in total 59.2% (45 of 76) UBC patients. Relative mean fold expression of CASC5 was significantly ($p < 0.01$) greater in the invasive patients incomparison to non-muscle-invasive patients (12.26 ± 9.53 *vs*. 4.64 ± 2.50, $p = 0.005$). Furthermore, relative mean fold expression of CASC5 was also significantly ($p < 0.05$) greater in the high grade UBC patients incomparison to low grade UBC patients (12.56 ± 10.64 *vs*. 6.55 ± 3.61, $p = 0.02$).

Among panel of 16 normal tissues, CASC5 mRNA expression was identified only in testis and placenta. However, over expression was noticed in testis only and relative mean fold expression level of CASC5 mRNA in placenta was 106-fold lower incomparison to testis.

The relative mean fold expression of CASC5 mRNA and its association with the UBC patient's clinicopathological characteristics are outlined in Table 2. No significant correlation was observed between CASC5 mRNA expression and clinicopathological characteristics such as patient's age, gender *etc.* in both the non-muscle-invasive and invasive patients. However, CASC5 mRNA relative mean fold expression was greater in older patients *vs*. younger patients, males *vs*. females, and in cigarette smokers *vs*. non-smokers.

Table 2. Correlation between relative mean fold CASC5 mRNA expression and clinical characteristics of UBC patients

Characteristics	Non-muscle-invasive Mean ± SD	*p* value	Muscle-invasive Mean ± SD	*p* value	Total (*n* = 45) Mean ± SD	*p* value
Age (years):		0.260		0.205		0.208
≤ 45	3.60 ± 1.52 (5)		9.50 ± 2.81 (12)		7.76 ± 3.70 (17)	
> 45	5.22 ± 2.82 (9)		14.00 ± 11.76 (19)		11.18 ± 10.58 (28)	
Sex:		0.594		0.443		0.718
Female	6.00 ± 0.00 (1)		9.20 ± 1.79 (5)		8.67 ± 2.07 (6)	
Male	4.54 ± 2.57 (13)		12.85 ± 10.31 (26)		10.08 ± 9.37 (39)	
Grade:		0.211		0.194		0.020
Low	4.10 ± 1.5 (10)		9.00 ± 3.43 (10)		6.55 ± 3.61 (20)	
High	6.00 ± 4.08 (4)		13.81 ± 11.09 (21)		12.56 ± 10.64 (25)	
Cigarette Smoking:		0.127		0.973		0.504
No	3.75 ± 1.67 (8)		12.33 ± 7.33 (12)		8.90 ± 7.12 (20)	
Yes	5.83 ± 3.06 (6)		12.21 ± 10.89 (19)		10.68 ± 9.93 (25)	

Numbers in parenthesis indicate the number of UBC patients.

3.2. Expression of CASC5 protein

IHC derived heterogeneous expression of CASC5 protein was noticed in 48.3% (14 of 29) of non-muscle-invasive and 83.3%% (30 of 36) muscle-invasive patients (Figure 1). Thus protein expression of CASC5 was recorded in 67.7% (44 of 65) UBC patients. Heterogenous expression of CASC5 protein was detected in 60.8% patients with low grade and 71.4% with high grade urothelial tumors respectively. There is also significant ($p < 0.01$) difference in frequency of CASC5 positive IHC expression between bladder tumor stages Ta, T1, T2-T4 (2 (4.5%) *vs*. 12 (27.3%) *vs*. 30 (68.2%)).

No significant expression of CASC5 protein was detected among BPH (Figure 1D). The IHC expression of CASC5 was correlated with the clinical characteristics of the UBC patients and is outlined in Table 3. No strong association was observed between protein expression of CASC5 and patient's clinicopathological characteristics such as age, gender, grade and stage of the disease. However, CASC5 protein expression was significantly ($p < 0.001$) associated with the cigarette smoking habit in both non-muscle-invasive and muscle-invasive patients.

