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Moracin M from *Morus alba* L. is a natural phosphodiesterase-4 inhibitor

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ABSTRACT

Phosphodiesterase-4 (PDE4) has been identified to be a promising target for treatment of asthma. Moracin M extracted from Chinese herbal drug 'Sang-Bai-Pi' (*Morus alba* L.) was studied for the inhibitory affinity towards PDE4. It inhibited PDE4D2, PDE4B2, PDE5A1, and PDE9A2 with the IC_{50} values of 2.9, 4.5, >40, and >100 μM, respectively. Our molecular docking and 8 ns molecular dynamics (MD) simulations demonstrated that moracin M forms three hydrogen bonds with Gln369, Asn321, and Asp318 in the active site and stacks against Phe372. In addition, comparative kinetics analysis of its analog moracin C was carried out to qualitatively validate their inhibitory potency as predicted by the binding free energy calculations after MD simulations.

The phosphodiesterase-4 (PDE4) enzymes play a vital role in regulating cyclic 3′,5′-adenosine monophosphate (cAMP) at cellular level by hydrolyzing cAMP to 5′-AMP, which acts as key regulators of many important biological processes.[1–5] A number of PDE4 inhibitors have been investigated as anti-inflammatory therapeutics since inhibition of PDE4 will lead to the accumulation of cAMP and thus attenuate inflammatory responses in multiple cell types.[5–7]

Herbal drug 'Sang-Bai-Pi' (*Morus alba* L.) is widely distributed in China and its root bark is clinically used as an anti-tussive and anti-asthmatic agent in traditional Chinese medicine.[8,9] Moracin M[10] is a phenolic component in the skin of *Morus alba* L. To understand the action mechanism of moracin M, our present work measured the inhibition of moracin M on PDEs and performed molecular docking and molecular dynamic simulations. The studies show that moracin M inhibits against PDE4D2 and PDE4B2 at micromolar level, but very weakly towards PDE5A1 and PDE9A2, suggesting it is a PDE4 inhibitor.

Isolation of moracin M and moracin C

The dried root bark of *Morus alba* L. (1 kg) was powdered and extracted with 95% ethanol at room temperature for 48 h. After removal of the solvent under reduced pressure, a brown extract was suspended with water, and sequentially partitioned with petroleum ether, ethyl acetate and *n*-butanol. The acetyl acetate extract (9 g) was subjected to column chromatography on silica gel (200–300 mesh) with increasing concentrations of acetyl acetate in petroleum ether. The fraction (petroleum ether–ethyl acetate 7/3, v/v) was collected and re-subjected to CC on silica gel to yield moracin C (10 mg) and moracin M (25 mg), which were determined by comparison with the NMR data published in the literature.[9]

Expression and purification of four proteins

The human PDE4D2, 4B2, 5A1, and 9A2 proteins were reported previously by Ke et al.[11–13] The cDNAs for expression of human PDE4D2 (catalytic domain, residues 86–413), PDE4B2 (catalytic domain and UCR2, residues 92–521), PDE5A1 (catalytic domain, residues 535–860), and PDE9A2 (catalytic domain, residues 181–506) were subcloned into the expression vector pET15b. All these resultant plasmids were transformed into *E. coli* strain BL21 (Codonplus) for over expression. The *E. coli* cells carrying these plasmids were grown in LB medium at 37 °C to OD_{600} = 0.7, and then 0.1 mM isopropyl β-D-thiogalactopyranoside was added for further growth at 16 °C for 20–40 h. These recombinant proteins were purified by Ni-NTA column (Qiagen). The purity of these recombinant proteins was greater than 95% as shown by SDS–PAGE. A typical batch of purification yielded over 50 mg of PDE4D2 (catalytic

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domain), 10 mg PDE4B2 (catalytic domain and UCR2), 30 mg of PDE9A2 (catalytic domain), and 7.8 mg of PDE5A1 (catalytic domain) from 1-l cell culture, respectively. Thrombin should be used to cleavage PDE5A1 protein to make it stable, but not PDE9A2.

Enzymatic assay

The inhibitory measurements of PDE inhibitors were carried out similarly as described in the literature.[11–13] The PDE enzymes were incubated with a reaction mixture containing 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and ^{3}H-cAMP or ^{3}H-cGMP (20000–30000 cpm/assay, GE Healthcare) at room temperature (25 °C) for 15 min. The reactions were terminated by addition of 200 μL of 0.2 M ZnSO4 and $Ba(OH)_2$. The reaction product ^{3}H-AMP or ^{3}H-GMP was precipitated out, while unreacted ^{3}H-cAMP or ^{3}H-cGMP remained in the supernatant. Radioactivity in the supernatant was measured in 2.5 ml Ultima Gold liquid scintillation cocktails (PerkinElmer) by a PerkinElmer 2910 liquid scintillation counter. For the IC_{50} measurement of inhibitors, at least eight concentrations of inhibitors were used, and the enzyme concentration that hydrolyzed up to 70% of the substrate was used for each inhibitory assay. Each measurement was repeated at least three times. The IC_{50} values were calculated by nonlinear regression. The potent inhibitors roflumilast, (S)-Bay 73-6691, and sildenafil purchased from Sigma served as references for the enzymatic assay of PDE4D2/PDE4B2, PDE9A2, and PDE5A1, respectively.

