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

EDL Ma *, CH Leung, P Chiu, YC Cheng

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.

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EDL Ma, CH Leung, P Chiu, YC Cheng

Department of Chemistry, Hong Kong Baptist University, Hong Kong

* Principal applicant and corresponding author: edmondma@hkbu.edu.hk

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 sorafenib, 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 (DOLHPIN) protocol[5] 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 1×10^5 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 Acceryls 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,

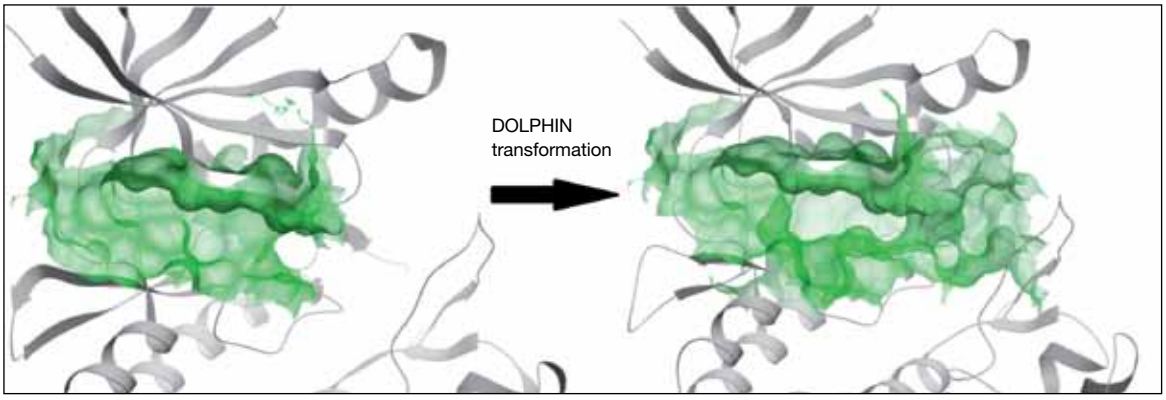

FIG 1. The molecular model of the inactive form of JAK2 generated using DOLPHIN: The active conformation of JAK2 (left) is converted to the inactive form (right) by removing DFG Phe and the next four residues in the sequence. The binding pocket is shown as a translucent green surface. (Reproduced with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry)

