Targeting AMPK signalling pathway with natural medicines for atherosclerosis therapy: an integration of in silico screening and in vitro assay

Tiantong Ou¹, Xumin Hou¹, Shaofeng Guan, Jinjie Dai, Wenzheng Han, Ruogu Li, Wenxia Wang, Xinkai Qu* and Min Zhang*

Cardiology Department of Shanghai Chest Hospital, Shanghai Jiaotong University, 241 West Huaihai Road, Shanghai 200030, P.R. China

(Received 25 February 2015; final version received 10 May 2015)

An integration of virtual screening and kinase assay was reported to identify AMPK kinase inhibitors from various natural medicines.

The activation of AMP-activated protein kinase (AMPK) signalling pathway plays a central role in the pathologic progression of atherosclerosis (AS). Targeting the AMPK is thus considered as a potential therapeutics to attenuate AS. Here, we report the establishment of a synthetic pipeline that integrates in silico virtual screening and in vitro kinase assay to discover new lead compounds of AMPK inhibitors. The screening is performed against a large-size pool of structurally diverse natural products, from which a number of compounds are inferred as promising candidates, and few of them are further tested in vitro by using a standard kinase assay protocol to determine their inhibitory potency against AMPK. With this scheme we successfully identify five potent AMPK inhibitors with IC₅₀ values at micromolar level. We also examine the structural basis and molecular mechanism of nonbonded interaction network across the modelled complex interface of AMPK kinase domain with a newly identified natural medicine.

Keywords: AMP-activated protein kinase; natural product; inhibitor; atherosclerosis

1. Introduction

Atherosclerosis (AS), also known as arteriosclerotic vascular disease, is a specific form of AS in which an artery wall thickens as a result of invasion and accumulation of white blood cells (Ross 1993). The disease is the leading cause of death worldwide resulting in 17 million deaths per year, affecting men and women in developed and developing countries (Lusis 2000). Recent
in vivo studies showed that the AMP-activated protein kinase (AMPK) pathway and its essential effector, the AMPK kinase, plays an important role in AS, due to its lasting effects on invasion and accumulation of white blood cells, leading to vascular remodelling and artery wall thickening (Li et al. 2011). The identification of the AMPK signalling cascade as an important regulator of AS pathologic progression and the development of selective AMPK inhibitors such as BML-275 and compound C provided the opportunity to gain new insights into the chemotherapeutics of AS and its complications (Motoshima et al. 2006). Natural products are considered as an important source of kinase inhibitors, which exhibit low toxicity and high biocompatibility and thus have been widely used as medicinal candidates with respect to signal transduction pathways (Liu et al. 2012). In order to exploit new therapeutics for AS, we herein reported a successful application of combining in silico virtual screening and in vitro kinase assay to discover new natural medicines with high inhibitory capability against AMPK. The structure-based virtual screening and rational drug design have been widely used to discover new enzyme-targeting natural products and bioactive small-molecule compounds (Leung et al. 2011, 2012; Ma et al. 2013). We also demonstrated that the identified compounds can potently bind to the active site of AMPK kinase domain through a complicated network of nonbonded interactions.

2. Results and discussion

2.1. Molecular docking and high-throughput virtual screening

The natural product library contained 24,709 structurally diverse, drug-like compounds, which were extracted from a variety of publicly available databases. First, we employed rigid DOCK method (Meng et al. 1992) to pre-evaluate the binding strength of these compounds to AMPK. In the procedure, the complex crystal structure of AMPK kinase domain with a cocrystallised inhibitor compound C was retrieved from the PDB database (Berman et al. 2000) under the accessible code 3AQV. Interaction modes and intermolecular affinities of the 24,709 natural product molecules with AMPK kinase domain were one-by-one calculated using DOCK, from which the top-5000 compounds were selected to define a promising set of AMPK binding candidates. Subsequently, we used the more reliable (but time-consuming) Autodock algorithm (Goodsell & Olson 1990) to redock the 5000 natural product compounds with consideration of the structural flexibility of ligand molecules and AMPK active-site residues. The redocking also suggested that the preliminary 5000 natural product have relatively high binding potency toward AMPK, from which the top-1000 compounds were extracted in terms of Autodock score. Furthermore, we used a quantitative structure–activity relationship (QSAR)-based scoring function (Wang et al. 2014) to rescore interaction strengths of the top-1000 compounds with AMPK kinase domain, and the results are shown in Figure 1. The QSAR-based scoring method gives a direct prediction of dissociation constant pK_d for each of the top-1000 compounds in binding to AMPK, and the obtained values distribute from 5 to 8, indicating that these potential compounds selected by docking methods were also predicted as promising candidates using the independent QSAR-based scoring approach.

