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Structure activity relationships of benzylproline-derived inhibitors of the glutamine transporter ASCT2

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ABSTRACT

The glutamine transporter ASCT2 has been identified as a promising target to inhibit rapid growth of cancer cells. However, ASCT2 pharmacology is not well established. In this report, we performed a systematic structure activity analysis of a series of substituted benzylproline derivatives. Substitutions on the phenyl ring resulted in compounds with characteristics of ASCT2 inhibitors. Apparent binding affinity increased with increasing hydrophobicity of the side chain. In contrast, interaction of the ASCT2 binding site with specific positions on the phenyl ring was not observed. The most potent compound inhibits the ASCT2 anion conductance with a K_i of 3 μM, which is in the same range as that of more bulky and higher molecular weight inhibitors recently reported by others. The experimental results are consistent with computational analysis based on docking of the inhibitors against an ASCT2 homology model. The benzylproline scaffold provides a valuable tool for further improving binding potency of future ASCT2 inhibitors.

Glutamine is transported across mammalian membranes by a variety of transporters from different families, including the alanine cysteine seine transporter, ASCT2 (reviewed in[1,2]). ASCT2 belongs to the solute carrier 1 (SLC1) family of transporters.[3] It transports neutral amino acids, including glutamine, across the plasma membrane in exchange with an intracellular neutral amino acid.[4,5] The process is dependent on Na^+, but not driven by the transmembrane concentration gradient of Na^+. Net exchange is electroneutral, although it was proposed that the actual translocation process is associated with charge movement.[5,6]

Glutamine is an important nutrient, in particular in rapidly-growing cancer cells, in which glutamine serves as a nitrogen source.[7] The increased nitrogen and carbon demand, together with the role of glutamine in the regulation of mammalian target of rapamycin complex 1 (mTORC1) signaling, creates glutamine "dependency" of certain types of cancer cells, resulting in an up-regulation of glutamine transport.[8] Such changes in metabolism of cells once they become cancerous are known as the Warburg effect, which has been shown as increased demand for glucose (glycolysis) as well as glutamine.[9]

Due to the increased nitrogen demand, glutamine transporters, in particular ASCT2, show dramatically increased expression levels in cancer cells.[10,11] For example, ASCT2 up-regulation was demonstrated in prostate cancer and triple negative breast cancer (TNBC).[10] Furthermore, it was shown that inhibition of ASCT2 expression through antisense RNA methods resulted in cell apoptosis, and even shrinkage of tumors.

Together, these results implicate ASCT2 as an important potential target for inhibiting the growth of cancer cells. However, at present the pharmacology of the ASCT2 substrate binding site and the knowledge of small molecule transport inhibitors are not very well developed. Several amino acid based inhibitors have been identified, including a series of serine derivatives,[11] and L-γ-glutamyl-nitroanilide, a commercially available ASCT2 inhibitor with mM affinity, and derivatives[12,13] (Fig. 1A). Based on these anilides, compounds with increased aromatic bulk were developed, which bind to ASCT2 with affinities in the low micromolar range.[14]

Recently, we have utilized an *in silico* screening approach to test a compound database for potential hits for ASCT2 binders. Among several other compounds, a proline derivative was identified, γ-2-fluorobenzyl proline, which inhibited ASCT2 with a 87 μM affinity.[15] This result was surprising, because proline (Fig. 1B) is not a known substrate/inhibitor of ASCT2 and does not induce any activity in ASCT2 at a concentration up to 1 mM (Fig. 2).

