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2-Substituted *N*γ-glutamylanilides as novel probes of ASCT2 with improved potency

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ABSTRACT

Herein, we report the discovery and structure–activity relationships (SAR) of 2-substituted glutamylanilides as novel probes of the steric environment comprising the amino acid binding domain of alanine–serine–cysteine transporter subtype 2 (ASCT2). Focused library development led to three novel, highly potent ASCT2 inhibitors, with *N*-(2-(morpholinomethyl)phenyl)-L-glutamine exhibiting the greatest potency in a live-cell glutamine uptake assay. This level of potency represents a three-fold improvement over the most potent, previously reported inhibitor in this series, GPNA. Furthermore, this and other compounds in the series exhibit tractable chemical properties for further development as potential therapeutic leads.

Emerging evidence implicates oncogenic signaling pathways with nutrient uptake in cancer cells. The natural amino acid glutamine is essential for cell growth and proliferation. In addition to glucose, cancer cells utilize glutamine as a carbon source for ATP production and biosynthesis. Mammalian cells can internalize glutamine through an evolutionary redundant repertoire of cell surface transporters, though a primary sodium-dependent transporter of glutamine, ASCT2 (gene symbol *SLC1A5*), stands out as a promising target for probe development. In cancer cells, *SLC1A5* expression is associated with oncogenic *MYC*[1,2] and *KRAS*,[3,4] suggesting its relevance in many clinically important tumors, including those of the lung, colon, and pancreas.[5–7] Demonstrating that ASCT2/SCL1A5 activity might be 'actionable' in variety of settings in oncology, Fuchs and co-workers first demonstrated that *SLC1A5* antisense RNA triggered apoptosis in human hepatocellular carcinoma cells.[8] Furthermore, Hassanein et al. more recently reported that *SLC1A5* was expressed in 95% of squamous cell carcinomas (SCC), 74% of adenocarcinomas (ADC), and 50% of neuroendocrine tumors. In those studies, siRNA down-regulation of ASCT2 in lung cancer cells resulted in significant growth inhibition.[9] Collectively, these studies suggest the potential fruitfulness of developing small molecules capable of inhibiting ASCT2 activity as precision cancer medicines.

To date, few pharmacological inhibitors of ASCT2 have been reported. Grewer and Grabsch described a series of serine and cysteine derivatives as inhibitors of ASCT2. The benzyl analogs of serine and cysteine were reported to have K_i values equal to 0.9 mM and 0.78 mM, respectively.[10] Further elaboration within this series led to serine biphenyl-4-carboxylate which inhibits ASCT2 function with an apparent affinity of 30 μM.[11] As an early entrant to the field, in 2004, Esslinger et al. described L-γ-glutamyl-*p*-nitroanilide (GPNA), a glutamine analog, as a commercially available probe of the ASCT2 amino acid binding site.[12] While this work illustrated that GPNA could inhibit glutamine uptake in cells at millimolar levels and ascribes certain potential electronic requirements possessed by GPNA and similar analogues from that series, this work did not address the steric requirements for binding to ASCT2 within this class of glutamine analogs.

To discover ASCT2 inhibitors with greater potency and to elucidate SAR around this target, we merged structure-based design

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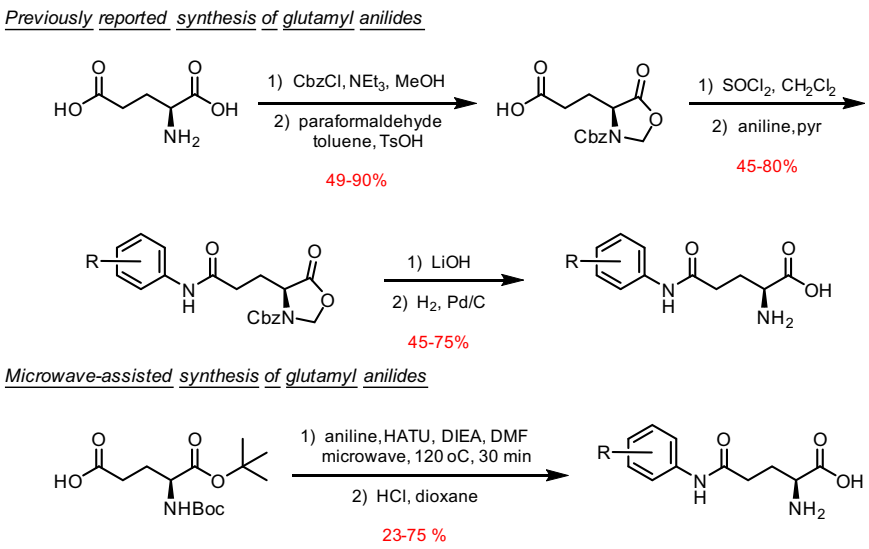


