Neurochemistry International xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Novel di-aryl-substituted isoxazoles act as noncompetitive inhibitors of the system x_c^- cystine/glutamate exchanger

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Excitatory amino acid
Transporter
xCT
Uptake
Glutathione

ABSTRACT

The system x_c^- antiporter is a plasma membrane transporter that mediates the exchange of extracellular L-cystine with intracellular L-glutamate. This exchange is significant within the context of the CNS because the import of L-cystine is required for the synthesis of the antioxidant glutathione, while the efflux of L-glutamate has the potential to contribute to either excitatory signaling or excitotoxic pathology. Changes in the activity of the transport system have been linked to the underlying pathological mechanisms of a variety of CNS disorders, one of the most prominent of which is its highly enriched expression in glial brain tumors. In an effort to produce more potent system x_c^- blockers, we have been using amino-3-carboxy-5-methylisoxazole propionic acid (ACPA) as a scaffold for inhibitor development. We previously demonstrated that the addition of lipophilic aryl groups to either the #4 or #5 position on the isoxazole ring markedly increased the inhibitory activity at system x_c^-. In the present work a novel series of analogues has been prepared in which aryl groups have been introduced at both the #4 and #5 positions. In contrast to the competitive action of the mono-substituted analogues, kinetic analyses indicate that the di-substituted isoxazoles block system x_c^--mediated uptake of ^{3}H-L-glutamate into SNB-19 cells by a noncompetitive mechanism. These new analogues appear to be the first noncompetitive inhibitors identified for this transport system, as well as being among the most potent blockers identified to date. These diaryl-isoxazoles should be of value in assessing the physiological roles and molecular pharmacology of system x_c^-.

1. Introduction

The system x_c^- (Sx_c^-) antiporter is a plasma membrane transporter present in multiple cell types that typically mediates the exchange of extracellular L-cystine (L-Cys_2) with intracellular L-glutamate (L-Glu) (for review see: Bridges et al., 2012a,b; Lewerenz et al., 2013). Functioning as an obligate exchanger, the antiporter utilizes the L-Glu concentration gradient generated by the Na-dependent excitatory amino acid transporters (EAATs) to drive the uptake of L-Cys_2. Once inside the cell, the L-Cys_2 is rapidly reduced to L-cysteine (L-CysH) where among many metabolic roles it typically serves as a rate-limiting precursor in the synthesis of glutathione. While studies in most cells have focused on the role of Sx_c^- in glutathione production and antioxidant protection, the requisite efflux of L-Glu through the antiporter carries with it added significance in the CNS, where this L-Glu has the potential to contribute to excitatory signaling and excitotoxic pathology. When both the import of L-Cys_2 and the export of L-Glu are taken into account, the Sx_c^- antiporter has been linked to a very wide array of physiological and pathological processes including: brain tumor growth (Watkins and Sontheimer, 2012), drug addiction (Madayag et al., 2010; Reissner and Kalivas, 2010), chemosensitivity and chemoresistance (Huang et al., 2005), viral pathology (Espey et al., 1998), oxidative protection (Shih et al., 2006), the operation of the blood brain barrier (Hosoya et al., 2002), neurotransmitter release (Baker et al., 2002), and synaptic organization (Augustin et al., 2007). Of particular interest, is the role of Sx_c^- in gliomas, where astrocytoma cells express markedly enriched levels of Sx_c^- and the obligate export of L-Glu that accompanies the import of L-Cys_2 (possibly to meet the increased synthetic demands for GSH) appears large enough to produce an excitotoxic necrosis

Abbreviations: ACPA, amino-3-carboxy-5-methylisoxazole propionic acid; 4-bis-TFM-HMICA, (*Z*)-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-methylisoxazole-3-carboxylic acid; 5-Benzyl-4-bis-TFM-HMICA, (*Z*)-5-benzyl-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)isoxazole-3-carboxylic acid; 5-Naphthyl-4-bis-TFM-HMICA, (*Z*)-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-(naphthalen-2-ylmethyl)isoxazole-3-carboxylic acid; 5-4-TFM-Benzyl-4-bis-TFM-HMICA, (*Z*)-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-(4-(trifluoromethyl)benzyl)isoxazole-3-carboxylic acid.

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http://dx.doi.org/10.1016/j.neuint.2013.11.012

that may aid tumor growth, migration and the production of peritumoral seizures (Lyons et al., 2007; Patel et al., 2004; Sontheimer, 2008; Ye and Sontheimer, 1999). Significantly, the development of more potent and selective blockers of Sx_c^- hold considerable potential to suppress the growth of primary brain tumors (Sontheimer and Bridges, 2012).

Sx_c^- is a eukaryotic heteromeric amino acid transporter (HAT) (*aka* glycoprotein-associated amino acid exchangers) classified within the amino acid, polyamine, and organic cation (APC) transporter super-family and L-amino acid transporter (LAT) family (Palacin et al., 2005; Verrey et al., 2003). As the HAT classification suggests, Sx_c^- is composed of a glycoslated "heavy chain" required for the trafficking and cell surface expression of the dimer (4F2hc *aka* CD98, SLC3 family) and a "light chain" that mediates transport activity (xCT, SLC7A11). Structural studies on the xCT subunit indicate that it possesses 12 transmembrane domains (TMDs), intracellular N and C termini, and a reentrant loop between TMD 2 and 3 that may participate in substrate binding and translocation (Gasol et al., 2004; Jimenez-Vidal et al., 2004). While *in vivo* Sx_c^- mediates the exchange of intracellular L-Glu and extracellular L-Cys_2, transport activity can be followed by quantifying the uptake of either radiolabeled substrate, with each acting as a competitive inhibitor of the other. When compared to the EAATs, Sx_c^- exhibits a distinct ionic dependency (Cl-dependent, Na-independent) and pharmacological specificity (Bridges et al., 2012b). Unfortunately, many of compounds initially identified as inhibitors of Sx_c^- are also well known for interacting with other components of the EAA system (e.g., quisqualate, ibotenate, serine-*O*-sulfate and bromo-homo-ibotenate), decreasing their utility for functional studies in more complex physiological preparations. For these reasons we have been pursuing the development of more potent and selective inhibitors of Sx_c^-.

