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In silico design and synthesis of piperazine-1-pyrrolidine-2,5-dione scaffold-based novel malic enzyme inhibitors

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Abstract—Fragment-based virtual library design and virtual screening have been conducted against malic enzyme (ME) homology model. Several scaffolds have been identified as promising motifs to target ME's NADP binding site. One small focused library has been synthesized and tested against ME. Several compounds from this library have shown sub-micromolar inhibitory activity against malic enzyme.

Malic enzyme has cytosolic and mitochondrial forms (c-ME and m-ME). Cytosolic malic enzyme (c-ME) is a lipogenic enzyme which generates NADPH substrates necessary for fatty acid synthesis in fatty tissues.[1–3] c-ME is highly regulated in fatty tissues and it is a key regulator of fatty acid synthesis. Therefore, c-ME is an ideal target for the development of new drugs to lower circulating lipid levels. The corresponding mitochondrial ME, m-ME, is localized in the vicinity of inner mitochondrial membrane and is capable of intercepting exogenous malate from malate dehydrogenase. This has been attributed as a major reason that some tumor cells use glutamine instead of glucose as energy source. Thus, m-ME is a potential target in cancer chemotherapy.[4] Therefore, inhibitors against c-ME and m-ME have potential as anti-diabetic/obesity and anticancer agents.

Malic enzyme (Fig. 1) is a tetrameric protein with a double dimer structure.[5,6] The tetrameric protein contains a total of eight Rossman-folds, a motif that is characteristic of NAD/P binding.[7,8] Each monomeric unit of the enzyme is composed of four structural domains designated as A, B, C, and D, which show a folding topology different from those of other oxidative decarboxylases (see Fig. 1).

Total of 13 structures of malic enzyme from different sources have been determined, including human m-ME and pigeon c-ME. The proposed catalytic mechanism involves Tyr-112 and Lys-183 as the general acid and base, respectively. In addition, a divalent metal ion Mn^{2+} is essential in helping the catalysis.[9]

The metal also helps to stabilize the structural integrity of the enzyme. In mammalian species, malic enzymes have three identifiable forms: cytosolic $NADP^+$-dependent (c-NADP-ME; EC 1.1.1.40), mitochondrial $NADP^+$-de-

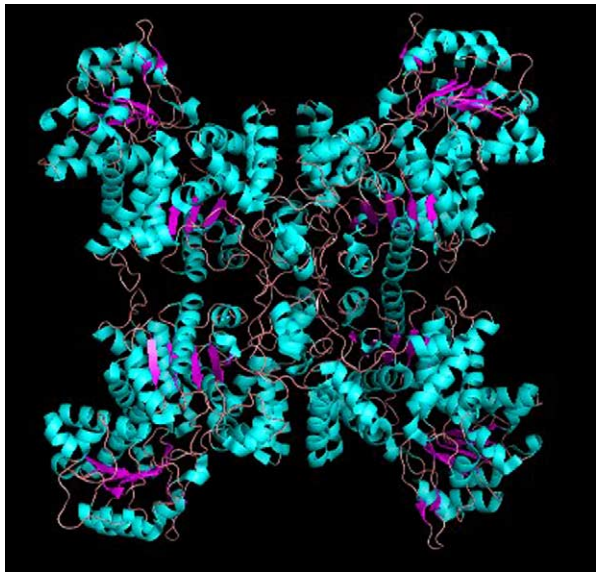

Figure 1. Tetrameric structure of malic enzyme.