Figure 1. Synthetic route towards 2-substituted *N*γ-glutamylanilides.

Table 1
SAR of *N*γ-glutamylanilides

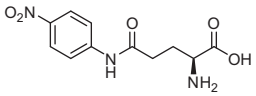
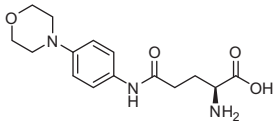
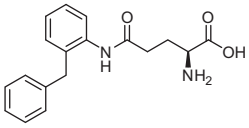
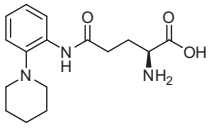
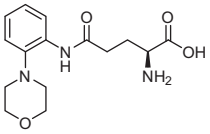
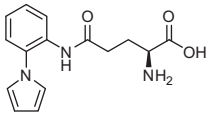
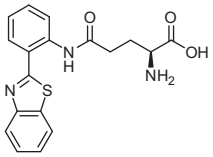
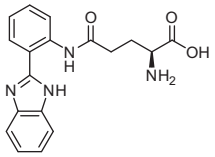
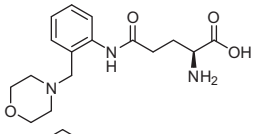
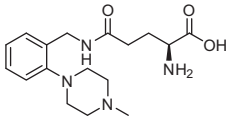
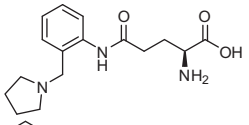
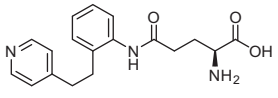
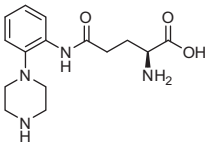
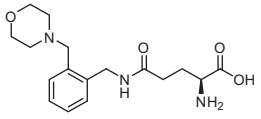
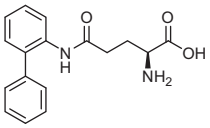
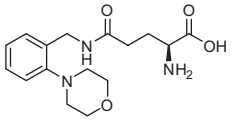
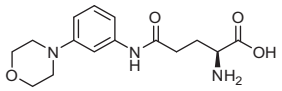
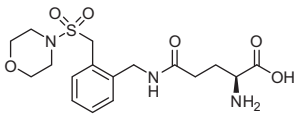
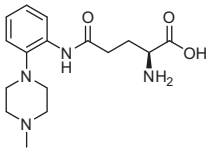
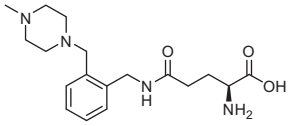
Compound		IC_{50}	Compound		IC_{50}
1		954 μM	**11**		Inactive
2		Inactive	**12**		776 μM
3		664 μM	**13**		Inactive
4		436 μM	**14**		Inactive
5		312 μM	**15**		Inactive
6		Inactive	**16**		832 μM
7		Inactive	**17**		Inactive
8		Inactive	**18**		Inactive

Table 1 (*continued*)

Compound		IC_{50}	Compound		IC_{50}
9		Inactive	**19**		677 μM
10		Inactive	**20**		697 μM

with technology-enabled medicinal chemistry and high-throughput screening to identify novel ASCT2 probes with improved potency. We also sought to explore the steric environment of the ASCT2 amino acid binding pocket to encourage future probe development. Since the crystal structure of human ASCT2 has not been elucidated, we employed computational approaches similar to the approach of Albers et al.[11] to explore potential points of intermolecular interaction and binding pockets accessible to candidate probes. From a homology model based on the open structure of the bacterial aspartate transporter GltPh in complex with inhibitor D,L-threo-benzyloxyaspartate (TBOA), PDB ID 2NWW, a number of targetable structural motifs were identified including a lipophilic pocket adjacent to the amino acid zwitterion binding site and potential hydrophilic points of contact within a loop region that was displaced by the inhibitor in the open form of the transporter. Based upon these structural elements, we expanded a focused library of candidate small molecules based on the *N*γ-glutamylanilide series to generate novel chemical matter to test the hypothesis that targeting at least a portion of these elements would result in ASCT2 inhibitors with greater potency. In support of this structure-based approach, we herein report several novel leads from this series that exhibit potency similar to or modestly greater than GPNA in live cell assays.