Not withstanding the issue of cross-reactivity, the actions of the isoxazoles and closely related heterocyclics mentioned above prompted the development of a series of derivatives based upon amino-3-carboxy-5-methylisoxazole propionic acid (ACPA) (Fig. 1). While ACPA exhibits little or no activity itself, the addition of lipophilic substituents to its isoxazole ring has yielded a growing library on increasingly potent Sx_c^- inhibitors (Matti et al., 2013; Patel et al., 2010). The more effective inhibitors within this series were based upon the introduction of benzyl or naphthyl-based aryl groups at two positions on the isoxazole ring: (i) replacing the methyl moiety at position #4 or (ii) replacing the ethyl amino acid group at position #5 via a hydrazone linkage. In all of those cases where detailed kinetic characterizations were carried out, the analogues acted as competitive blockers of the Sx_c^--mediated uptake of ^{3}H-L-Glu. These results support the conclusion that there are lipophilic (or aryl-binding) domains adjacent to the substrate site on the transport protein. To further assess the relative positions of these lipophilic domains, several 4,5-di-substituted ACPA derivatives were prepared to test whether the aryl groups were interacting with one or two distinct sites (Patel et al., 2010). While considerably less active as Sx_c^- inhibitors than a number of the mono-substituted isoxazoles, the observed inhibitory activity was consistent with the presence of two lipophilic (or aryl-binding) "pockets" on the antiporter. In the present work we have continued optimizing aryl group substituents at the 4 and 5 positions of the isoxazole ring of the ACPA template to generate some of the most potent inhibitors of Sx_c^- yet identified. Further, kinetic analyses indicate that unlike the parent mono-substituted derivatives, these "hybrid" di-substituted isoxazoles act as noncompetitive inhibitors. These findings identify a new pharmacological strategy with which to regulate Sx_c^- activity, as well as raise interesting questions as to the position of the lipophilic domains relative to the substrate-binding site on the transporter.

2. Methods and materials

2.1. Chemistry: synthesis

The novel analogues reported in this study were prepared from the bromo acetal **6** shown in Scheme 1 (Nelson et al., 2008). Suzuki–Miyaura palladium (McDaniel et al., 2011) catalyzed coupling with the corresponding arylboronic acids put the C-5 aryl in place, **7**–**9**, hydrolysis of the acetal, hydrazone condensation (Patel et al., 2010), and hydrolysis of the C-3 ester under basic conditions to arrive at the products **2**–**4** was then accomplished as previously described (Matti et al., 2013). To enhance solubility DMSO was included in the preparation of stock solutions of the inhibitors. The concentration of DMSO present following dilution into the assay solutions was $\leqslant$0.5% vol./vol. Previous studies confirmed that this amount of DMSO had no effect on transport rates.

2.2. Cell culture

SNB-19 glioma cells, purchased from American Type Culture Collection (Manassas, VA), were grown in DMEM/F-12 medium (pH 7.4) containing 1 mM pyruvate and 16 mM $NaHCO_3$ and

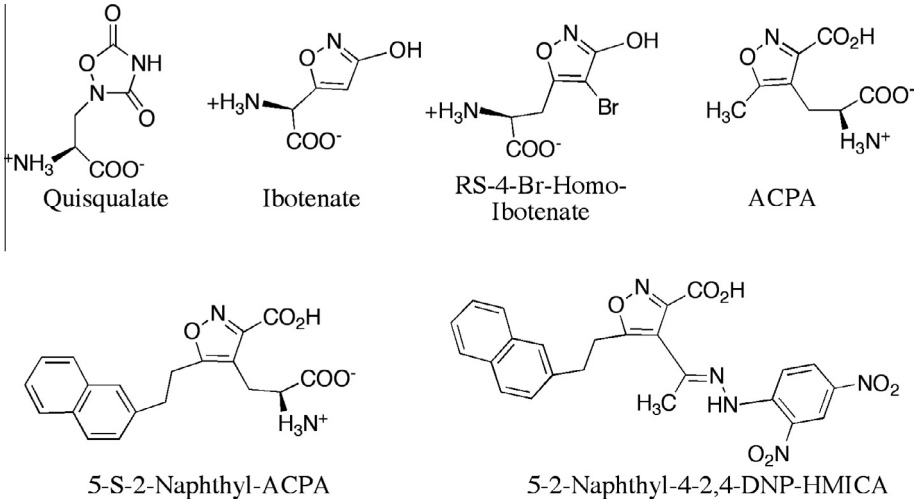

Fig. 1. Structures of isoxazole-based ligands.