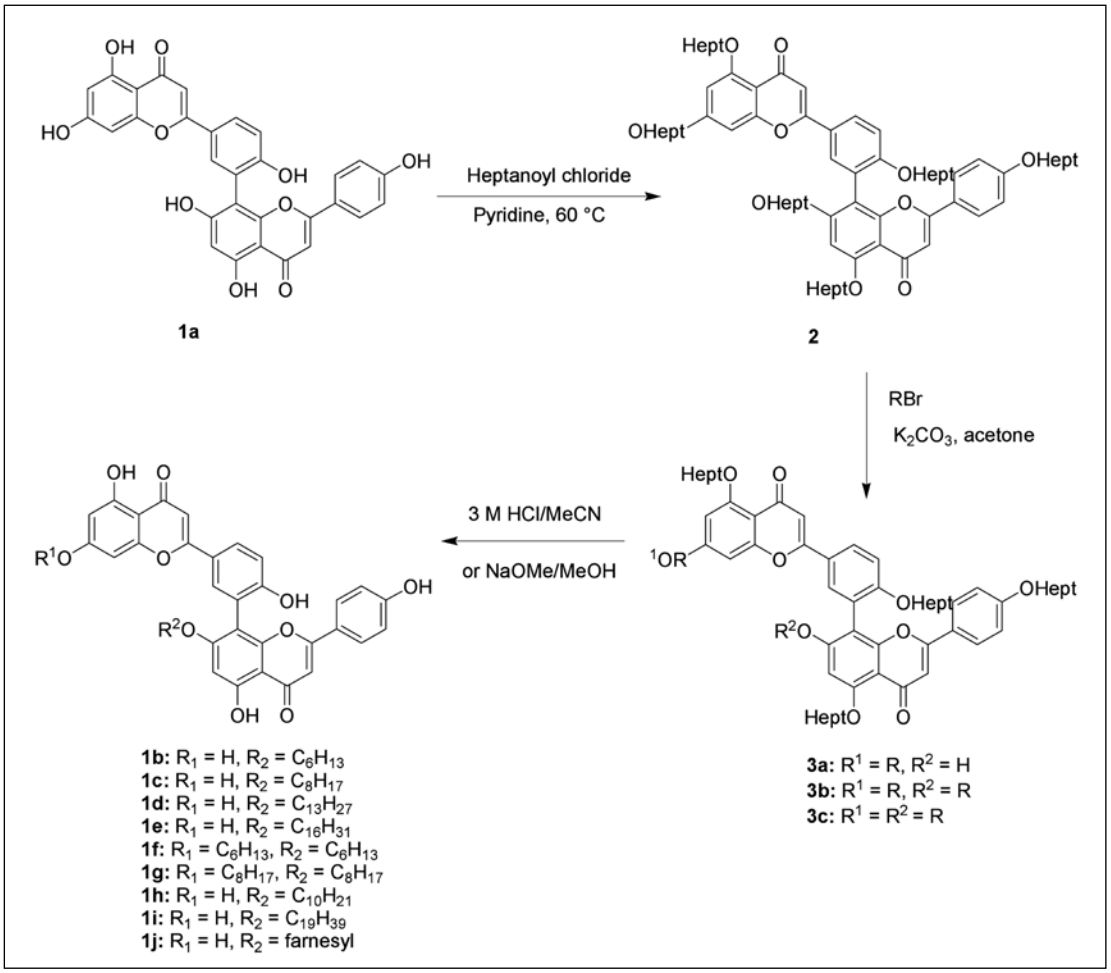

FIG 2. Synthetic procedure for the preparation of top-scoring compounds (Reproduced with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry)

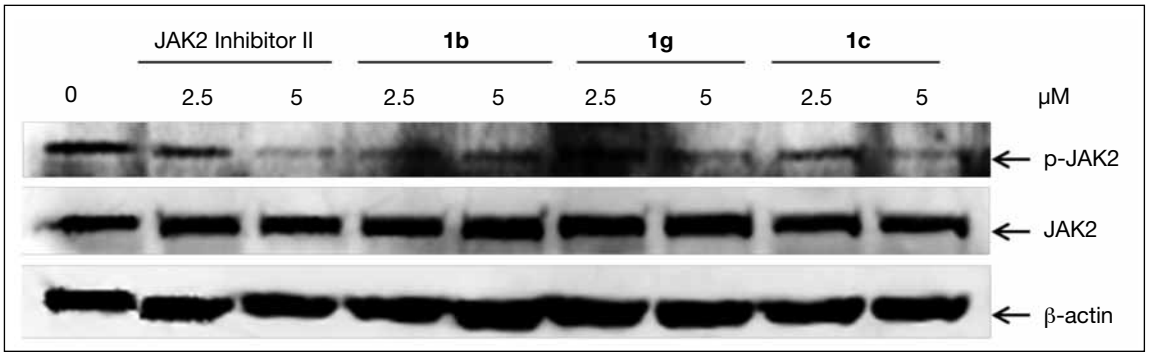

FIG 3. HEL cells were incubated with the compounds for 16 hours and protein lysates were electrophoresed and immunoblotted using anti-phospho-JAK2 Y1007/Y1008 antibody or anti-JAK2 antibody. Equal protein loading was confirmed by total β-actin levels. Representative gel photograph images of triplicate experiments are shown. Western blot analysis of the effect of compounds **1a**, **1c**, **1g** and JAK2 Inhibitor II on JAK2 autophosphorylation *in cellulo*. Estimated IC_{50} 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 ^{1}H and ^{13}C 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 IC_{50} 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 alkyl 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 EC_{50} 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 IC_{50} 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** *in silico*, 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

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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.

References

1. Leung N, Chu C, Tam JS. Viral hepatitis C in Hong Kong. Intervirology 2006;49:23-7.
2. Ferenci P. Peginterferon and ribavirin in HCV: improvement of sustained viral response. Best Pract Res Clin Gastroenterol 2008;22:1109-22.
3. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958-65.
4. Waris G, Turkson J, Hassanein T, Siddiqui A. Hepatitis C Virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. J Virol 2005;79:1569-80.
5. Kufareva I, Abagyan R. Type-II kinase inhibitor docking, screening, and profiling using modified structures of active kinase states. J Med Chem 2008;51:7921-32.