Keywords: Enzyme inhibitor design; Virtual screening.
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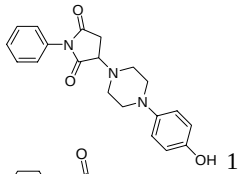
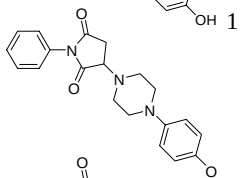
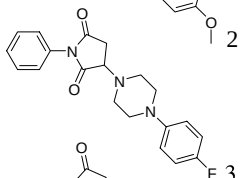
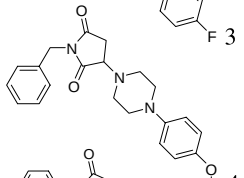
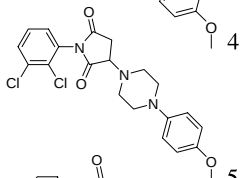
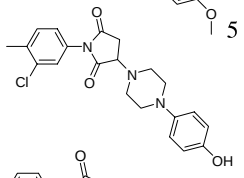
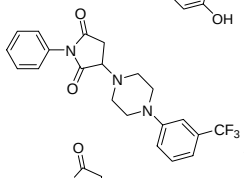
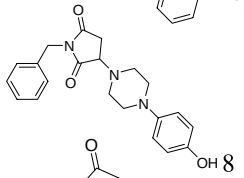
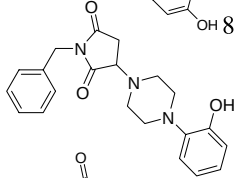
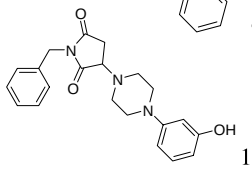
pendent (m-NADP-ME; EC 1.1.1.38), and mitochondrial $NAD(P)^+$-dependent malic enzymes (m-NAD-ME; EC 1.1.1.39). ATP has been shown to be a weak inhibitor of MEs and co-crystal structures shown ATP can bind at NAD/P binding pocket. Also small molecules, like oxalic acid, demonstrated strong inhibitory activity against c-ME and m-ME via chelating to Mn^{2+}.

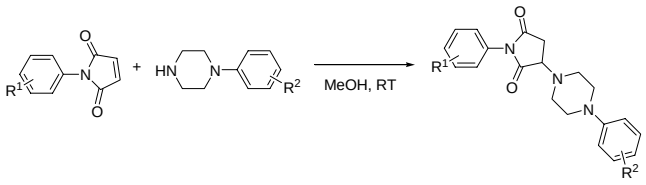
The availability of crystal structures and small molecule inhibitors of both c-ME and m-ME makes a structure-based drug discovery approach possible. We have applied the virtual screening approach to identify original leads from our fragment-based virtual library. The virtual library was generated from a set of 45 core scaffolds having two attachment points each and 50 possible side chains, resulting in a total of approximately 112,000 compounds (all library generation performed using the SYBYL Legion[10] module). The cores and side chains were chosen by focusing on NAD(P)H-like features, in order to pick similar interactions as NADP and oxalate in the active site. Another very important factor in the choice of scaffold cores and side chains was the presence of favorable ADMET properties. The library was filtered through various hard and soft filters implemented in in-house SYBYL SPL scripts. Some of the hard filtering criteria included, structure, formal charge, and ring count. Similarly, soft (or voting) filtering criteria included: the Lipinski's rule of five, molecular weight, *c*Log *P*, a number of hydrogen bond donors and acceptors, topological polar surface area (tPSA), and a number of rotatable bonds. Post-filtering, we obtained a set of approximately 82,000 compounds. OptiSim[11] was applied to reduce the dataset size without compromising on diversity. Specifically, each one of the final 10,000 compounds selected is less similar (more diverse) than 0.85 according to a MACCS 2D Tanimoto correlation coefficient metric.

Library docking to the homology model was performed using the FlexX module.[12] We employed the FlexX scoring function to select the best conformer for each compound with the restriction that conformers completely exterior to the volume near the metal were excluded from consideration. This choice of scoring function for this study was guided in part by the ability of FlexX to successfully identify the crystallographic binding conformation (pose) of docked substrate and NADP in the pigeon cytoplasmic malic enzyme [1GQ2]. The FlexX scoring function did the best at this electronic assay of the functions tried, possibly since FlexX is very much an enthalpic type function which likes charge interactions that are a key feature of this system. Additionally, post-docking filtering was performed using the consensus scoring approach with five different scoring functions provided within the SYBYL CSCORE module, wherein a compound was allowed to pass the filter if the top scoring pose for a given compound was scored in the top 50% of scores for all five scoring functions. This is a standard practice in the use of the C-SCORE module.[13]