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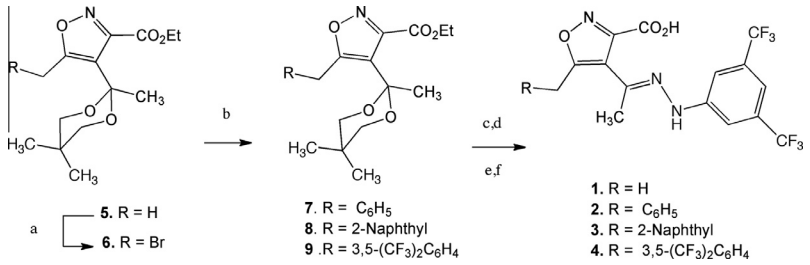

Scheme 1. Reagents and reaction conditions: (a) NBS CCl_4, (b) $ArB(OH)_2$, PdL_4, $CsCO_3$, (c) TsOH, Acetone, (d) Bis-3,5-trifluoromethylphenylhydrazine, (e) 3 N NaOH, (f) HCl.

supplemented with 10% fetal calf serum. The cells were cultured in 150 cm^2 flasks (Corning) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. In the ^{3}H-L-Glu uptake experiments, cells were seeded in 12 well culture plates (Costar) at a density of 5×10^4 cells/well and maintained for 3 days until 80–90% confluent. Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

2.3. Glutamate uptake assay

Uptake of ^{3}H-L-Glu into cultured cells was quantified using a modification of the procedure of Martin and Shane as previously described by Patel et al. (2010). Briefly, after removal of culture media, wells were rinsed three times and pre-incubated in 1 ml Na^+-free HEPES buffered (pH 7.4) Hank's balanced salt solution (HBHS) at 30 °C for 5 min. The Na^+-free buffer contained: 137.5 mM choline Cl, 5.36 mM KCl, 0.77 mM KH_2PO_4, 0.71 mM $MgSO_4 \cdot 7H_2O$, 1.1 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES. Uptake was initiated by aspiration of the pre-incubation buffer and the addition of a 500 µl aliquot of Na^+-free transport buffer containing ^{3}H-L-Glu (4–16 mCi/ml) mixed with L-Glu (10–500 µM, final concentration). In those assays that evaluated inhibitor activity, the 500 µl aliquot of transport buffer contained both the ^{3}H-L-Glu and potential inhibitors to ensure simultaneous addition. Following a 5 min incubation at 30 °C, the assays were terminated by three sequential 1 ml washes with ice cold buffer after which the cells were dissolved in 1 ml of 0.4 M NaOH for 24 h. An aliquot (200 µl) was then transferred into a 5 ml glass scintillation vial and neutralized with the addition of 5 µl glacial acetic acid followed by 3.5 ml Liquiscint© scintillation fluid (National Diagnostics) to each sample. Incorporation of radioactivity was quantified by liquid scintillation counting (LSC, Beckman LS 6500). Values are reported as mean ± S.E.M. and are corrected for non-specific uptake (e.g., leakage and binding) by subtracting the amount of ^{3}H-L-Glu accumulation at 4 °C.

2.4. Kinetic analyses

Michaelis–Menten and Lineweaver–Burk (LWB) plots and associated kinetic parameters (K_m and V_{max}) for transport inhibitors were estimated using a non-linear curve fitting analysis (KaleidaGraph 4.1.3). K_i determinations from LWB and Eadie–Hofstee replots were calculated using linear-regression analysis (KaleidaGraph 4.1.3).

3. Results

3.1. Inhibition of Sx_c^--mediated uptake of ^{3}H-L-glutamate

The inhibitory activity of the compounds was determined by quantifying the ability of the analogues to reduce the accumulation of ^{3}H-L-Glu into human SNB-19 glioblastoma cells under Cl-dependent (Na-free) conditions. A number of glioma cell lines, including SNB-19, express markedly higher levels of Sx_c^- and reduced levels of the sodium-dependent EAATs than do primary astrocytes, making them well suited for pharmacological assays (Ye et al., 1999). The compounds were initially screened at a single concentration of substrate (100 µM ^{3}H-L-Glu) and isoxazole (500 µM) to confirm to inhibitory activity. As reported in Table 1, the analogues almost completely blocked the uptake of the ^{3}H-L-Glu into the cells under these conditions. (The data are reported as % of control uptake, thus the smaller the number the greater the level of inhibition.) The activity for 4-bis-TFM-HMICA (Compound #1, Table 1) has been previously reported, although its kinetic mechanism had not been examined in detail (see below). These initial screenings confirmed that the introduction of aryl groups to the isoxazole scaffold as either mono-substitutions at the 4/5 positions or di-substitutions at both could produce potent inhibitors of Sx_c^-.

3.2. Competitive inhibition of Sx_c^- by the mono-substituted isoxazoles

The inhibitory action of 4-bis-TFM-HMICA was characterized in greater detail using a standard Michaelis–Menten analysis in which the concentration of both the isoxazole derivative and substrate (^{3}H-L-Glu) were systematically varied. A representative series of plots from assays in the presence of 4-bis-TFM-HMICA are depicted in Fig. 2, which includes both a V vs. S plot (Panel A) and a $1/V$ vs. $1/S$ Lineweaver–Burk (LWB) plot (Panel B). The pattern of inhibition displayed by 4-bis-TFM-HMICA is representative of a competitive mechanism and is consistent with the inhibitory action of related isoxazoles that have been modified with aryl groups at the C4 position of the heterocyclic ring (Matti et al., 2013; Patel et al., 2010). Replots of the slopes of the lines from the LWB plot vs. [I] (Fig. 2, Panel C) were used to determine K_i. An average of $n = 3$ such analyses yielded a K_i of 61 ± 5 µM for 4-bis-TFM-HMICA (Table 1). A replot of averaged $K_{m,apparent}$ vs. [I] was also linear and yielded a similar K_i value (≈80 µM), as would be expected for a competitive inhibitor (plot not shown) (Segel, 1993).