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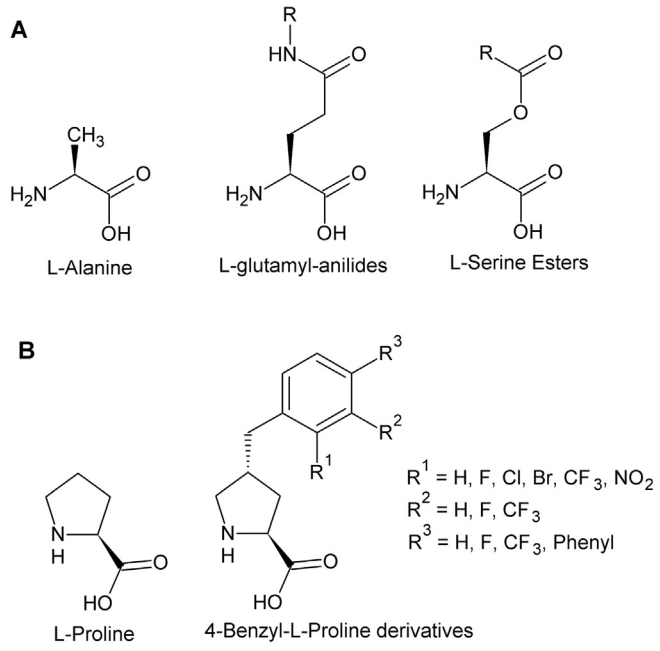

Fig. 1. (A) Structures of previously identified ASCT2 substrates (alanine) and inhibitors (glutamyl-anilide and serine ester derivatives). (B) Proline and substituted benzylproline derivatives investigated in this study.

In the present work, we aimed at using benzylproline as a scaffold for systematic structure activity analysis (Fig. 1B), altering substituents on the benzyl ring. We tested a number of benzylproline derivatives with substitutions at the 2, 3, and 4 positions of the benzyl ring (Fig. 1B). Through this approach, we identified a new ASCT2 inhibitor, which binds to the *apo* (unbound) transporter with a 3 μM apparent affinity. Interestingly, the position of the substituent on the phenyl ring had only a minor effect on inhibitory potency. In contrast, the ability of the substituent to affect hydrophobicity played a major role. Our new results add to the understanding of the molecular parameters that govern inhibitor interaction with the ASCT2 binding site.

The first strategy was to test whether the nature of the substituent on the 2-position of the phenyl ring affected binding potency. To test this question, we determined kinetic parameters for ligand interaction with ASCT2 for six γ-benzylproline derivatives with varying 2-substituents, ranging from hydrogen to halogens and the methyl group (structures shown in Fig. 1B, R^1). Only the (R) enantiomers were experimentally tested. To determine kinetic parameters of binding, we recorded currents in response to compound application to ASCT2-expressing cells. Rat ASCT2 was transiently expressed in HEK293T cells, as was shown previously.[5,11,16] HEK293T cells do not express detectable levels of ASCT2 before transfection with ASCT2 cDNA-containing plasmids. All six compounds showed characteristics of ASCT2 inhibitors, because they blocked the permanent ASCT2 leak anion current (Fig. 2). In the presence of intracellular anion (SCN^-), this leak anion current is inward directed (SCN^- outflow). Therefore, application of blockers reduces the inward leak anion current, generating apparent outward current (Fig. 3A middle panels and right panel), as reported previously for other ASCT2 blockers.[11,15,16] In contrast, transported substrates, such as alanine, activate a substrate-dependent anion current, which in the presence of intracellular anion (SCN^-) is inward directed.[5] Thus, alanine and other transported substrates induce inward current (SCN^- outflow) under these conditions (Fig. 3A, left panel). This characteristic behavior of ASCT2 substrates/inhibitors has been demonstrated in several reports, and is caused by the kinetic relationship between substrate transport and the visitation of anion conducting states along the transport pathway.[5,11,15,16]

The apparent affinity of ASCT2 for the 2-substituted benzylproline derivatives, which was determined by measuring the dose response relationships of the outward currents (Fig. 3B), varied over almost 2 orders of magnitude, with γ-benzylproline (H-substituent) having the lowest affinity (highest K_i, 2.0 ± 1.5 mM), whereas the 2-bromo derivative displayed the lowest K_i of 25 ± 15 μM (highest affinity, Fig. 3B). The apparent affinity data for these compounds are summarized in Table 1. These results show that halogen substituents, as well as a methyl group in 2 position result in a significant increase in apparent affinity over the H-substituted compound.