Next, from the top-1000 natural products we selected 10 compounds with QSAR score > 7 to test their inhibitory activity against AMPK using a standard kinase assay protocol. The protocol was modified from a previous report of Onyenwoke et al. (2012), which measured the concentration of compounds at which the AMPK’s enzymatic activity can be inhibited by 50% (i.e. IC_50 value). The ten selected natural products are baicalin, emodin, tetrahydropapaverine, balanol, gossypol, phloretin, cercosporamide, kinetin, hesperetin and hordenine; these compounds are originally from different sources, such as fungal, plant, fish and animal, and their structures are also diverse, ranging from flavone to diphenyl. Consequently, five compounds were determined to have high inhibitory potency against the AMPK (IC_50 <
10 μM), and other five are moderate (10 μM < IC₅₀ < 100 μM) or modest (IC₅₀ > 100 μM) AMPK inhibitors. Here, the five high-activity natural products are tabulated in Table 1, from which it is evident that these compounds are structurally diverse, although they can all bind effectively to AMPK active site. Interestingly, the emodin, balanol and cercosporamide have already been previously found as the good inhibitors of protein kinase CK2, G protein-coupled receptor kinase and Mnk kinase, respectively. Therefore, it is reasonable that these five high-activity compounds can also bind to and inhibit AMPK kinase potently, if considering that all kinases share high similarity in sequence, structure and function.

2.2. Structural and energetic analysis of AMPK–natural product interactions

In order to deeply understand molecular mechanism and energetic property of the binding of newly identified compounds to AMPK, we further performed atomistic molecular dynamics (MD) simulations and post molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) analysis to investigate the modelled complex structures of AMPK kinase domain with the five high-activity natural products (Zhou et al. 2013). The MD is exhaustive but can obtain accurate dynamics profile for biomolecular systems. Here, 10-ns simulations were imposed on each of the five complexes to generate trajectory files, from which the dynamics snapshots were extracted and used to carry out MM/PBSA calculations of the total binding free energy \( \Delta G_{\text{total}} \) of AMPK–natural product binding. Here, the \( \Delta G_{\text{total}} \) is computed as follows: \( \Delta G_{\text{total}} = \Delta E_{\text{int}} + \Delta G_{\text{slv}} \), where \( \Delta E_{\text{int}} \) is direct interactions (such as electrostatic and van der Waals) between the AMPK kinase domain and its natural product ligand, and calculated using molecular force field approach, while \( \Delta G_{\text{slv}} \) is solvent effect on the binding and treated with Poisson–Boltzmann surface area model. Consequently, the \( \Delta E_{\text{int}}, \Delta G_{\text{slv}} \) and \( \Delta G_{\text{total}} \) values of the five natural products binding to AMPK kinase domain were visualised in Figure 2. As can be seen, the direct interaction contributes significantly to the binding, with \( \Delta E_{\text{int}} \) at \( \approx -50 \) kcal/mol, whereas the solvent effect appears to be very unfavourable to the binding, with a large free energy penalty \( \Delta G_{\text{slv}} = \approx 40 \) kcal/mol. By comparing between the direct interactions \( \Delta E_{\text{int}} \) and indirect solvent effects \( \Delta G_{\text{slv}} \) of the five natural products it is found that the two energetic terms exhibit a good balance between them, that is, weak hydrophobicity is always associated with strong nonbonded force. In this way, direct interaction can be effectively counteracted by solvent effect.