3.3. Noncompetitive inhibition of Sx_c^- by di-substituted isoxazoles

The di-substituted isoxazole analogues with aryl groups appended at both the #4 and #5 position were similarly assayed to determine a mechanism of inhibition. Representative plots are shown in Fig. 3 for 5-4-TFM-Benzyl-4-bis-TFM-HMICA (Compound #4, Table 1), the most potent of the blockers examined. In contrast to mono-substitutions made at either of these positions, all three di-substituted analogues exhibited a pattern of inhibition consistent with noncompetitive inhibition. Both the V vs. S and LWB plots demonstrate that the inhibitors produced a decrease in V_{max} with little or no change in K_m, as would be expected of noncompetitive inhibitors. Again, replots of the slopes from the LWB graphs were linear and used to determine K_i values (Table 1). A representative slope replot for 5-4-TFM-Benzyl-4-bis-TFM-HMICA is included in Fig. 3, Panel C (average $K_i = 3 \pm 1$ µM, $n = 5$). Competitive and

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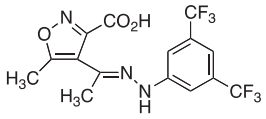
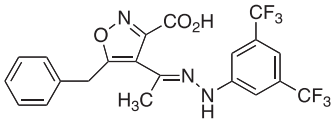
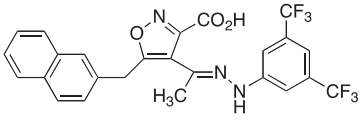
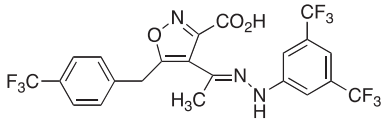
Table 1
Percent of control uptake of ^{3}H-L-Glu and K_i values for inhibition of Sx_c^-.

Compound (500 μM)	Sx_c^--mediated ^{3}H-L-Glu uptake screening assay (% of control)	K_i value from LWB slope replots	Inhibitory mechanism
 1 4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-methylisoxazole-3-carboxylic acid ***4-bis-TFM-HMICA***	14 ± 4 (n = 3)	61 ± 5 μM (n = 3)	Competitive
 2 5-benzyl-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)isoxazole-3-carboxylic acid ***5-Benzyl-4-bis-TFM-HMICA***	6 ± 1 (n = 3)	22 ± 2 μM (n = 3)	Noncompetitive
 3 4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-(naphthalen-2-ylmethyl)isoxazole-3-carboxylic acid ***5-Naphthyl-4-bis-TFM-HMICA***	14 ± 4 (n = 3)	13 ± 1 μM (n = 3)	Noncompetitive
 4 4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-(4-(trifluoromethyl)benzyl)isoxazole-3-carboxylic acid ***5-4-TFM-Benzyl-4-bis-TFM-HMICA***	6 ± 9 (n = 5)	3 ± 1 μM (n = 5)	Noncompetitive

SNB-19 cells were assayed for ^{3}H-L-glutamate (25–500 μM) uptake under Cl-dependent (Na-free) conditions in the presence of a range of inhibitor concentrations (0–100 μM). K_i values were determined directly from a replot of LWB slope vs. [I] values using linear regression fitting (KaleidaGraph 4.1.3). Values are reported as mean ± SEM ($n \geqslant 3$). The type of inhibition observed was determined based on Lineweaver–Burk and Eadie–Hofstee replots of ^{3}H-L-glutamate uptake saturation curves.

noncompetitive inhibitors can also be distinguished by replots from LWB graphs of either $K_{m,\text{apparent}}$ vs. [I], linear for competitive mechanisms, or $1/V_{\text{max,apparent}}$ vs. [I], linear for noncompetitive inhibition (Segel, 1993). In the instance of 5-4-TFM-Benzyl-4-bis-TFM-HMICA the replot of $1/V_{\text{max,apparent}}$ vs. [I] was indeed linear and yielded a K_i of ≈8 μM (plots not shown). If both the slope replot and the $1/V_{\text{max,apparent}}$ vs. [I] replot yield similar K_i values, as is the case for 5-4-TFM-Benzyl-4-bis-TFM-HMICA, the analogue is considered to be acting as a "pure" noncompetitive inhibitor, where the binding of the compound does not alter the binding affinity of the substrate (Segel, 1993). Interestingly, while the K_i values determined by these two replot methods for 5-4-TFM-Benzyl-4-bis-TFM-HMICA and 5-Naphthyl-4-bis-TFM-HMICA (Compound #3, Table 1) were not markedly different, the K_i values for 5-Benzyl-4-bis-TFM-HMICA (Compound #2, Table 1) generated from the $1/V_{\text{max,apparent}}$ vs. [I] replots was ≈60 μM (average n = 3), a substantial increase over the ≈20 μM K_i determined by the slope replot method (plots not shown). This would suggest that in contrast to the other noncompetitive inhibitors, the binding of 5-Benzyl-4-bis-TFM-HMICA to Sx_c^- also decreased the affinity with which the transporter binds L-Glu.