While no significant correlation of K_i value with substituent size (as quantified through Taft steric parameter[17]) or electron withdrawing/donating properties of the substituent (Hammett substituent constant[18]) were observed, K_i decreased with increasing hydrophobicity of the benzylproline side chain (Fig. 4, quantified

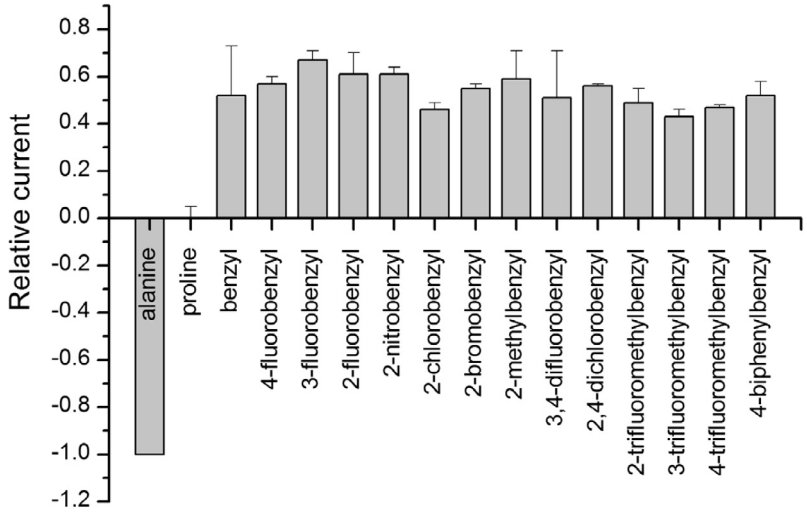

Fig. 2. All 4-substituted proline derivatives display inhibitory behavior. Current responses, I_{max}, in the presence of saturating concentrations of alanine and proline derivatives relative to a response to saturating [alanine]. Saturating current was calculated from the response at the tested concentration (1 mM) and the known K_m value, $I/I_{max} = c/(c + K_m)$ with c = concentration. Outward (positive) current reflects inhibitory behavior due to inhibition of the leak anion conductance.

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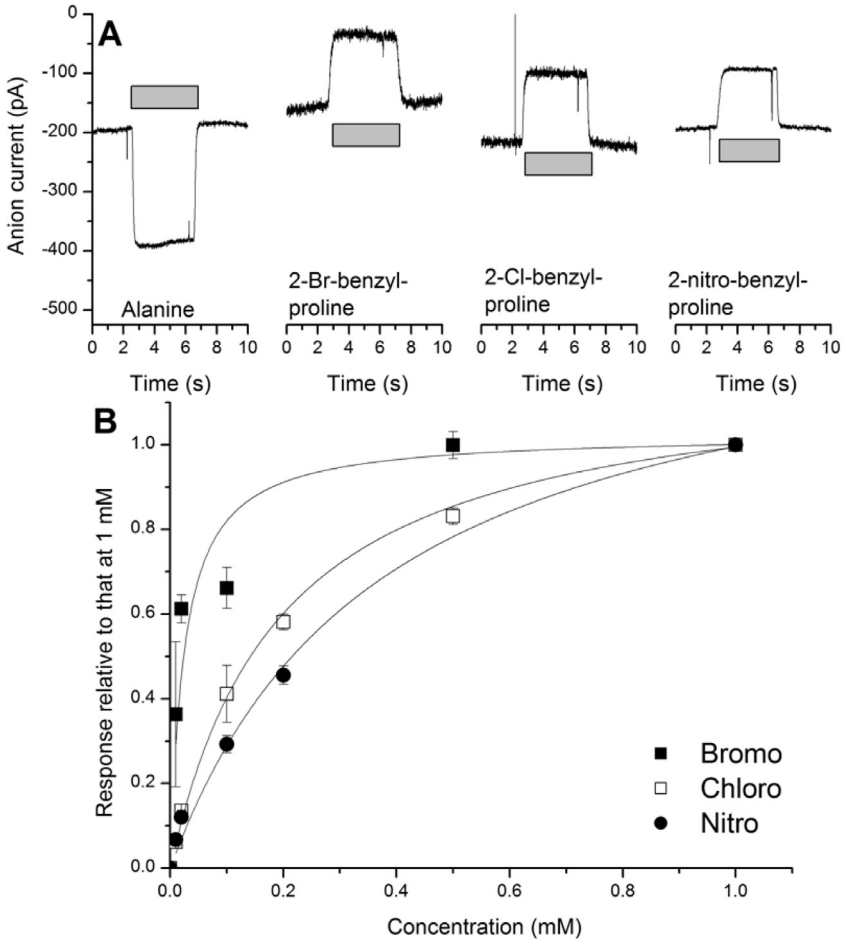