A large proportion of top-scoring compounds identified from the virtual screening effort dock in the homology model active site in a manner similar to the docking of NADP in the 1GQ2 active site, thus validating, in silico, the structural basis for designing NAD(P)H focused compound libraries. Here we report our preliminary c-ME assay results of one top-scoring scaffold, the combination of 2,5-dioxopyrrolidine and piperazine, from our approach (Table 1).[14]

Table 1. SAR of 2,5-dioxopyrrolidines

Structure	IC_{50}(μM)
 1	0.15
 2	>20
 3	10
 4	>20
 5	>20
 6	0.56
 7	3.15
 8	0.45
 9	>20
 10	10



Scheme 1. Synthesis of malic enzyme inhibitors.

Both piperazine and 2,5-dioxopyrrolidine have been the essential sub-structure motifs in various drugs.[15] The combination of these two substructures has generated an interesting scaffold with drug-like features. This fragment-based drug design approach has been known and successfully applied in many drug discovery projects.[16] We have built a small focused library based on this scaffold (Scheme 1). Then the compounds were screened against malic enzyme by measuring the production of NADPH. NADPH has a maximal absorption wavelength at 340 nm. In traditional direct assay, ME activity is determined by measuring the activity in a continuous spectrophotometric assay in which the reduction of NADP is monitored at 37 °C or room temperature. The activity of the malic enzyme is related to the rate of increase in the absorbance. Unfortunately, when NADP and malic acid were used at concentrations close to their K_m values a traditional continuous assay did not provide us with desired sensitivity and robustness, required for a screening of inhibitors. Therefore, we have developed a coupled assay in 96 and 384 plate formats.[17] Assay was based on the capability of NADPH formed during enzymatic reaction to transfer hydrogen and electrons to tetrazolium salts. In our experiments, we used tetrazolium salt MTS which is readily reduced by NADPH to the colored formazan in the presence of an electron mediator PMS. Absorbance can be detected using a more favorable wavelength of 492 nm. This coupled assay offers an improved signal:noise ratio and allows us to use substrates at K_m range. Assay was performed as described in notes.

Electrostatic contacts which provide molecular specificity include: (1) direct coordination of the phenol hydroxyl to the Mn^{2+}, (2) favorable electrostatic between a piperazine ring nitrogen and bound water 4124, and (3) a hydrogen bond between the A312 amide hydrogen and one of compound **1**'s carbonyl oxygens. Hydrophobic interactions include: (1) L419 and V392 with the phenol ring, (2) L310, G311, and A393 with the phenyl group, and (3) contact of the phenyl group with the long alkyl chain portion of K346. Compound **1**'s overall chemical geometry is curved and relaxed, including a chair conformation for the piperazine ring. Compound **1**'s second carbonyl oxygen is exposed to the solvent and not directly involved in any hydrogen bond to the active site (see Fig. 2).

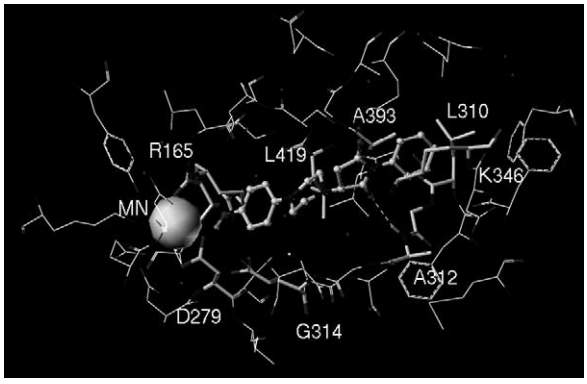


Figure 2. Hypothetic interaction of compound **1** at human cME active site.

The difference of inhibitory activities of compounds **9** and **10** can be explained perfectly by this mode. The importance of the position of the phenol OH group for metal chelating effect and the piperazine ring for perfect geometry to project hydrophobic moiety into lipophilic pocket plays a big role for the activity of this series of compounds.