Initially, we developed an improved synthetic scheme to yield target *N*γ-glutamylanilides. The previously reported synthesis of GPNA and related analogs required 6 steps starting from L-glutamate in overall yields ranging from 10% to 54%.[12] In order to achieve a more facile synthesis, we took advantage of microwave-assisted organic synthesis (MAOS), which has been shown to improve reaction yields and shorten reaction times.[13–15] Utilizing this approach, we were able to rapidly generate *N*γ-glutamylanilide analogs in just two steps starting from the commercially available Boc-L-glutamic acid-*tert*-butyl ester with typical yields ranging from 23% to 75% over two steps (Fig. 1).[16]

Initial compound libraries focused on **2**, **3**, and **4** substituted *N*γ-glutamylanilides with aryl, alicyclic, and heterocyclic substitutions (Table 1). To evaluate the biological activity, compounds were initially screened at a single concentration for their ability to inhibit ^{3}H-glutamine uptake in live HEK-293 cells, an established model suitable for evaluating ASCT2 activity.[18,19] Full concentration response curves were developed for compounds that exhibited evidence of glutamine inhibition; inactive compounds were not pursued further. From early library development efforts, we were able to prioritize the 2-substitution as a determinant of ASCT2 activity among this series. For example, *N*-(2-morpholinophenyl)-L-glutamine (Table 1, compound **3**)[17] emerged as a potential lead compound of interest, exhibiting a potency roughly equivalent to that of GPNA (Table 1, compound **1**). In contrast, the analogous 4-morpholinophenyl and 3-morpholinophenyl anilides proved to be inactive, leading us to pursue 2-substituted glutamylanilides for the remainder of this study. Further development of the 2-substituted series led to three novel compounds with modestly

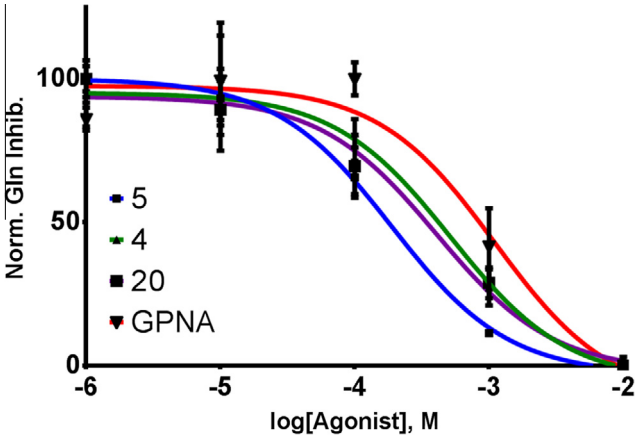


Figure 2. Concentration response curves for compounds **4**, **5**, **20**, and GPNA.