UBC is a frequent genitourinary malignancy worldwide with approximately 160,000 deaths per year worldwide (*23*). It has the highest lifetime treatment costs per patient of all cancers, from diagnosis to death (*24,25*). Although existence of improved surgical procedures and role of pelvic lymphadenectomy has well understood, invasive UBC patient's long term prognostification remains poor after treatment. Molecular mechanisms contributing UBC progression and metastasis also remain unexplained (*26*). Thus, UBC presents a major clinical challenge due to confined treatment choice to prevent recurrence and poor prognosis (*27*). Therefore, need of hour is to identify new biomarker which can be employed in early diagnosis as well as in therapeutic approach for UBC. Thus, we have evaluated mRNA and protein expression of a CT gene named CASC5 in UBC towards identification of newer diagnostic biomarker and development of active immunotherapy in carcinoma of urinary bladder (CaUB).

Recently, a number of studies showed CASC5 is abundantly expressed in testis, various human cancer

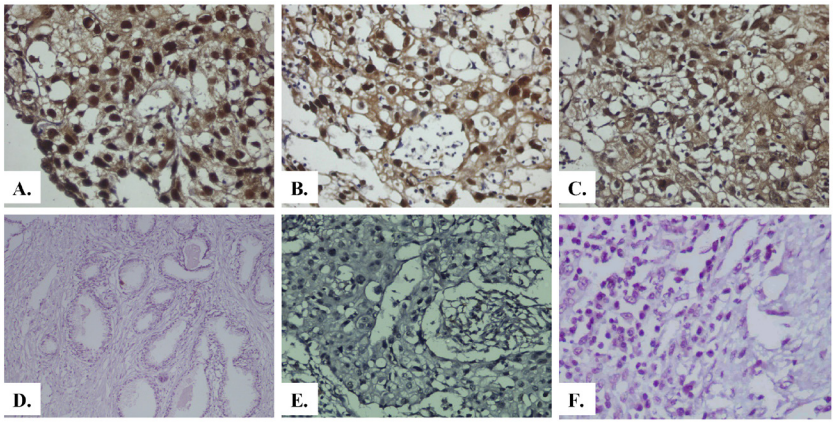

Figure 1. Heterogenous expression of CASC5 protein in surgically resected UBC (40×). UBC tissues were stained with rabbit polyclonal antibody against CASC5. **(A)** NMIBC showing strong positive expression. **(B)** MIBC showing strong positive expression. **(C)** MIBC showing strong positive expression. **(D)** BPH showing negative expression. **(E)** MIBC showing negative expression. **(F)** NMIBC tissue showing negative expression.

Table 3. Correlation between CASC5 protein expression and clinical characteristics of UBC patients

Characteristics	Non-muscle-invasive ($n = 29$)			Muscle-invasive ($n = 36$)			Total ($n = 65$)		p value
	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	p value	Negative n (%)	Positive n (%)	
Age (years):			0.924			0.750			0.133
≤ 45	3 (20.0%)	3 (21.4%)		0 (0.0%)	11 (36.7%)		3 (14.3%)	14 (31.8%)	
> 45	12 (80.0%)	11 (78.6%)		6 (100%)	19 (63.3%)		18 (85.7%)	30 (68.2%)	
Sex:			NA			0.193			0.587
Female	0 (0.0%)	0 (0.0%)		1 (16.7%)	1 (3.3%)		1 (4.8%)	1 (2.3%)	
Male	15 (100%)	14 (100%)		5 (83.3%)	29 (96.7%)		20 (95.2%)	43 (97.7%)	
Grade:			0.550			1.000			0.384
Low (G1)	8 (53.3%)	9 (64.3%)		1 (16.7%)	5 (16.7%)		9 (42.9%)	14 (31.8%)	
High (G2-G3)	7 (46.7%)	5 (35.7%)		5 (83.3%)	25 (83.3%)		12 (57.1%)	30 (68.2%)	
Stage:			0.411			NA			0.007
Ta	4 (26.7%)	2 (14.3%)		0 (0.0%)	0 (0.0%)		4 (19.0%)	2 (4.5%)	
T1	11 (73.3%)	12 (85.7%)		0 (0.0%)	0 (0.0%)		11 (52.4%)	12 (27.3%)	
T2-T4	0 (0.0%)	0 (0.0%)		6 (100%)	30 (100%)		6 (28.6%)	30 (68.2%)	
Cigarette Smoking:			0.013			$p < 0.001$			$p < 0.001$
No	13 (86.7%)	6 (42.9%)		6 (100%)	4 (13.3%)		19 (90.5%)	10 (22.7%)	
Yes	2 (13.3%)	8 (57.1%)		0 (0.0%)	26 (86.7%)		2 (9.5%)	34 (77.3%)	

