**Structure-based discovery and development of natural products as Type II JAK2 inhibitors for the treatment of hepatitis C**

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**KEY MESSAGES**

1. Hepatitis C is a highly infectious disease that imposes a high health, social and financial burden on the population in Hong Kong and worldwide.

2. A new platform for the virtual screening of JAK2 Type II inhibitors was constructed and utilised to identify amentoflavone as a lead scaffold for the development of new inhibitors.

3. Novel natural inhibitors were developed and showed anti-JAK2 activity and anti-viral activity in cellular systems.

4. The compounds potentially represent a novel therapeutic approach to the treatment of hepatitis C, and may supplement existing regimens for hard-to-treat HCV genotypes in Hong Kong.

**Introduction**

Hepatitis C virus (HCV) can cause a highly infectious liver disease. Intravenous drug users, transfusion-dependent children with haematological disease, HIV-positive patients, and hepatocellular carcinoma patients are at high risk of HCV infection, with an associated incidence of 46.0%, 16.3%, 7.9%, and 7.3%, respectively.1 Worldwide, HCV infects 3 000 000 to 4 000 000 people each year and has been estimated to infect 200 million people, or 3% of the global population.

Chronic HCV disease can result in liver fibrosis and cirrhosis and finally liver damage and/or liver cancer, life-threatening oesophageal varices, and gastric varices. Existing treatment of chronic HCV disease involves a combination of pegylated interferon-alpha-2a or pegylated interferon-alpha-2b (also known as peginterferon), and ribavirin.2 Around 50% of HCV genotype 1 patients, which accounts for about 80% and 60% of HCV patients in the US and in Hong Kong, respectively, do not respond to this therapy.2,3

Activation of STAT-3 by HCV non-structural proteins results in constitutive activation of STAT-3 in HCV-replicon-expressing cells. STAT-3 activity and HCV RNA production significantly decrease following treatment of HCV-infected cells with Janus kinase 2 (JAK2) inhibitor AG490.4 This demonstrates that JAK2 inhibitors are useful for inhibition of HCV translation and replication, potentially supplementing existing treatment of HCV. Although many potent JAK2 inhibitors have been developed as possible anticancer agents, none of them has been certified by the US Food and Drug Administration.

Type II inhibitors, including imatinib (Gleevec) and soralafenib, target kinases in the inactive conformation. As the inactive forms of kinases display a wide structural and chemical heterogeneity, Type II inhibitors have the potential to achieve greater selectivity for target kinases. This project proposed to select small molecules as Type II JAK2 inhibitors from databases of natural products using ligand docking-based virtual screening methods, as a new therapeutic avenue to combat HCV infection. Ligands with high efficacy and selectivity were rapidly identified using a computer-aided approach that reduced the number of compounds for in vitro evaluation.

**Methods**

This study was conducted from December 2011 to November 2013.

**Molecular docking and virtual screening**

We used the deletion-of-loop Asp-Phe-Gly-in (DOLPHIN) protocol5 to change an active ‘DFG-in’ structure of JAK2 (Protein Data Bank code: 2B7A) into a Type II-compatible conformation that was capable of molecular docking. We conducted in silico screening of 150 000 natural products and natural product-like compounds on the DOLPHIN kinase model using the internal coordinate mechanics (ICM) method [ICM-Pro 3.6-1d molecular docking software (Molsoft)] to select natural product scaffolds as Type II JAK2 inhibitors. Molecular docking was conducted using the virtual library screening (VLS) module in the Molsoft programme.
Quantification of HCV RNA

The HCV genotype 1b subgenomic replicon cell line with a luciferase reporter (Huh-luc.neo-ET) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1 mM non-essential amino acids, and 250 µg/ml G418 (Invitrogen). The cells were seeded at a density of 1×10⁵ cells per well in a 6-well plate with the treatment of the candidate compounds or DMSO. Cells were harvested after incubation for 72 hours and subjected to RNA quantification to evaluate the effect of the candidate compounds on viral replication.