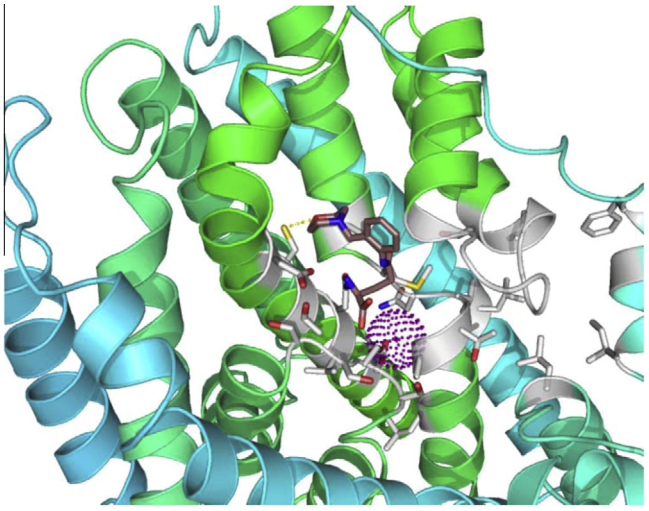


Figure 3. Docking of potential leads into ASCT2 homology model. The most potent lead, compound **5** (brown all atom colored, capped sticks) fits the homology model (protein ribbons) generated for the inhibited form of human ASCT2 and is consistent with the displacement of a key loop region (grey, right). The docked pose shown represents the best scoring SurflexDock conformation (Total score, Table S1) for compound **5** (IC_{50} = ~312 μM) that contains a morpholino moiety occupying an hydrophobic pocket adjacent to the amino acid zwitter ion binding site. One of the two reported sodium binding sites in the SLC1A5 family (purple van der Waals dotted surface) is shown centered beneath the amino acid binding site. A potential weak hydrogen bond between the morpholine oxygen of compound **5** to ASCT2 residue Cys 467 sulfhydryl side chain (yellow dashed line) is highlighted.

greater potency than GPNA, N^5-(2-(benzo[*d*]thiazol-2-yl)phenyl)-L-glutamine (Table 1, compound **4**),[20] *N*-(2-(morpholinomethyl) phenyl)-L-glutamine (Table 1, compound **5**),[21] and *N*5-(2-((4-methylpiperazin-1-yl)methyl)benzyl)-L-glutamine (Table 1, compound

20). Furthermore, **4** novel compounds among the series exhibited potencies equivalent to GPNA. Full Concentration response curves for compounds **4**, **5**, and **20** are shown in Figure 2.

Biologically active compounds were also evaluated computationally in the open human ASCT2 model. The best scoring poses for the most potent compounds identified demonstrated a compatible fit with the human ASCT2 model and, interestingly, a tendency to exhibit points of interaction with both the amino acid zwitterion binding site and an adjacent hydrophobic pocket (Fig. 3).[22]

In summary, we report three novel *N*γ-glutamylanilides as inhibitors of cellular glutamine uptake via ASCT2 with modestly greater potency than GPNA. Evaluation of this chemical series within the context of ligand docking to a homology model of human ASCT2 revealed reasonable compatibility with the ASCT2 binding site based on SurflexDock Total Scores. Based upon our data, we anticipate that compounds with the greatest potency may interact with multiple structural elements within the ASCT2 binding site, including the amino acid zwitterion binding site and the adjacent hydrophobic pocket. Ongoing efforts employing a combination of these effects may lead to compounds with even greater potency. Uniquely, previous work in the *N*γ-glutamylanilide series suggested that reduction of the glutamine amide pK_a was required for ASCT2 inhibition;[10] we did not observe this trend in our study. Advances from these studies will be reported in due course.