In conclusion, we have demonstrated that potent ME inhibitors can be discovered using fragment-based virtual library and virtual screening. This approach can be equally applied to other inhibitor discovery programs.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.10.065.

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14. General experimental procedure for preparation of pyrrolidine-2,5-dione. All reactions were performed in oven-dried glassware unless otherwise noted. Flash chromatography was performed on an Isco Optix 10 parallel purification system. The NMR spectra were recorded in DMSO-d_6 on a Varian Mercury 400 (^{1}H NMR at 400 MHz; ^{13}C NMR at 100 MHz) spectrometer. Mass spectra were obtained on a Thermo Finnigan LCQ Advantage MAX spectrometer. Analytical HPLC was performed on an automated Gilson system using a

gradient of 10% MeCN to 90% MeCN(0.1% FA) over 10 min on a C18 column (3 × 50 mm). An oven-dried disposable culture tube (5 mL) was charged with phenyl maleimide (0.2 mmol, 1.0 equiv), phenyl piperazine (0.2 mmole, 1.0 equiv) and methanol (2.0 mL). The tubes were then placed on a Bhodan Miniblock synthesizer and shaken for 20 h at room temperature. The TLC was taken before silica gel (~300 mg) was added to the reaction mixture. The solvent was evaporated using a SpeedVac system. Solid loading to the CombiFlash system and the reaction mixture was purified by silica gel column (4 g of silica gel) eluting with a linear gradient of hexane and ethyl acetate. Detector wavelength was set to 254 nm and the UV active fractions were collected and conformed by MS analysis. The solvent was removed by the SpeedVac system to generate the final products.3-(4-(4-Hydroxyphenyl)piperazin-1-yl)-1-phenylpyrrolidine-2,5-dione (**1**) ^{1}H NMR(DMSO-d_6) δ 7.50–7.46 (m, 2 H), 7.43–7.41 (m, 1H), 7.26–7.24 (m, 2H), 6.76 (dd, J = 7.2, 2 Hz, 2H), 6.62 (dd, J = 6.8, 1.6 Hz, 2H), 4.09–4.06 (m, 1H), 3.33–3.29 (m, 4H), 3.02–2.94 (m, 4H), 2.65–2.62 (m, 2H); MS m/z 352 $(M+H)^+$. 3-(4-(4-Methoxyphenyl)piperazin-1-yl)-1-phenylpyrrolidine-2,5-dione (**2**) ^{1}H NMR($CDCl_3$) δ 7.50–7.46 (m, 2H), 7.42–7.41 (m, 1H), 7.28 (dd, J = 7.2, 1.6 Hz, 2H), 6.92–6.90 (m, 2H), 6.86–6.84 (m, 2H), 4.03–4.00 (m, 1H), 3.77 (s, 3H), 3.15–2.92 (m, 8H), 2.84–2.80 (m, 2H); MS m/z 366 $(M+H)^+$.3-(4-(4-fluorophenyl)piperazin-1-yl)-1-phenylpyrrolidine-2,5-dione (**3**) ^{1}H NMR($CDCl_3$) δ 7.50–7.45 (m, 2H), 7.43–7.41 (m, 1H), 7.30–7.27 (m, 2H), 7.00–6.95 (m, 2H), 6.92–6.90 (m, 2H), 4.03–4.00 (m, 1H), 3.18–3.04 (m, 7H), 2.92–2.82 (m, 3H); MS m/z 354 $(M+H)^+$. 1-Benzyl-3-(4-(4-methoxyphenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**4**) ^{1}H NMR($CDCl_3$) δ 7.40–7.37 (m, 2H), 7.31–7.29 (m, 2H), 6.86–6.82 (m, 4H), 4.67 (s, 2H), 3.86–3.83 (m, 1H), 3.76 (s, 3H), 3.08–3.00 (m, 4H), 2.94–2.73 (m, 4H), 2.66–2.64 (m, 2H); MS m/z 380 $(M+H)^+$. 