Numbers in parenthesis indicate the number of UBC patients.

derived cell lines, primary cancers, spermatocytes, and in spermatogenesis (*15,18,19*). Nevertheless, the role of CASC5 in UBC remains unknown. To the best of our knowledge, our study has first time demonstrated mRNA and protein expression of CASC5 in UBC. It is notable that we detected its mRNA and protein expression in independent cohort of UBC patients. So far, only two studies have recorded CASC5 mRNA expression in primary human tumors, cancer cell lines including three UBC cell lines (*15,16*). In contrast, our study characterized frequent expression of both CASC5 mRNA and protein expression in large number of bladder tumors irrespective of their stages and grades. This aspect of our study is a crucial step towards characterization of new CT genes as a diagnostic biomarker as well as potential target for UBC specific immunotherapy.

Under qRT-PCR, CASC5 mRNA over expression was recorded in bladder tumor tissue, testis and a very low level in placenta tissue and obtained results are in line with previous report (*15*). Furthermore, heterogenous expression of CASC5 protein was also seen in clinical tissue specimens of TCC patients, but, no signified expression was noted among BPH tissues. These attributes propose that CASC5 could potentially be a transcriptional factor which may translocate between nucleus and cytoplasm. Therefore unsurprisingly, heterogenous expression pattern of CASC5 protein was observed in CaUB. These results are undeviating with past findings that observed heterogenous expression pattern of various other CT genes in breast cancer and UBC (*28,29*). Thus in present study not only CASC5 mRNA expression is noticeable, also protein expression which is derived from IHC analysis of CASC5 in UBC of non-muscle-invasive and invasive nature. In this study, the overall frequency of CASC5 mRNA/protein expression was observed in 59.2% / 67.7% tissue specimens using qRT-PCR and IHC. Our present result originated by qRT-PCR and IHC are in agreement with the past studies that investigated CASC5 expression on smaller cohorts of tumors by RT-PCR and microarray, which reported expression of the transcript in 36-89% of tumors respectively (*15,30*).

In our study CASC5 mRNA was not found to be significantly correlated with cigarette smoking habit of the UBC patients; however, higher relative mean fold expression was observed in tumors derived from patients with smoking habit than the ones from non-smokers patients. Furthermore, IHC expression of CASC5 was significantly related with the smoking habit in non-muscle-invasive and muscle-invasive UBC patients. Thus our IHC findings were consistent with those of the previous study that CASC5 is the first CT gene, the expression of which is significantly related to smoking habits of the cancer patients. Thus CASC5 mRNA and protein expression pattern, which is investigated through qRT-PCR and IHC, may have an important role at the transcription level and translational events of CaUB and may have an important function in molecular initiation of UBC.

Our research study had a few limitations. First, relatively less number of bladder tumor tissue specimens was used to assess mRNA and protein expression of CASC5 in UBC. Furthermore, mRNA and IHC expression of CASC5 was analyzed on different bladder tumor tissues collected from two independent cohorts of UBC patients. Moreover, smaller number of UBC patients did not granted to perform sub analyses.