Acknowledgment

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16. *General procedure for the synthesis of N©—glutamylanilides*: To a microwave vial containing a solution of Boc-L-glutamic acid *tert*-butyl ester (0.165 mmol, 1.0 equiv) and HATU (0.165 mmol, 1.0 equiv) in DMF (1.65 mL) was added the amine followed by DIPEA (57.5 μL, 2.0 equiv). The vial was sealed and heated under microwave irradiation for 30 min at 120 °C. Upon completion, the reaction was partitioned between water and CH_2Cl_2, extracted 3× with CH_2Cl_2, dried over anhydrous Na_2SO_4, and concentrated under vacuum. Compounds were purified via reverse phase chromatography (5–95% acetonitrile/water) to afford the *N*-Boc-glutamylanilide-*tert*-butyl esters. The compounds were transferred to vials followed by the addition of 2.0 mL of 4.0 M HCl in dioxane. The reaction stirred at 40 °C for 4 h. The reactions were concentrated under vacuum to afford the title compounds which were used without further purification.
17. The compound was prepared according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.85 (d, J = 7.9 Hz, 1H); 7.62–7.50 (m, 3H); 4.19–4.09 (m, 5H); 3.78–3.71 (m, 4H); 3.05–2.89 (m, 2H); 2.45–2.27 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 175.69; 171.37; 132.17; 132.07; 129.32; 127.35; 123.22; 73.56; 72.45; 62.18; 55.93; 53.24; 43.75; 32.65; 26.59.
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19. Live-cell glutamine uptake assays featuring HEK293 cells were carried out in 96 well plates (CulturPlate-96, Perkin Elmer). Cells were plated at a density of 35,000 cells per well 24 h prior to carrying out the assay. Each set of conditions was carried out in at least triplicate. For the assay, cells were washed three times with 100 μL of assay buffer at pH 6.0 (containing 137 mM NaCl, 5.1 mM KCl, 0.77 mM KH_2PO_4, 0.71 mM $MgSO_47H_2O$, 1.1 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES). ^{3}H-glutamine (500 nM) in the same buffer was added concomitantly with inhibitor and allowed to incubate for 15 min at 37 °C. Following incubation period, the ^{3}H-glutamine/inhibitor is removed and the cells were washed three times with buffer. The cells were then lysed by the addition of 50 μL 1 M NaOH. For reading, 150 μL of scintillation fluid (Microscint 40, Perkin Elmer) was added and the plates were counted on a scintillation counter (Topcount, Perkin Elmer). Fifty percent inhibitory concentrations (IC_{50}) were calculated (6 concentrations) using GraphPad Prism version 6.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Reported data is the mean of $n \geqslant 3$ biological replicates.
20. The compound was prepared according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 8.62 (d, J = 8.1 Hz, 1H); 8.12 (d, J = 8.1 Hz, 1H); 8.06 (d, J = 7.8 Hz, 1H); 8.01 (dd, $J1$ = 7.9 Hz, $J2$ = 1.4 Hz, 1H); 7.59 (td, $J1$ = 7.1 Hz, $J2$ = 1.2 Hz, 1H); 7.52 (qd, $J1$ = 8.4 Hz, $J2$ = 1.5 Hz, 2H); 7.28 (td, $J1$ = 7.0 Hz, $J2$ = 1.1 Hz, 1H); 4.16 (t, J = 6.5 Hz, 1H); 2.95–2.84 (m, 2H); 2.47–2.29 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 171.05; 170.06; 168.39; 152.56; 137.00; 133.31; 131.55; 129.77; 126.62; 125.90; 123.87; 122.36; 121.42; 121.12; 119.91; 52.00; 32.95; 25.57.
21. The compound was prepared according to the general procedure. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 7.64 (d, J = 7.61, 1H); 7.56 (td, $J1$ = 7.6 Hz, $J2$ = 1.1 Hz, 1H); 7.46–7.40 (m, 2H); 4.39 (s, 2H); 4.13 (t, J = 6.6 Hz, 1H); 4.05 (dd, $J1$ = 12.7 Hz, $J2$ = 2.4 Hz, 2H); 3.82 (t, J = 12.1 Hz, 2H); 3.42 (d, J = 12.3 Hz); 2.96–2.81 (m, 2H); 2.41–2.24 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ (ppm): 173.34; 169.98; 136.88; 133.03; 131.06; 127.37; 127.19; 124.24; 63.59; 56.59; 51.89; 51.52; 51.48; 30.99; 25.26.
22. A model of an inhibitor-bound conformation of human ASCT2 was used as a target for ligand docking of proposed compounds in the 2-substituted *N*γ-glutamylanilide series using SurflexDock v.2.706 from Biopharmics (Jain et al. *J. Med. Chem.* **2003**, *46*, 499–511) as implemented in Tripos' SYBYL-X v2.1 (Certera, 1699 South Hanley Rd. St. Louis, MO 63144-2917; http://www.certara.com). Additionally, ll compounds were experimentally tested for their potency in inhibition of the uptake of ^{3}H-glutamine in a plate-based assay. Compounds with potency values equal to or better than the inhibitor GPNA were retained and assessed for their fit into the homology model of human ASCT2 to facilitate design of further ligand series in an attempt to discover structure activity relationships for development of potent inhibitors of ASCT2-mediated transport of ^{3}H-glutamine. Two-dimensional structures for all ligands were generated in ChemDraw and imported into Tripos Sybyl for conversion into three-dimensional structures using CONCORD and docking using SurflexDock (referenced above). Figures for docked complexes were generated and ray-traced using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4, Schrödinger, LLC.).