Results

Construction of virtual screening platform for the discovery of Type II JAK2 inhibitors using the DOLPHIN protocol

At the onset, no X-ray crystal structure of JAK2 in an inactive conformation could be used for virtual screening. We successfully changed a DFG-in structure of JAK2 into a Type II-compatible conformation that could be used for molecular docking through the DOLPHIN algorithm. The molecular model of JAK2 obtained after the DOLPHIN transformation is displayed in Fig 1.

High-throughput virtual screening of a natural product and natural product-like database

To identify useful natural product scaffolds as Type II inhibitors of JAK2, we conducted in silico screening of natural products and natural product-like databases using the JAK2 model obtained by DOLPHIN through the ICM method in parallel with the molecular dynamics-based Accelrys Discovery Studio 3.0. During the screening campaign, 150,000 natural products or natural product-like compounds were screened against our molecular model of inactive form of JAK2 obtained by the DOLPHIN algorithm, the compounds of which were from the Analyticon Discovery NATx and MEGabolite databases, the ZINC natural product database and the Hongcam natural products database. The top ten highest-scoring compounds were evaluated in an ELISA in order to determine their ability to inhibit JAK2 phosphorylation in vitro. From these experiments, amentoflavone 1a, a biflavonoid from the Chinese plant Gingko biloba, was chosen as a promising candidate for further biological investigation.

In silico design and synthesis of amentoflavone derivatives

In our preliminary molecular modelling analysis, the deletion of the DFG loop in the DOLPHIN model of JAK2 kinase showed a large hydrophobic pocket, not present in the active DFG-in form of the kinase that was partially occupied by amentoflavone 1a. We anticipated that the presence of one or more aliphatic side chains to the biflavonoid scaffold of 1a would lead the molecule to occupy the hydrophobic pocket vacated by the DFG loop, thus producing more potent and selective inhibitors of JAK2. According to this result, we designed >50 derivatives of amentoflavone 1a and docked these compounds against the DFG-out model of JAK2 kinase in silico. We found that analogues 1b-j with long, alkyl chains emerged as top-scoring compounds. We synthesised the compounds 1b-j in three steps from amentoflavone 1a using a protection-transetherification-deprotection sequence (Fig 2). These derivatives included both monoalkylated (1b-e, 1h, 1i) and dialkylated (1f, 1g) analogues,
FIG 2. Synthetic procedure for the preparation of top-scoring compounds (Reproduced with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry)

FIG 3. HEL cells were incubated with the compounds for 16 hours and protein lysates were electrophoresed and immunoblotted using anti-phospho-JAK2 Y1007/Y1008 antibody or anti-JAK2 antibody. Equal protein loading was confirmed by total β-actin levels. Representative gel photograph images of triplicate experiments are shown. Western blot analysis of the effect of compounds 1a, 1c, 1g and JAK2 Inhibitor II on JAK2 autophosphorylation in cellulo. Estimated IC50 values: 1c=2.5 μM and 1g=5 μM. (Reproduced with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry)
as well as an alkenyl analogue (1j). These novel derivatives were fully characterised by 1H and 13C NMR spectroscopy and high-resolution mass spectrometry.

**JAK2 inhibitory activity of top-scoring candidates in cellulo**

We next evaluated the effect of the amentoflavone derivatives 1a-j on JAK2 autophosphorylation using a Western blot assay in human erythroleukaemia cells (HEL), which harbour a V617F mutation in JAK2 leading to constitutive JAK2 activity. Among the ten compounds tested, significant inhibition of JAK2 autophosphorylation was observed for the monoalkylated analogue and 1c and the dialkylated analogue 1g, with estimated IC50 values of 2.5 and 5 μM (Fig 3). The potencies observed for 1c and 1g were comparable with those of the positive control compound JAK2 inhibitor (1,2,3,4,5,6-hexabromocyclohexane). Encouragingly, these two compounds exhibited superior efficacy against JAK2 autophosphorylation in comparison with the parent compound amentoflavone 1a, demonstrating the application of our structure-based techniques to develop more potent JAK2 inhibitors.