Our present study concludes upregulated mRNA and protein expression of CASC5 in CaUB, which demonstrates that CASC5 is a potential CT gene in UBC. Our study further proposes that it could be a productive target for peptide vaccines development specifically in CaUB. However, larger prospective studies are required to validate these results before these antigens can be proven for peptide vaccine mediated immunotherapy.

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Communication

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Telepharmacy in mountainous depopulated areas of Japan: An exploratory interview study of patients' perspectives

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SUMMARY Japan has an ageing population and geographical impediments to healthcare access, so an experimental trial of telepharmacy has recently been implemented in remote islands or remote areas of Japan prior to the formal implementation. This exploratory study was conducted to understand patients' perspectives on telepharmacy in a mountainous depopulated area away from urban areas of Japan. Semi-structured interviews were conducted with four elderly patients, who were all of the patients receiving telepharmacy in Toyone village, Japan, at the time of the survey. The transcribed interview data were qualitatively analyzed by coding and categorization. The subjects thought telepharmacy would be advantageous to overcome poor access to a clinic and to improve convenience in processes ranging from medical examination to obtaining prescribed medicines. However, they pointed out the low digital literacy of the elderly. Also, they had low expectations for pharmacists, because they had previously had no relationship with pharmacists due to lack of pharmacies in the area. To promote telepharmacy, efforts to eliminate resistance to smartphones and to provide support for smartphone operations are needed among the elderly. Work is also needed to establish how pharmacists should best be involved in patient care and health support in remote areas. Our findings suggest that telepharmacy is useful in remote areas of Japan, but in locations where there is no existing relationship with pharmacists, it would be desirable for pharmacists to be actively involved with the community to maximize its effectiveness.

Keywords telemedicine, telepharmacy, elderly, mountainous depopulated area

Japan has an ageing population as well as geographical impediments to healthcare access, such as mountainous depopulated areas remote from urban areas. Telemedicine by doctors has been used as one approach to solve such problems in Japan, in common with other countries. Furthermore, telemedicine in areas other than rural and remote areas has been permitted since 2015, and insurance coverage of telemedicine was started in 2018 in Japan (*1*). In contrast, telepharmacy by pharmacists was not allowed until recently, since pharmacists' medication instructions were legally required to be given face-to-face based on the Act on Securing Quality, Efficacy and Safety of Products Including Pharmaceuticals and Medical Devices (PMD Act) in Japan. With the amendment of the law, telepharmacy finally became available in September 2020.

Before formal introduction of telepharmacy, it was first trialed for residents of remote islands or remote areas, and for those who use telemedicine in the National Strategic Special Zones (*2*). Three regions of the National Strategic Special Zones, Aichi Prefecture, Yabu City in Hyogo Prefecture, and Fukuoka City in Fukuoka Prefecture, became the first regions in Japan to provide telepharmacy. Various clinical benefits of telepharmacy, such as improved access to healthcare (*3*), economic benefits (*4*), improved patient satisfaction (*5*), and effective patient counselling (*6*) have already been reported (*7*) in other countries. Therefore, the purpose of this study was to understand patients' perspectives on telepharmacy in a mountainous area remote from urban areas, and to obtain basic data for improving the practice and dissemination of telepharmacy in Japan.

Exploratory semi-structured interviews with patients who received telepharmacy service in Toyone Village, Aichi Prefecture were conducted in October 2019 by an interviewer and a sub-interviewer who specialize in pharmacy, based on an interview guide consisting of four open-ended questions. The survey items included

1) background of starting telepharmacy, 2) recent experience of telepharmacy, 3) opinions regarding the advantages and disadvantages of telepharmacy, and 4) opinions regarding the future of telepharmacy. The interviews were audio-recorded with the permission of the participants. After transcribing the whole interview contents, we separated the subjects' remarks by meaning, and assigned codes to remarks expressing facilitators and barriers to telepharmacy service (coding). Next, we integrated codes that conceptually have the same meaning and named them as categories (categorization). Coding and categorization were repeated and appropriate changes and corrections were made during the analysis process based on discussions among the authors. The interviews and analysis were all conducted in Japanese. All methods were carried out in accordance with the Declaration of Helsinki. This study was approved by the Research Ethics Review Committee, Keio University Faculty of Pharmacy (No.191003-1). Informed consent for study participation was obtained from all subjects.