As the identification of the di-substituted isoxazoles as noncompetitive inhibitors was unexpected, the kinetic data was also analyzed using the Eadie–Hofstee method as a second graphical approach. As depicted in Fig. 3 (Panel D) for 5-4-TFM-Benzyl-4-bis-TFM-HMICA, the plots of V vs. $V/[S]$ for it and 5-Naphthyl-4-bis-TFM-HMICA yielded a series of parallel lines, a pattern indicative of noncompetitive inhibition. The Eadie–Hofstee plot for 5-Benzyl-4-bis-TFM-HMICA generated non-intersecting lines consistent with mixed inhibition (plot not shown). This pattern of mixed inhibition is in agreement with the analysis described above in which the binding of the inhibitor also reduces the ability

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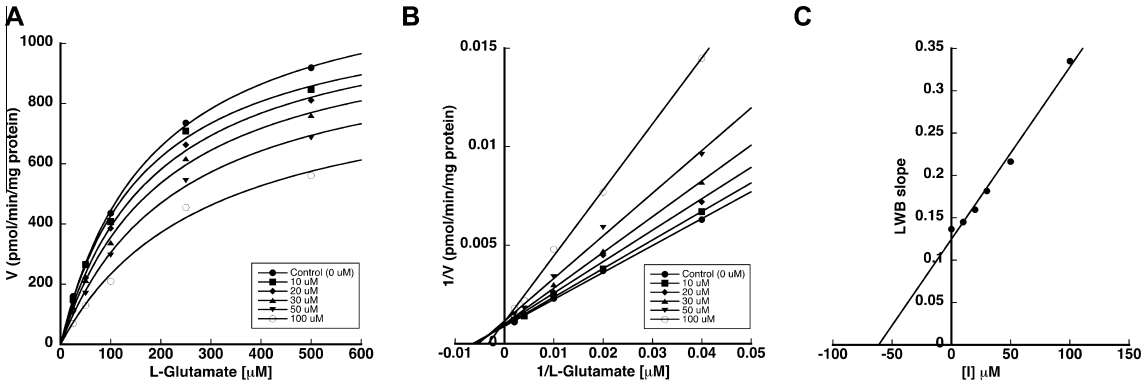

Fig. 2. Representative kinetic analyses and K_i determination for 4-bis-TFM-HMICA. SNB-19 cells were assayed for ^{3}H-L-glutamate uptake under Cl-dependent (Na-free) conditions in the presence of a range of inhibitor concentrations. Data are plotted as pmol/mg/mg protein and have been corrected for non-specific uptake and leakage. *Panels A and B*, K_m (≈150 μM) and V_{max} (≈1100 pmol/min/mg protein) values were determined by non-linear curve fitting of the saturation curves and linear regression analysis of LWB plots (KaleidaGraph 4.1.3). *Panel C*, K_i (70 μM) values were determined by linear regression of LWB slope vs. [*I*] replot.

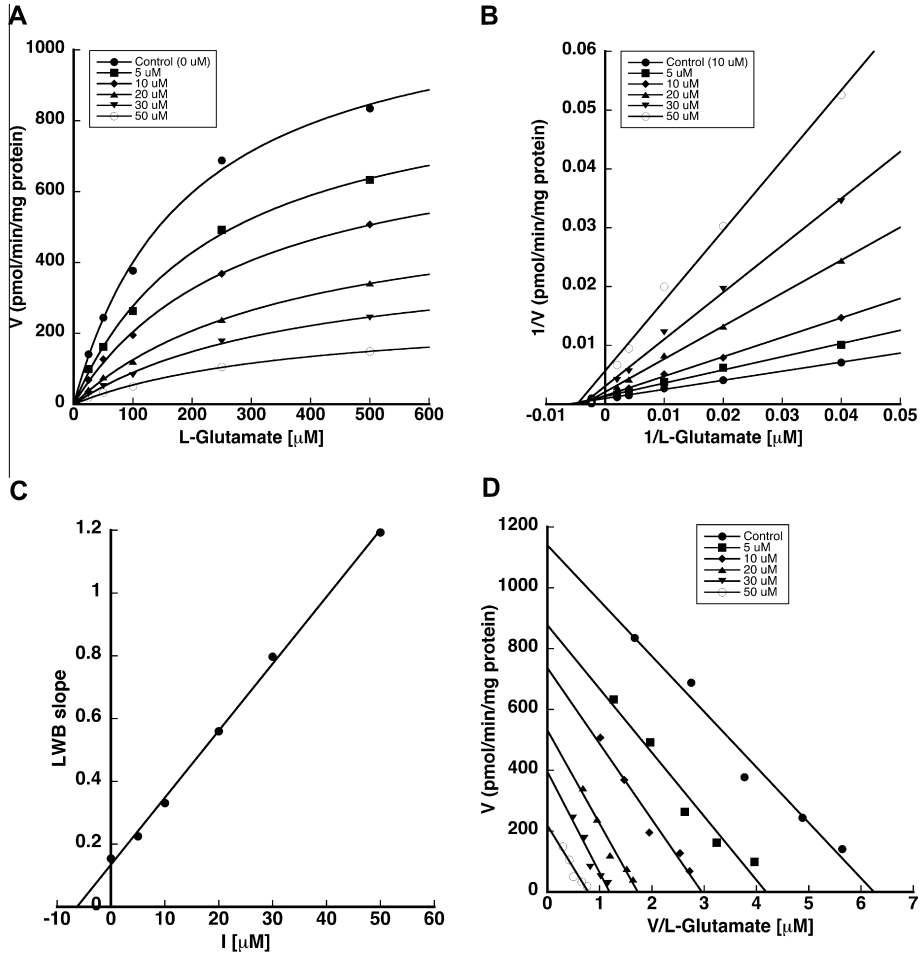

Fig. 3. Representative kinetic analyses of 5-4-TFM-Benzyl-4-bis-TFM-HMICA displaying noncompetitive inhibition. *Panel A*, Michaelis–Menten analysis; *Panel B*, LWB replot; *Panel C*, LWB slope vs. [*I*] replot; *Panel D*, Eadie–Hofstee replot. K_m (≈160 μM), V_{max} (≈1100 pmol/min/mg protein) and K_i (3 μM) values for plots shown were determined using KaleidaGraph (4.1.3).

of the transporter to bind L-Glu as a substrate. When the K_m/V_{max} (equivalent to a LWB slope) and $1/V_{max,apparent}$ values garnered from the Eadie–Hofstee graphs were replotted vs. [*I*], the resulting K_i values were very similar to those determined from replots of the LWB graphs (plots not shown).