**Molecular modelling analysis of candidate compounds**

Our molecular modelling results showed that 1c was situated snugly in the binding pocket of the inactive form of JAK2 DOPHLIN model. The amentoflavone moiety was located at the ATP binding pocket, whereas the alky1 side chain protruded inward into the hydrophobic pocket beside the ATP binding site. The ICM binding score for 1c to the inactive conformation of JAK2 was calculated to be –38.46 kcal/mol, showing the strong binding interaction between 1c and the binding site. Moreover, the interaction between 1c and the active conformation of JAK2 was relatively weak (–13.36 kcal/mol). These results indicate that compound 1c may be used as a potential JAK2 Type II inhibitor.

**Anti-viral activity of the candidate compounds**

We then tested the HCV anti-viral activity of selected amentoflavone analogues in the Huh-Luc/neo-ET cell line. The results demonstrated that compound 1c led to a decrease in the HCV RNA level, with an EC50 value of 3.1±0.8 μM. Notably, 1c was also the most potent compound in the JAK2 autophosphorylation assay. We anticipated that the inhibition of JAK2 signalling in the infected cells was at least in part due to the HCV anti-viral activity of compound 1c, thus causing reduced STAT-3 activity and HCV RNA production.

**Cytotoxicity evaluation of candidate compounds**

The cytotoxicity of the amentoflavone analogues against HEL cells was determined by MTT assay. Most of the amentoflavone analogues were relatively non-toxic. Importantly, derivatives 1c and 1g showed the greatest activity against JAK2 autophosphorylation, and displayed IC50 values of >100 μM. This suggests that their ability to inhibit JAK2 and HCV viral activity, and thus potentially HCV viral replication, may be achieved at concentrations that are non-toxic to normal cells. This property is significant if these amentoflavone derivatives are to be further developed as therapeutic agents for the treatment of hepatitis C, as adverse side effects may be minimised.

**Discussion**

To date, no protective vaccine for HCV is available. Current standard therapy for HCV infection using pegylated interferon alpha combined with ribavirin shows some side effects, such as fatigue, flu-like symptoms, and gastrointestinal symptoms, and results in a sustained virological response in only a small number of patients. Moreover, HCV genotype 1 infection, which accounts for the majority of HCV infection in Hong Kong, is resistant to conventional therapy. In recent years, combination therapy of boceprevir or telaprevir with pegylated interferon and ribavirin has shown to improve virological response rates, but anaemia and drug-drug interactions are potentially treatment-limiting adverse effects. The development of alternative HCV inhibitors is needed.

The HCV life cycle is closely related to host cell factors, and identification of inhibitors of such host factors (such as JAK2) offers an alternative approach to the development of anti-HCV compounds. A potential advantage of targeting host proteins such as JAK2 for the treatment of HCV is that the therapy would be less affected by drug resistance arising from the mutation of viral proteins.

In this project, we successfully applied the DOLHPIN protocol to convert the X-ray co-crystal structure of JAK2 in the active conformation into an inactive conformation, and screened 150 000 compounds from a natural product and natural product-like library using this model. From the result of in vitro testing assay of the initial hit-list, amentoflavone 1a was selected as a promising candidate for further optimisation. After screening over 50 rationally-designed derivatives of amentoflavone 1 insilico, high-scoring amentoflavone derivatives 1b-j were synthesised in three steps using efficient organic chemistry techniques. In a cellular assay, derivatives 1c and 1g showed potent activity against JAK2 autophosphorylation. Furthermore, compound 1c displayed anti-viral activity in a HCV...
replicon cell line. Molecular modelling analysis indicated that the compounds may be used as Type II inhibitors of JAK2. We envisage that these non-toxic JAK2 inhibitors have significant potential to be further developed as JAK2 inhibitors for the treatment of hepatitis C.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#11101212).

Results of this study have been published in Ma DL, Chan DS, Wei G, et al. Virtual screening and optimization of Type II inhibitors of JAK2 from a natural product library. Chem Commun (Camb) 2014;50:13885-8.

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