As might be expected, two natural products with the highest inhibitory activities, i.e. balanol and emodin (IC₅₀ = 0.89 and 0.27 μM, respectively), were also predicted to exhibit strong
binding capability toward AMPK ($\Delta G_{\text{total}} = -16.2$ and $-18.4$ kcal/mol, respectively). However, these two compounds were found to have different mechanisms to obtain the high affinity. According to MM/PBSA analysis, the emodin is a small molecule composed of a large hydrophobic core (terphenyl) and few polar substituents (hydroxyl and carbonyl), which only causes low solvent penalty, albeit its direct nonbonded force is also weak when binding to AMPK. Instead, the large balanol molecule can form strong direct interaction with AMPK, but this effect would be largely cancelled by the significant solvent penalty.

Table 1. The five high-activity natural products.

| Natural product | Structure | Predicted pK_d | Experimental IC_50 (µM) |
|-----------------|-----------|----------------|-------------------------|
| Baicalin        | ![Baicalin Structure](image) | 7.56           | 5.6                     |
| Emodin          | ![Emodin Structure](image) | 7.14           | 0.89                    |
| Gossypol        | ![Gossypol Structure](image) | 7.71           | 7.8                     |
| Balanol         | ![Balanol Structure](image) | 7.64           | 0.27                    |
| Cercosporamide  | ![Cercosporamide Structure](image) | 7.42           | 3.2                     |
Next, the modelled complex structures of AMPK kinase domain with balanol and emodin were examined in detail. As can be seen from Figure 3, both compounds adopt a rational mode to interact with AMPK active site. The balanol molecule is relatively bulky which can thus only partially contact the active site. However, this molecule possesses many polar groups that can form an intensive hydrogen bonding network with the active-site residues Thr21, Lys31, Glu94, Val96, Ser97, Gly99, Glu100 and Asp157 of AMPK kinase domain, which is thought to confer substantial stability and specificity for the AMPK–balanol complex architecture. In contrast, the small emodin molecule is tightly bound within the active pocket of AMPK to constitute a number of effective hydrophobic and van der Waals contacts between them, although this system can only form a single hydrogen bond at complex interface. Therefore, it is suggested that the balanol appears to have higher selectivity for AMPK as compared to emodin, albeit both the two compounds exhibit strong binding capability toward the kinase.

3. Experimental

3.1. Natural product library

A total of more than 20,000 natural products were extracted from a variety of natural product databases. First, we carried out structural similarity analysis and drug likeness evaluation to define a structurally diverse, drug-like natural product library. This library includes more than 5000 compounds and is considered as candidate set that will be used in subsequent docking calculations.

3.2. Molecular docking

The native ligand compound C and water molecules were manually removed from the complex crystal structure of AMPK with compound C (PDB:3AQV), and the structure was further examined and repaired using WHATIF server (Vriend 1990). The active site of AMPK kinase was defined by the cocrystallised compound C. Molecular docking methods DOCK (Meng et al. 1992) and Autodock (Goodsell & Olson 1990) were then used to model the intermolecular interactions between the AMPK kinase domain and natural product candidates in the library. In the procedure, the rigid DOCK method was first applied to perform fast screening against the original library to

Figure 2. The direct interaction, indirect solvent effect and total binding free energy of the five high-activity natural products to AMPK.
obtain a preliminary set of AMPK binders, which were further re-examined with the flexibility AutoDock docking with respect to their binding capability toward AMPK active site.

### 3.3. QSAR-based scoring

A kinase-inhibitor affinity scoring method was used to evaluate the binding strength of natural products to AMPK (Wang et al. 2014). This method was developed from a number of kinase-inhibitor systems with known complex crystal structures and experimental binding affinity values through a QSAR approach, which can be readily used to predict the interaction strength of diverse kinases with various small-molecule ligands. In this study, the QSAR-based scoring was implemented based on the docked complex structures of AMPK kinase domain with natural products.

### 3.4. Kinase assay

Kinase assays were performed as previous description (Onyenwoke et al. 2012). Briefly, AMPK activity assays with GST-AMPK were performed at room temperature in a 25-μL reaction mixture containing 3–12 μg of protein in kinase buffer 50 mM HEPES, pH 7.0, 75 mM NaCl, 5 mM sodium acetate, 5 mM magnesium chloride, 1 mM dithiothreitol, 8% glycerol, 0.1 mM EDTA, 200 μM AMP and ATP and 2 μCi of [γ-32P]ATP. Compounds were assayed over
different concentrations to derive dose–response curves, where data were normalised and expressed as percentage inhibition, and then curve fitting was performed to determine inhibitory IC<sub>50</sub> value for each compound.