1-(2,3-Dichlorophenyl)-3-(4-(4-methoxyphenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**5**) ^{1}H NMR($CDCl_3$) δ 7.58 (dd,J = 8.0, 1.2 Hz, 1H), 7.33 (t, J = 8.0, 1H), 7.13 (dd, J = 7.6, 1.2 Hz, 1H), 6.91 (dd, J = 6.6, 2.4 Hz, 2H), 6.84 (dd, J = 6.8, 2.8 Hz, 2H), 4.13–4.09 (m, 1H), 3.77 (s, 3H), 3.17–3.01 (m, 8H), 2.88–2.84 (m, 2H); MS m/z 434 $(M+H)^+$. 1-(3-Chloro-4-methylphenyl)3-(4-(4-hydroxyphenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**6**) ^{1}H NMR($CDCl_3$) δ 7.34–7.30 (m, 2H), 7.11–7.09 (m, 1H), 6.90 (br s, 2H), 6.78 (d, J = 8.8 Hz, 2H), 4.01–3.98 (m, 1H), 3.15–3.01 (m, 7H), 2.92–2.81 (m, 3H), 2.40 (s, 3H); MS m/z 400 $(M+H)^+$. 1-Phenyl-3-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**7**) ^{1}H NMR($CDCl_3$) δ 7.51–7.47 (m, 2H), 7.44–7.40 (m, 1H), 7.36–7.34 (m, 1H), 7.30–7.28 (m, 2H), 7.12–7.06 (m, 3H), 4.04–4.01 (m, 1H), 3.33 (br s, 4H), 3.15–3.08 (m, 3H), 2.90–2.85 (m, 3H); MS m/z 404 $(M+H)^+$. 1-Benzyl-3-(4-(4-hydroxyphenyl)piperazin-1-yl)-1-pyrrolidine-2,5-dione (**8**) ^{1}H NMR(DMSO-d_6) δ 8.82 (s, 1H), 7.35–7.24 (m, 4H), 6.76 (dd, J = 6.6, 2.4 Hz, 2H), 6.63 (dd, J = 6.6, 2.0 Hz, 2H), 4.56 (s, 2H), 4.03–4.00 (m, 1H), 2.93–2.87 (m, 8H), 2.80–2.74 (m, 2H); MS m/z 366 $(M+H)^+$. 1-Benzyl-3-(4-(2-hydroxyphenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**9**) ^{1}H NMR($CDCl_3$) δ 7.40–7.38 (m, 2H), 7.34–7.28 (m, 3H), 7.15–7.13 (m, 1H), 7.09–7.07 (m, 1H), 6.96–6.94 (m, 1H), 6.88–6.84 (m, 1H), 4.68 (s, 2H), 3.89–3.85 (m, 1H), 3.00–2.92 (m, 7H), 2.80–2.69 (m, 3H); MS m/z 366 $(M+H)^+$. 1-Benzyl-3-(4-(3-hydroxyphenyl)piperazin-1-yl)pyrrolidine-2,5-dione (**10**) ^{1}H NMR($CDCl_3$) δ 7.39–7.36 (m, 2H), 7.34–7.28 (m, 3H), 7.10 (t, J = 8.4 Hz, 1H), 6.50–6.48 (m, 1H), 6.42–6.41 (m, 1H), 6.36–6.34 (m, 1H), 4.67 (s, 2H), 3.86–3.82 (m, 1H), 3.20–3.17 (m, 4H), 2.95–2.84 (m, 3H), 2.74–2.65 (m, 3H); MS m/z 366 $(M+H)^+$.

15. For detailed therapeutic information of 2,5-dioxopyrrolidine-based drugs, please consult Merck Index and MDL's MDDR database.
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17. Procedure for malic enzyme activity assay. Assay in a 96-well format was performed as follows: the reaction mixture containing 85 mM Tris (pH7.5), 2 mM malic acid, 5 mM $MnCl_2$, and appropriate amounts of enzyme was pre-incubated with compounds for 45 min at room temperature. Then reactions were initiated by addition of 124 μ M NADP. After 30 min at room temperature, the reaction was stopped with 0.5 M EDTA. Fifty microliters of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium/phenazinemethosulfate (MTS/PMS) was added, and absorbance was measured at 492 nm.