Fig. 3. Benzyl-proline derivatives substituted in the 2 position of the phenyl ring inhibit ASCT2 activity. (A) Typical whole-cell current recording traces from ASCT2-transfected HEK293T cells in the presence of 1 mM alanine (left panel) and 1 mM of 2-Br, 2-Cl, and 2-nitro-benzylproline. Timing of substrate/inhibitor application is indicated by the gray bars. (B) Dose response relationships for the three inhibitors shown in (A). All responses were normalized to the response at 1 mM of each compound. Experiments were performed at 0 mV transmembrane potential in the presence of 140 mM external NaCl, 135 mM internal NaSCN, and 10 mM internal alanine.

Table 1
K_i and I_{max} values for the benzylproline derivatives (I_{max} are relative to the current at saturating alanine concentration). Errors represent ± S.D. All compounds were the hydrochloride salts.

Compound	K_i (μM)	Relative I_{max}
(*R*)-γ-benzyl-L-proline hydrochloride	2000 ± 1500	0.52 ± 0.21
(*R*)-γ-(4-fluoro-benzyl)-L-proline	190 ± 110	0.57 ± 0.03
(*R*)-γ-(3-fluoro-benzyl)-L-proline	177 ± 31	0.67 ± 0.04
(*R*)-γ-(2-fluoro-benzyl)-L-proline	83 ± 20	0.61 ± 0.09
(*R*)-γ-(2-nitro-benzyl)-L-proline	373 ± 65	0.61 ± 0.03
(*R*)-γ-(2-chloro-benzyl)-L-proline	195 ± 15	0.46 ± 0.03
(*R*)-γ-(2-bromo-benzyl)-L-proline	25 ± 15	0.55 ± 0.02
(*R*)-γ-(2-methyl-benzyl)-L-proline	38 ± 25	0.59 ± 0.12
(*R*)-γ-(3,4-difluoro-benzyl)-L-proline	73 ± 55	0.51 ± 0.20
(*R*)-γ-(2,4-dichloro-benzyl)-L-proline	30 ± 14	0.56 ± 0.01
(*R*)-γ-(2-trifluoromethyl-benzyl)-L-proline	220 ± 36	0.49 ± 0.06
(*R*)-γ-(3-trifluoromethyl-benzyl)-L-proline	77 ± 36	0.43 ± 0.03
(*R*)-γ-(4-trifluoromethyl-benzyl)-L-proline	360 ± 60	0.47 ± 0.01
(*R*)-γ-(4-biphenylmethyl)-L-proline	3 ± 2	0.57 ± 0.08