The participants were four patients with chronic diseases (a female in the 60s, two males in the 60s, a female in the 90s) who were receiving telemedicine and telepharmacy services in Toyone village. They were all of the patients in the village who had received telepharmacy service at the time of the survey.

Three main categories of facilitators of telemedicine and telepharmacy were identified based on subjects' statements (Table 1). As facilitators, subjects stated that telemedicine and telepharmacy contributed to improving accessibility for both medical staff and patients in an area where access to medical institutions is poor (Table 1-A). They pointed out that residents who live far from the clinic in the village would have difficulty going there, since there is little public transportation in this area. They felt that telemedicine and telepharmacy would make up for poor transportation in the area and reduce the burden of regularly going to distant medical institutions. The doctor in the village clinic travels about 40 minutes by car to another clinic in a neighboring village for an hour of medical examination every Tuesday. One subject expected that telemedicine would reduce the doctor's time and effort required for this. Some patients who have chronic diseases such as hypertension and dyslipidemia, and whose symptoms are stable, felt that telemedicine and telepharmacy services would allow them to obtain medicines while at home without going to the clinic. Subjects were aware of the advantage that the time required from examination to obtaining medicines could be shortened (Table 1-B). Some subjects felt that they did not have time to visit the clinic because they worked during the day. Furthermore, the clinic in the study area was open only on weekday mornings. It seems to be a great advantage for such patients that they can receive examinations wherever they are, at home or at work, and do not have to worry about running out of medicine (Table 1-C).

Three main categories of barriers to telemedicine and telepharmacy were identified based on subjects' statements (Table 2). Of the four subjects, three in their 60s, who usually use smartphones, did not feel any particular difficulty or inconvenience regarding smartphone use for communication. However, they were worried that older generations than theirs would be unfamiliar with smartphones and find them challenging to use (Table 2-A). In fact, the subject in her 90s said that she could manage to operate the tablet device if instructed by her family or care staff, but she could not operate it herself. Since many elderly people live alone in the study area, she speculated that some might find it difficult to use telemedicine and telepharmacy. To overcome this, it would be desirable to provide opportunities for the elderly to learn how to handle devices such as smartphones and tablet terminals and to familiarize them with the devices. Besides, the subjects pointed out telecommunication system issues (Table 2-B), such as "telecommunication status is sometimes bad" and "the voice is sometimes hard to hear". At the time of the study, telepharmacy was operated only within the National Strategic Special Zone, and some

Table 1. Facilitators of telemedicine and telepharmacy for patients

Category	Code
A).Improving accessibility for remote area residents to a village clinic	• Reducing the burden of going to a distant village clinic regularly. • Making it easier to have an examination for the elderly visiting a distant village clinic. • Making up for poor transportation in the area of residence. • Unnecessary for doctors to go to the neighboring village for examination.
B).Improving convenience from taking a medical examination to obtaining prescribed medicines	• Availability of prescribed medicines at home. • Reducing the time required from taking a medical examination to obtaining prescribed medicines.
C).Possibility of balancing work and hospital visits	• Possibility of seeing a doctor anywhere, such as at home or at work. • Reducing the burden of having time to obtain medicines during work. • Reducing worry about running out of prescribed medicines due to being unable to visit a village clinic. • No need to visit a village clinic in time for its limited opening hours.

Table 2. Barriers to telemedicine and telepharmacy for patients

Category	Code
A).Low digital literacy for the elderly	• Difficulty in operating devices such as smartphones and tablets. • Limitation of operating devices by themselves without help. • Unfamiliar with devices.
B).Immature telecommunication system	• Poor transmission status in mountainous areas. • Difficult to hear the voice.
C).Issues for the implementation phase	• Need for development of new rules or regulations. • No established delivery system of prescribed medicines. • Inconvenience of not being able to receive telemedicine and telepharmacy on their own devices. • Much labor and time needed for receiving instructions and preparation prior to telemedicine and telepharmacy.

issues for the implementation phase were also pointed out (Table 2-C).