4. Discussion

To the best of our knowledge, the diaryl-substituted isoxazoles described here represents the first noncompetitive blockers to be identified for the Sx_c^- transport system. This mechanism of action

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was not anticipated, as these compounds emerged during the course of structure–activity-relationship (SAR) studies aimed at the optimization of rationally designed competitive inhibitors. Thus, the isoxazole scaffold was selected for analogue development because of the previously characterized inhibitory activity of a number of closely related compounds, including quisqualate, ibotenate, and bromo-homoibotenate (Bridges et al., 2012b). Employing amino-3-carboxy-5-methylisoxazole (ACPA) as a starting point, it was found that the inhibitory activity increased as aryl groups were systematically introduced at either the 5 position on the isoxazole ring (replacing the methyl group of ACPA) or the 4 position of the isoxazole via a hydrazone linkage (replacing α-amino acid moiety) (Matti et al., 2013; Patel et al., 2010). Of these derivatives, S-2-naphthyl-ethyl-ACPA emerged as one of the more potent Sx_c^- inhibitors. Detailed kinetics analysis similar to those employed in the present study confirmed that it competitively inhibited the Sx_c^--mediated uptake of ^{3}H-L-Glu into SNB-19 cells with a K_i of about 50 μM (Patel et al., 2010). Given the structural similarities between the analogues, it was assumed at that time that 4-bis-TFM-HMICA, in which the aryl substitution was made at the 4 position of the isoxazole ring was also acting as a competitive manner. That this was indeed the case is confirmed in the present report, where Michaelis–Menten and LWB analyses demonstrated it competitively inhibited the uptake of ^{3}H-L-Glu into SNB-19 cells with a K_i of about 60 μM. The results also confirm the utility and potency of 4-substituted aryl isoxazole as inhibitors. These SAR data were particularly valuable, as the results suggest that there are lipophilic (or aryl-binding) pockets adjacent to the substrate binding site on Sx_c^- and that the presence of these domains can be exploited, much in the same manner as has been done with the EAATs (Bridges and Patel, 2009), to develop more potent and specific inhibitors.

SAR-based comparisons between the 4- and 5-aryl substituted isoxazole raised intriguing questions as to whether the aryl groups were interacting with the same domains on the transporter (necessitating a change in the manner in which the isoxazole ring was accommodated) or that there are two distinct lipophilic domains present on Sx_c^-. This issue was directly addressed through the synthesis and testing of comparable isoxazoles that were modified at both positions. Although markedly less potent than the monosubstituted isoxazoles, the ability of these first "hybrid" analogues to also block the Sx_c^--mediated uptake of ^{3}H-L-Glu into SNB-19 cells supported the conclusion that there were at least two distinct lipophilic (aryl-binding) domains on the transporter, although the mechanism of inhibition remained to be elucidated (Patel et al., 2010). These results prompted the optimization of the di-substituted isoxazoles and the preparation of the three analogues characterized in the present study. As initial screening assays suggested that these new isoxazoles were among the best inhibitors yet developed for Sx_c^- (Table 1), kinetic studies were carried out to determine K_i values. Surprisingly, the Michaelis–Menten and LWB analyses of the concentration dependence with which the di-substituted isoxazoles inhibited the Sx_c^--mediated uptake of ^{3}H-L-Glu into SNB-19 cells revealed a noncompetitive mechanism rather than a competitive one. Similarly, when the uptake rates were analyzed using Eadie–Hofstee plots as a second approach, the resulting pattern of lines was again indicative of a noncompetitive inhibitor. The Lineweaver–Burk plots were further analyzed by repotting both the slope and $1/V_{max,apparent}$ ($1/V_{intercept}$) vs. [I]. If a compound is acting as "pure" noncompetitive inhibitor, then these two replots should both be linear and yield the same value for K_i (i.e., $-X$ intercept). This was the case for 5-4-TFM-Benzyl-4-bis-TFM-HMICA, where a K_i value of about 5 μM places it among the most potent Sx_c^- inhibitors yet identified (Bridges et al., 2012b). Mechanistically, the data suggest this inhibitor can bind to either the empty or substrate-occupied transporter to produce a "dead end" complex (i.e., an inactive transporter) and that is does so in a manner that does not alter the binding of the substrate (e.g., L-Glu). Among the three isoxazoles examined, only 5-Benzyl-4-bis-TFM-HMICA exhibited a substantial difference between the two replots methods, yielding a K_i of about 20 μM from the slope replot and 60 μM from the $1/V$ intercept replot. While there are different types of mixed inhibition involving multiple sites of interaction that could produce such results, the most straightforward interpretation suggests that 5-Benzyl-4-bis-TFM-HMICA is noncompetitively inhibiting Sx_c^- with a K_i of ≈20 μM, but that when it is bound the analogue also reduces the affinity of the transporter for its substrate (Segel, 1993). Such an interpretation is also supported by the fact that the other closely related di-substituted isoxazoles are both more potent and act as "pure" noncompetitive inhibitors.