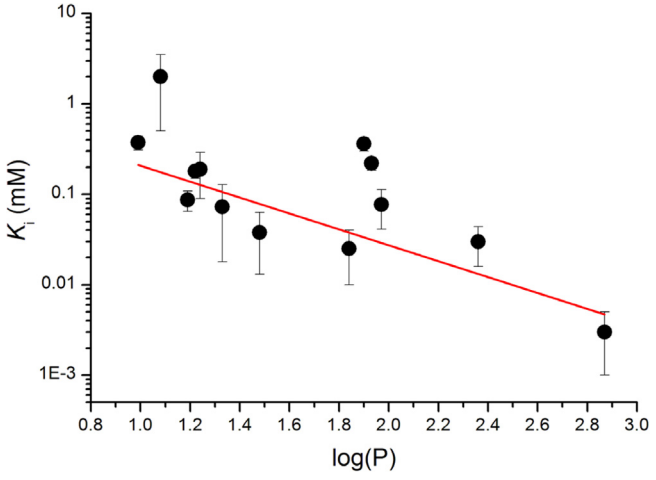

Fig. 4. Inhibitor affinity correlates with the hydrophobicity of the substituent. The log(K_i) is plotted as a function of log(P) of the side chain. log(P) was calculated according to.[22] R^2 for the linear regression (solid line) is 0.73. Pearson's r value is −0.85, indicating good correlation.

by log(*P*), with *P* = octanol/water partition coefficient of the side chain).

Next, we tested the effect of the position of a fluorine substituent on the benzyl ring. All three tested derivatives (2-fluoro, 3-fluoro, and 4-fluoro γ-benzylproline) showed inhibitory behavior (Fig. 2). As illustrated in Suppl. Fig. 1 and Table 1, the position of the fluorine substituent did not have a large effect on apparent affinity, although binding potency was most favorable with the fluorine atom in the 2 position on the benzyl ring. This result indicates that the main effect of the fluorine substituent on inhibitor potency is the general increase of hydrophobicity, rather than

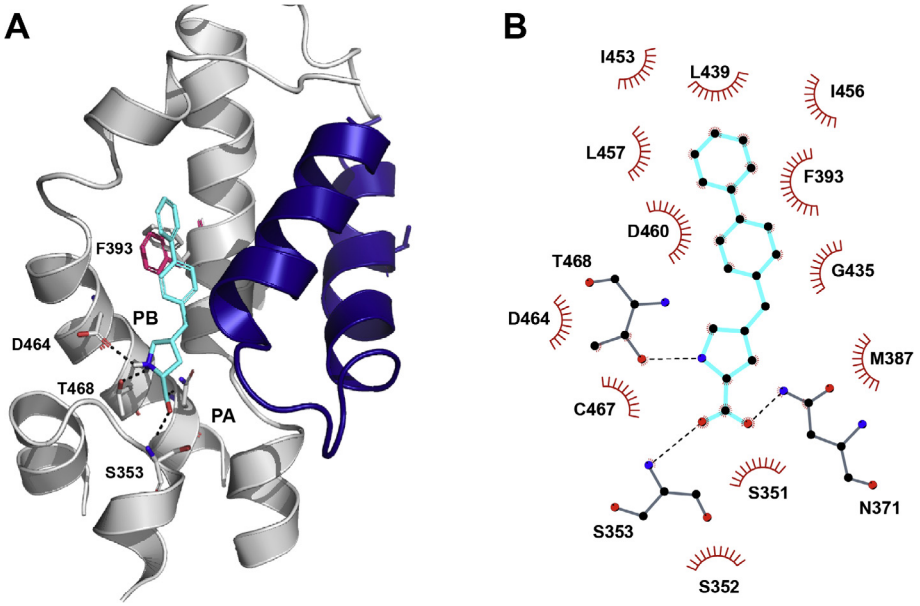

Fig. 5. Predicted binding pose of γ-(4-biphenylmethyl)-L-proline in the homology model of the human ASCT2. (A) The coordinates of γ-(4-biphenylmethyl)-L-proline are visualized in cyan sticks. The ASCT2 binding site is shown in gray cartoons, with the open HP2 loop in dark blue. The residues forming hydrogen bonds with the ligand are shown in sticks. The regions of each pocket PA and PB are labeled. Two orientations of Phe393 are displayed: in gray, the phenyl side chain of Phe393 is perpendicular to the vertical axis of the binding site; in pink, Phe393 is flipped parallel to the vertical axis, as a result of the IFD. (B) The docking pose of γ-(4-biphenylmethyl)-L-proline is shown in a two-dimensional representation generated by ligplot, where the residues forming hydrogen bonds are colored in gray, and the residues establishing hydrophobic interactions with the ligand are shown as red spoked arcs.

localized and specific molecular interactions of the fluorine atoms with the ASCT2 ligand binding site.