As for communication with doctors or pharmacists online, the patients felt that they could communicate as smoothly as in face-to-face communication. However, they were also concerned about whether telemedicine and telepharmacy could provide the same level of care as face-to-face contact. Moreover, depending on their age and symptoms, some patients preferred face-to-face examination. They believed that they would be more reassured if they were examined in person and that face-to-face talking with a doctor was an important part of treatment for the elderly.

In the study area, patients receive medicine at the clinic after examination and receive explanations about the medicine from the doctor or nurses, and so they had never communicated with a pharmacist. The lack of opportunities to contact pharmacists seemed to be directly linked to their low awareness and low expectations for pharmacists. One participant felt that it was bothersome to tell the pharmacist what he/she had already told the doctor. One subject with experience of prescribing errors showed some understanding of the importance of medication instruction. One subject thought that many patients, including herself, often do not understand much about the medicines they are taking, and that it was good to have opportunities to ask pharmacists to confirm that their drugs were suitable.

There had been little contact between patients and pharmacists so far in the study area. Therefore, the subjects' awareness of pharmacists and their function was low. No one had any experience of consulting with a pharmacist. It may not be easy to give meaningful medication instructions in such a situation. To make the most of telepharmacy in regions where there is no pharmacy nearby, it may be necessary to educate patients about pharmacist's abilities. It would probably be helpful for pharmacists at urban pharmacies in charge of telepharmacy to visit the relevant areas regularly to carry out health support activities and communicate with residents.

As mentioned above, in the area surveyed, there was no pharmacy nearby, and this is not uncommon in underpopulated areas. Therefore, patients' unfamiliarity with medication counselling by pharmacists may have affected their perspectives. This is a limitation of the study.

In Japan, telepharmacy in urban areas will be expanded following an amendment to the PMD Act in September 2020. The COVID-19 pandemic has also greatly affected the implementation of telepharmacy in Japan. As an extraordinary and temporary measure during the COVID-19 pandemic, remote medical counselling by pharmacists, including by telephone, has also been permitted since April 2020, before the official introduction in Japan. It will be important to identify the differences between patients' perspectives of telepharmacy in mountainous areas and urban areas to improve the delivery of telepharmacy in Japan.

This exploratory interview survey extracted patients' perspectives on telepharmacy in a remote mountainous area, where there is no pharmacy nearby. The results suggest telepharmacy is useful in areas with poor medical resources and offers improved convenience in receiving care, but it would be desirable for pharmacists to be more actively involved with the community to increase its effectiveness.

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Conflict of Interest: Kyowa Chemical Co., Ltd. runs a pharmacy that conducted telepharmacy in the present study. IY and Nasu T are executive officers, Nagai T is an employee, MK is CEO/COO of Kyowa Chemical Co., Ltd., respectively. Minacolor Inc. provided a system for telepharmacy. NS and KS are an employee and a representative director of Minacolor Inc. The authors report no other conflicts of interest in this work.

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Letter

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Entomophthoramycosis: An unusual cause of facial disfigurement

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SUMMARY Entomophthoramycosis is a rare fungal infection of nose, paranasal sinuses and subcutaneous tissues found in tropical and subtropical region. From India very few cases have been reported. Here we report a case of Entomophthoramycosis due to *Conidiobolus coronatus* from the eastern India who presented with slowly growing rhinofacial swelling and right sided nasal obstruction due to intranasal mass. The case was diagnosed by typical histopathological findings of broad aseptate hyphae with surrounding eosinophilic granular material (Splendore Hoeppli phenomenon) on microscopy of nasal biopsy material and confirmed by PCR assay of DNA and sequencing from biopsy tissue. Treatment with saturated solution of potassium iodide and itraconazole was successful and clinical cure was attained in 8 months.