Preparation of the systems and molecular docking

The X-ray structure (PDB code: 1XOQ, chain A) of catalytic domain of human PDE4D in complex with roflumilast[14] was used as the initial structure. Adding hydrogen atoms and charges to this system used a standard method. All ionizable residues of this system were set at their default protonation states at a neutral pH. A charge of +2 was give to the zinc and magnesium ions. The docking procedure was carried out by using the CDOCKER protocol[15,16] implemented in Accelrys Discovery Studio 2.5.5.[17] The ligand-binding site of PDE4D was defined by using roflumilast[14] as a reference ligand. The input site sphere, where roflumilast and moracin M were docked into, was then defined with a radius of 15 Å from the center of the ligand-binding site. The ligand conformation was obtained by simulated annealing method, that is, first heated to 700 K (2000 steps) and then annealed to 300 K (5000 steps). The value of the Grid Extension was 8 Å. For each ligand structure, fifty random conformations were generated. Orientations to refine were set to fifty also. Protein residues in the interaction sphere were allowed to move during minimization. From the top 20 poses with relatively higher docking scores, we chose the optimal one based on both the docking score and cluster popularity. As a result, the PDE4D/roflumilast and PDE4D/moracin M complexes were prepared for molecular dynamics simulations.

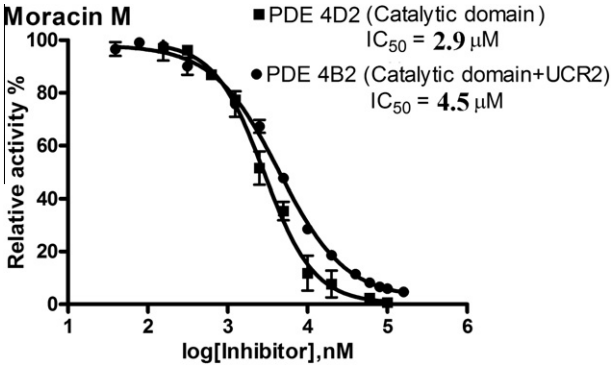


Figure 2. Inhibition of phosphodiesterase-4 by moracin M.

MD simulations and binding free energy calculation

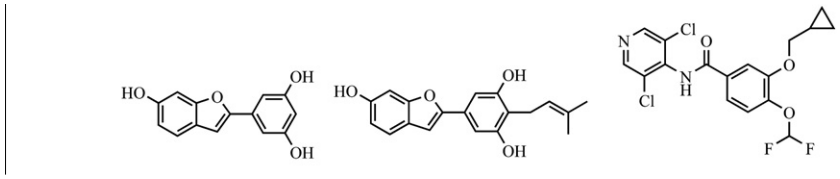
AMBER 10[18] was employed for all the MD simulations. Since the theories and procedures of AMBER MD simulations and MM-PBSA binding free energy calculations[16,19,20] are well documented, the detailed information about the MD simulations and binding free energy calculations in this study is provided in Appendix 1.

Moracin M is a PDE4 inhibitor

The IC_{50} values (0.46 nM and 0.67 nM, Fig. 1) of the reference ligand roflumilast towards PDE4D2 and PDE4B2 were similar to those (0.68 nM and 0.84 nM) reported in the literature.[14] Moracin M showed in vitro inhibition of 2.9, 4.5, >40, and >100 μM towards PDE4D2, PDE4B2, PDE5A1, and PDE9A2 (Figs. 1 and 2), respectively. Moracin M exhibited at least 30 times greater selectivity for PDE4D2 than for PDE9A2, and approximately 13 times than for PDE5A1.

Both docking and MD simulation methods could produce reliable results for 1XOQ

To confirm the reliability of the CDOCKER docking approach, roflumilast was extracted from the X-ray structure 1XOQ of PDE4D and was docked back into the same receptor via a variety of docking conditions and scoring parameters. The root-mean-square deviations (RMSDs) between the docked and crystal poses of roflumilast were calculated. The RMSDs for the top 20 docked poses ranged from 0.65 to 1.7 Å, which confirmed that the CDOCKER



IC_{50}	Moracin M	Moracin C	Roflumilast
PDE4D2	2.9 μM	26.0 μM	0.46 nM
PDE4B2	4.5 μM	18.1 μM	0.67 nM
PDE5A1	> 40 μM	> 50 μM	
PDE9A2	>100 μM	>100 μM	

Figure 1. Chemical structures and inhibitory affinities of three phosphodiesterase-4 inhibitors.