Two di-substituted chloro and fluoro derivatives were also included in the analysis (Table 1). While both compounds showed inhibitory activity, their K_i value of the 3,4-difluoro derivative was not dramatically improved over the mono-substituted analog. This finding was not unexpected, since the two side chains have very similar hydrophobicity. In contrast, adding a second Cl substituent in the 4 position (2,4 dicholoro-γ-benzylproline) resulted in a 6-fold increase in apparent affinity, presumably because the di-substituted side chain has a significantly higher log(*P*) value than the mono-substituted compound.

To further test the correlation of side chain hydrophobicity with inhibitor potency, we tested two compounds with substituents that increase the log(*P*) value, phenyl and trifluoromethyl groups. While the trifluoromethyl substituted compounds showed no improvement in K_i over the other mono-substituted benzylproline derivatives (Table 1), the 4-phenyl-substitued derivative was found to have an apparent inhibition constant of 3 ± 2 μM (Supplementary Fig. 2, Table 1), an about 8-fold higher affinity compared to any other ASCT2 inhibitor we have tested so far.[11,15]

To visualize the binding mode of the most potent newly identified ASCT2 inhibitor γ-(4-biphenylmethyl)-L-proline, we conducted *in silico* Induced Fit Docking (IFD,[19]) of this compound against an ASCT2 homology model (Fig. 5).[16] This ASCT2 model was built based on the outward open conformation of Glt_{Ph},[20] in which hairpin loop 2 (HP2) is propped open by the bound, bulky inhibitor TBOA (DL-*threo*-β-Benzyloxyaspartic acid), thus preventing translocation of the C-terminal transport domain across the membrane. The outward-open ASCT2 model reveals two hydrophobic pockets PA and PB, which can be targeted with small molecule inhibitors (Fig. 5).[21] The hydrophobic side chains of the original inhibitor γ-2-fluorobenzylproline as well as the newly discovered ligands are predicted to interact with PB. Interestingly, during IFD, in which the flexibility of the binding site is introduced, Phe393 is reoriented, thereby making additional accessible volume in PB and facilitating the binding of the hydrophobic bulk of the biphenylmethyl substituent. The carboxy and pyrrolidine groups of the newly discovered ligands are predicted to form polar interactions with key binding site residues, including Ser353, Asn371, and Thr468, which are also predicted to make similar polar interactions with known ligands (Fig. 5).[15]

In summary, our work highlights the usefulness of substituted benzylprolines as a scaffold for the development of ASCT2 inhibitors with improved binding potency. The newly-identified biphenylmethyl derivative interacts with rat ASCT2 with a low micromolar apparent affinity. To our knowledge only one compound has been reported with higher affinity (1.3 μM,[14]), based on a substituted diaminobutanoic acid. However, this compound has substantially higher molecular weight, as well as hydrophobicity. If the γ-(4-biphenylmethyl)-L-proline scaffold can be used to further improve affinity, this would provide further advance in the ongoing work to generate ASCT2 inhibitors with sub-micromolar binding affinity.

Our results also provide a quantitative basis for our understanding of the molecular parameters that govern interaction of the inhibitors with the ASCT2 binding site. They suggest that specific atomic interactions between substituents of the hydrophobic moiety and the ASCT2 binding site are less important than the overall hydrophobicity of the side chain (Fig. 4). Future structure function analysis can exploit this finding by increasing the hydrophobicity of the side chain further, as well as by integrating a specific side chain hydrogen bonding interactions, as proposed in a previous report.[12] This hydrogen bond interaction is missing in the proline derivatives reported here, and, if additive with the hydrophobic effect, may further strengthen ASCT2 interaction with the bound ligand. Therefore, the prospects of developing ASCT2 inhibitors with sub-micromolar affinity are encouraging.

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A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.12.063.

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