Keywords Entomophthoramycosis, *Conidiobolus coronatus*, Splendore Hoeppli phenomenon

Entomophthoramycosis is a rare fungal infection of tropical and subtropical region. The disease usually affects adult males involved in agricultural works (*1*). It is caused by the fungi of the order Entomophthorales under the class Zygomycetes. However, unlike order Mucorales (another zygomycosis), entomophthoramycosis occurs predominantly in immunocompetent patients, is non angioinvasive and has chronic course (*2*). It has two genera, *Conidiobolus* and *Basidiobolus*. The former usually involves the rhinofacial area whereas the latter mostly involves the subcutaneous structures of trunk, arms or the gastrointestinal tract (*1*). Here we present a case of rhinofacial entomophthoramycosis caused by *Conidiobolus coronatus* in an immunocompetent host from Eastern India.

A 55-year old man, farmer, resident of West Bengal, presented with gradually increasing swelling of nose and face and right sided nasal obstruction for last three months. Initially there was a small mass inside the right nasal cavity which gradually increases in size causing nasal obstruction. After few days there was gradually increasing painless swelling of the dorsum of the nose, forehead, bilateral cheeks and the upper lip. It caused significant disfigurement of the face (Figure 1). He also complained of two episodes of epistaxis. He did not give any history of trauma. He consulted several doctors but without any improvement he came to our outpatient department. On examination the rhinofacial swelling was firm to hard in consistency. There was mild tenderness over both the maxillary sinuses. All other physical examinations were within normal limits. Routine blood examinations like complete blood count, liver and kidney function tests, fasting blood sugar, HbA1c, HIV showed no abnormality. Computed tomography of paranasal sinus showed polypoidal mucosal thickening in maxillary, ethmoid, sphenoid and frontal sinuses. Bilateral turbinates were hypertrophied. Diffuse soft tissue thickening was noted in right nasolabial area and over maxilla. Magnetic resonance imaging also confirmed these findings. It also showed an enhancing altered signal intensity lesion in mid portion and adjacent bilateral fronto-naso-ethmoidal-maxillary areas and upper lip, mildly extending into the nasal cavities. Endoscopic examination revealed polypoid growth in the right nasal cavity. Histopathology of the biopsy material showed epitheloid granulomas with foreign body giant cells in haematoxylin eosin (H&E) stain (Figure 2A). Broad thin walled aseptate fungal hyphae were also seen by Periodic Acid Schiff stain (Figures 3A and 3B). Each hyphal filament was enveloped by eosinophilic granular material known as Splendore Hoeppli phenomenon (Figure 2B). No vascular involvement was noted. Though culture of the biopsy material in Sabouraud's dextrose agar media yielded no growth but based on the clinical presentation and typical histopathological findings we suspected the case as entomophthoramycosis probably caused by *Conidiobolus*. Later PCR assay of DNA from biopsy tissue and sequencing identified the

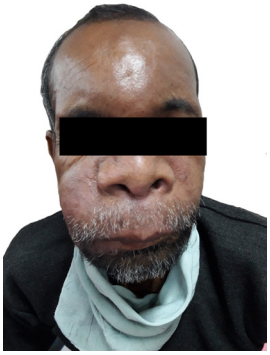

Figure 1. Rhinofacial lesion at the time of presentation.

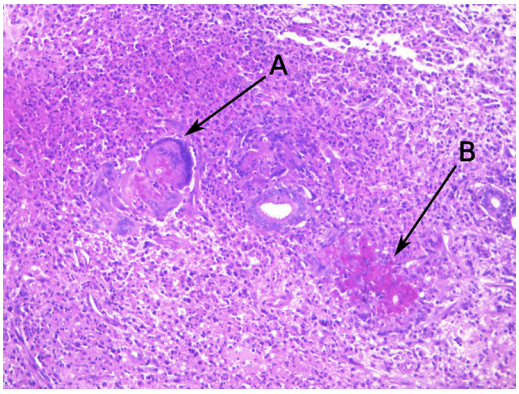

Figure 2. Nasal biopsy Histopathology stained by Haematoxylin-Eosin Stain with 20X magnification. A: Epitheloid granuloma with giant cell reaction; **B:** fungal filament enveloped by eosinophilic Splendore Hoeppli phenomenon.