The switch from a competitive to a noncompetitive mechanism observed with the di-substituted isoxazoles raises intriguing questions as to the molecular relationships between the potential sites of action on Sx_c^-. Based upon the competitive action of the monosubstituted isoxazoles, such as 4-bis-TFM-HMICA, it was hypothesized that the isoxazole portion of the molecule was acting as an L-Glu mimic and interacting with substrate binding domains, while the trifluoromethyl-substituted benzyl group was interacting with an adjacent lipophilic (aryl-binding) domain. As a consequence of occupying some portion of the substrate site, it competitively blocks the binding of L-Glu. The 5-monosubstituted isoxazole would be hypothesized to bind in an analogous manner; only it would be interacting with a different lipophilic domain, the presence of which is supported by the inhibitory action of the di-substituted "hybrid" isoxazoles. If this is occurring, the present demonstration of the noncompetitive action of the di-substituted analogues may reflect the optimal binding of the two aryl groups to their respective lipophilic (aryl-binding) domains in a manner that still inhibits uptake, but also repositions the isoxazole ring such that it is no longer directly precludes the binding of L-Glu. This, in turn, would lead to the hypothesis that the substrate binding and lipophilic domains are located in close proximity to one another. The competitive action of the other isoxazoles would also be consistent with this model. Further, lipophilic (aryl-binding) domains have been identified in a number of other transport systems that are in close proximity to the substrate binding domains, including the EAATs and the serotonin transporter (SERT) (Bridges and Patel, 2009; Leary et al., 2011; Zhou et al., 2009b). However, the possibility that the aryl-substituted isoxazoles are acting at a site well removed from the substrate-binding site cannot be excluded. In such an instance, analogue binding would have to produce conformational changes that inhibit transport activity in a manner that may or may not also block L-Glu binding, reflecting competitive and noncompetitive mechanisms, respectively. Whichever mechanisms are ultimately resolved, the marked increase in the potency of the present inhibitors, likely also reflect the presence of the trifluoromethyl groups on the aryl substituents. The role of fluorine in enhancing the binding affinity of the ligands likely arises from either the proper filling of apolar pockets, multipolar C–F···H–N, C–F···C=O, and C–F···H–Cα interactions or polar interactions with electropositive side chains (Bissantz et al., 2010; Muller et al., 2007; Zhou et al., 2009a; Zurcher and Diederich, 2008). It is entirely plausible that with three trifluoromethyl groups present on the most potent inhibitor, 5-4-TFM-Benzyl-4-bis-TFM-HMICA, that both types of interactions contribute to the enhanced binding affinity. Future work within this evolving library of compounds will focus on the continued optimization of these aryl group interactions. The protein domains with which these ligands interact have been postulated to represent either intermediate binding sites guiding substrate permeation (e.g., "vestibule sites") or potential allosteric regulatory sites (e.g., "halogen

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binding pockets") (Singh et al., 2008; Zhou et al., 2009b). Ligands occupying these sites may prevent the transporter from accessing one or more conformational states within the alternate access mechanism that are required for substrate translocation. Ironically, further inhibitor development will likely include exploring different linkers between the aryl groups that now, in the face of a noncompetitive mechanism, may no longer require the inclusion of a L-glutamate (or L-cystine) mimic.

Acknowledgments

This work was supported in part by NIH NINDS Grants R21NS067466 and P30-NS055022.

References

Augustin, H., Grosjean, Y., Chen, K., Sheng, Q., Featherstone, D., 2007. Nonvesicular release of glutamate by glial xCT transporters suppresses glutamate receptor clustering *in vivo*. J. Neurosci. 27, 111–123.

Baker, D.A., Xi, Z.X., Hui, S., Swanson, C.J., Kalivas, P.W., 2002. The origin and neuronal function of *in vivo* nonsynaptic glutamate. J. Neurosci. 22 (20), 9134–9141.

Bissantz, C., Kuhn, B., Stahl, M., 2010. A medicinal chemist's guide to molecular interactions. J. Med. Chem. 53, 5061–5084.

Bridges, R.J., Patel, S.A., 2009. Pharmacology of glutamate transport in the CNS: substrates and inhibitors of excitatory amino acid transporters (EAATs) and the glutamate/cystine exchanger system x_c^-. In: Napier, S. (Ed.), Topics in Medicinal Chemistry. Springer, NY, pp. 187–222.

Bridges, R.J., Lutgen, V., Lobner, D., Baker, D.A., 2012a. Thinking outside the cleft to understand synaptic activity: contribution of the cystine–glutamate antiporter (system x_c^-) to normal and pathological glutamatergic signaling. Pharmacol. Rev. 64 (3), 780–802.

Bridges, R.J., Natale, N.R., Patel, S.A., 2012b. System x_c^- cystine/glutamate antiporter, an update on molecular pharmacology and roles within the CNS. Br. J. Pharmacol. 165 (1), 20–34.

Espey, M.G., Kustova, Y., Sei, Y., Basile, A.S., 1998. Extracellular glutamate levels are chronically elevated in the brains of LP-BM5-infected mice: a mechanism of retrovirus-induced encephalopathy. J. Neurochem. 71 (5), 2079–2087.

Gasol, E., Jimenez-Vidal, M., Chillaron, J., Zorzano, A., Palacin, M., 2004. Membrane topology of system x_c^- light subunit reveals a re-entrant loop with substrate-restricted accessibility. J. Biol. Chem. 279 (30), 31228–31236.

Hosoya, K., Tomi, M., Ohtsuki, S., Takanaga, H., Saeki, S., Kanai, Y., et al., 2002. Enhancement of L-cystine transport activity and its relation to xCT gene induction at the blood–brain barrier by diethyl maleate treatment. J. Pharm. Exp. Ther. 302 (1), 225–231.