4. Conclusions
An integration of in silico screening and in vitro assay was purposed to identify AMPK kinase inhibitor from a large library of natural products with diverse structures, high drug-likeness and relatively low flexibility. The screening was performed against a structurally diverse, drug-like natural product library, from which several available compounds were tested to determine their inhibitory potencies against AMPK by using a standard kinase assay protocol. With this scheme we successfully identified two potent AMPK inhibitors balanol and emodin. Structural examination suggested that complicated network of nonbonded interactions such as hydrogen bonding, hydrophobic forces and van der Waals contacts across the complex interfaces of AMPK kinase with the screened compounds. This study would help to establish an integrative approach to rational kinase inhibitor discovery by exploiting numerous existing natural products.

Supplementary material
Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2015.1050672.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This work was supported by the National Natural Science Foundation of China [grant number 81370400]; and the Foundation of Shanghai Committee of Science and Technology [grant number 14JC1405600].

Note
1. Both these authors contributed equally to this work.

References
Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. 2000. The protein data bank. Nucleic Acids Res. 28:235–242. doi:10.1093/nar/28.1.235.
Goodsell DS, Olson AJ. 1990. Automated docking of substrates to proteins by simulated annealing. Proteins. 8:195–202. doi:10.1002/prot.340080302.
Leung CH, Chan DS, Yang H, Abagyan R, Lee SM, Zhu GY, Fong WF, Ma DL. 2011. A natural product-like inhibitor of NEDD8-activating enzyme. Chem Commun. 47:2511–2513. doi:10.1039/c0cc04927a.
Leung CH, Zhong HJ, Yang H, Cheng Z, Chan DS, Ma VP, Abagyan R, Wong CY, Ma DL. 2012. A metal-based inhibitor of tumor necrosis factor-α. Angew Chem Int Ed Engl. 51:9010–9014. doi:10.1002/anie.201202937.
Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, et al. 2011. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metab. 13:376–388. doi:10.1016/j.cmet.2011.03.009.
Liu J, Hu Y, Waller DL, Wang J, Liu Q. 2012. Natural products as kinase inhibitors. Nat Prod Rep. 29:392–403. doi:10.1039/c2np00097k.
Lusis AJ. 2000. Atherosclerosis. Nature. 407:233–241. doi:10.1038/35025203.
Ma DL, Chan DSH, Leung CH. 2013. Drug repositioning by structure-based virtual screening. Chem Soc Rev. 42:2130–2141. doi:10.1039/c2cs35357a.
Meng EC, Shoichet BK, Kuntz ID. 1992. Automated docking with grid-based energy evaluation. J Comp Chem. 13:505–524. doi:10.1002/jcc.540130412.
Motoshima H, Goldstein BJ, Igata M, Araki E. 2006. AMPK and cell proliferation – AMPK as a therapeutic target for atherosclerosis and cancer. J Physiol. 574: 63–71.

Onyenwoke RU, Forsberg LJ, Liu L, Williams T, Alzate O, Brenman JE. 2012. AMPK directly inhibits NDPK through a phosphoserine switch to maintain cellular homeostasis. Mol Biol Cell. 23:381–389. doi:10.1091/mbc.E11-08-0699.

Ross R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 362:801–809. doi:10.1038/362801a0.

Vriend G. 1990. WHAT IF: a molecular modeling and drug design program. J Mol Graph. 8:52–56. doi:10.1016/0263-7855(90)80070-V.

Wallace AC, Laskowski RA, Thornton JM. 1995. LIGPLOT: a program to generate schematic diagrams of protein–ligand interactions. Protein Eng. 8:127–134. doi:10.1093/protein/8.2.127.

Wang B, Shen W, Yang H, Shen J, Sun T. 2014. Targeting EGFR mutants with non-cognate kinase inhibitors in non-small cell lung cancer. Med Chem Res. 23:4510–4530. doi:10.1007/s00044-014-1012-2.

Zhou P, Wang C, Ren Y, Yang C, Tian F. 2013. Computational peptidology: a new and promising approach to therapeutic peptide design. Curr Med Chem. 20:1985–1996. doi:10.2174/0929867311320150005.