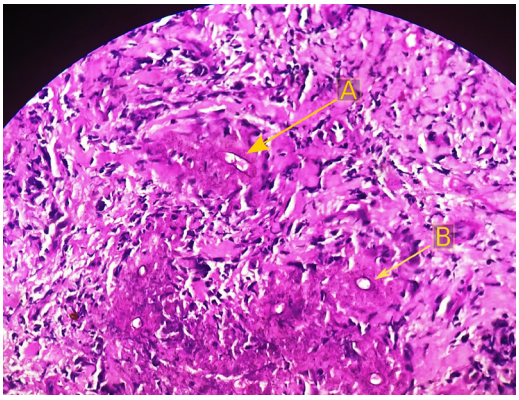

Figure 3. Nasal biopsy histopathology stained by Periodic Acid Schiff stain with 40X magnification showing fungal filaments.

fungus as *Conidiobolus coronatus*. We started treatment with itraconazole 200 mg per capsule, one capsule thrice daily for 3 days then continued as twice daily dose and saturated solution of potassium iodide (SSKI) at a dose of 5 drops thrice daily. It was gradually increased up to 30 drops thrice daily. Each drop of freshly prepared SSKI contains approximately 65 mg of potassium iodide. After one month the facial swelling reduced significantly and consistency became softer. After 8 months of therapy the lesions were dramatically improved.

Entomophthoramycosis is a chronic granulomatous subcutaneous infection which is acquired by inhalation or minor trauma. Conidiobolomycosis is mainly caused by *Conidiobolus coronatus* whereas Basidiobolomycosis is caused by *Basidiobolus ranarum* (*3*). Conidiobolomycosis usually presents with unilateral nasal obstruction, nasal discharge, epistaxis, sinus tenderness and extensive facial swelling resulting in facial disfigurement. Conidiobolomycosis is diagnosed by characteristic rhinofacial swelling and typical histopathological findings of broad, aseptate or sparsely septated fungal hyphae surrounded by eosinophilic granular material known as Splendore Hoeppli phenomenon on microscopy of biopsy and confirmed by PCR assay and sequencing of DNA from biopsy tissue (*3*). Often culture is negative. During microscopy entomophthoramycosis must be differentiated from mucormycosis (*4*). Splendore Hoeppli phenomenon is very common in entomophthoramycosis but it is uncommon in mucormycosis. Vascular involvement is characteristic of mucormycosis whereas vessels are spared in entomophthoramycosis. Entomophthoramycosis occurs in immunocompetent individuals and has slow clinical course, whereas mucormycosis is seen in immunocompromised patients and has very rapid, aggressive course. Treatment options for entomophthoramycosis include SSKI, cotrimoxazole, amphotericin B and azole group of antifungals with varying clinical outcome and success. Currently combination of a SSKI and itraconazole appears to be the preferred drugs for rhinofacial conidiobolomycosis (*5*). The first case of entomophthoramycosis in humans was reported in 1965 (*6*). A review article on *Conidiobolus* showed that infection usually starts in the nasal mucosa of the inferior turbinate, then it gradually progresses to involve the dorsum of nose, forehead, cheeks and upper lip (*7*). The appearance of the patient has often been described as tapir or hippopotamus. Entomophthoramycosis is a rare disease and very few cases were reported from India (*8,9*).

As cases of entomophthoramycosis are very rare it is easy to misdiagnosis a case. A very high index of clinical suspicion is essential to correctly diagnose a case of entomophthoramycosis in our clinical practice.

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

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