Huang, Y., Barbacioru, C., Sadee, W., 2005. Cystine–glutamate transporter SLC7A11 in cancer chemosensitivity and chemoresistance. Cancer Res. 65 (16), 7446–7454.

Jimenez-Vidal, M., Gasol, E., Zorzano, A., Nunes, V., Palacin, M., Chillaron, J., 2004. Thiol modification of cysteine 327 in the eighth transmembrane domain of the light subunit xCT of the heteromeric cystine/glutamate antiporter suggests close proximity to the substrate binding site/permeation pathway. J. Biol. Chem. 279 (12), 11214–11221.

Leary, G.P., Holley, D.C., Stone, E.F., Lyda, B.R., Kalachev, L.V., Kavanaugh, M.P., 2011. The central cavity in trimeric glutamate transporters restricts ligand diffusion. Proc. Natl. Acad. Sci. USA 108 (36), 14980–14985.

Lewerenz, J., Hewett, S.J., Huang, Y., Lambros, M., Gout, P.W., Kalivas, P.W., et al., 2013. The cystine/glutamate antiporter system x_c^- in health and disease: from molecular mechanisms to novel therapeutic opportunities. Antioxid. Redox Signal. 18 (5), 522–555.

Lyons, S.A., Chung, W.J., Weaver, A.K., Ogunrinu, T., Sontheimer, H., 2007. Autocrine glutamate signaling promotes glioma cell invasion. Cancer Res. 67, 9463–9471.

Madayag, A., Kau, K.S., Lobner, D., Mantsch, J.R., Wisniewski, S., Baker, D.A., 2010. Drug-induced plasticity contributing to heightened relapse susceptibility: neurochemical changes and augmented reinstatement in high-intake rats. J. Neurosci. 30 (1), 210–217.

Matti, A.A., Mirzaei, J., Rudolph, J., Smith, S.A., Newell, J.L., Patel, S.A., et al., 2013. Microwave accelerated synthesis of isoxazole hydrazide inhibitors of the system x_c^- transporter: initial homology model. Bioorg. Med. Chem. Lett. 23 (21), 5931–5935.

McDaniel, S.W., Keyari, C.M., Rider, K.C., Natale, N.R., Diaz, P., 2011. Suzuki–Miyaura cross-coupling of benzylic bromides under microwave conditions. Tetrahedron Lett. 52 (43), 5656–5658.

Muller, K., Faeh, C., Diederich, F., 2007. Fluorine in pharmaceuticals: looking beyond intuition. Science 317 (5846), 1881–1886.

Nelson, J.K., Twamley, B., Villalobos, T.J., Natale, N.R., 2008. The catalytic asymmetric addition of alkyl- and aryl-zinc reagents to an isoxazole aldehyde. Tetrahedron Lett. 49 (41), 5957–5960.

Palacin, M., Nunes, V., Jimenez-Vidal, M., Font-Llitjos, M., Gasol, E., Pineda, M., et al., 2005. The genetics of heteromeric amino acid transporters. Physiology 20, 112–124.

Patel, S.A., Warren, B.A., Rhoderick, J.F., Bridges, R.J., 2004. Differentiation of substrate and non-substrate inhibitors of transport system x_c^-: an obligate exchanger of L-glutamate and L-cystine. Neuropharmacology 46, 273–284.

Patel, S.A., Rajale, T., O'Brien, E., Burkhart, D.J., Nelson, J.K., Twamley, B., et al., 2010. Isoxazole analogues bind the system x_c^- transporter: structure–activity relationship and pharmacophore model. Bioorg. Med. Chem. 18, 202–213.

Reissner, K.J., Kalivas, P.W., 2010. Using glutamate homeostasis as a target for treating addictive disorders. Behav. Pharmacol. 21 (5–6), 514–522.

Segel, I., 1993. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, second ed. Wiley.

Shih, A., Erb, H., Sun, X., Toda, S., Kalivas, P., Murphy, T., 2006. Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. J. Neurosci. 41, 10514–10523.

Singh, S.K., Piscitelli, C.L., Yamashita, A., Gouaux, E., 2008. A competitive inhibitor traps LeuT in an open-to-out conformation. Science 322 (5908), 1655–1661.

Sontheimer, H., 2008. A role for glutamate in growth and invasion of primary brain tumors. J. Neurochem. 105 (2), 287–295.

Sontheimer, H., Bridges, R.J., 2012. Sulfasalazine for brain cancer fits. Expert Opin. Investig. Drugs 21 (5), 575–578.

Verrey, F., Closs, E.I., Wagner, C.A., Palacin, M., Endou, H., Kanai, Y., 2003. CATs and HATs: the SLC7 family of amino acid transporters. Eur. J. Physiol. 447 (5), 532–542.

Watkins, S., Sontheimer, H., 2012. Unique biology of gliomas: challenges and opportunities. Trends Neurosci. 35 (9), 546–556.

Ye, Z.C., Sontheimer, H., 1999. Glioma cells release excitotoxic concentrations of glutamate. Cancer Res. 59, 4383–4391.

Ye, Z., Rothstein, J.D., Sontheimer, H., 1999. Compromised glutamate transport in human glioma cells: reduction–mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine–glutamate exchange. J. Neurosci. 19 (24), 10767–10777.

Zhou, P., Zou, J., Tian, F., Shang, Z., 2009a. Fluorine bonding – how does it work in protein–ligand interactions? J. Chem. Inf. Model. 49 (10), 2344–2355.

Zhou, Z., Zhen, J., Karpowich, N.K., Law, C.J., Reith, M.E., Wang, D.N., 2009b. Antidepressant specificity of serotonin transporter suggested by three LeuT-SSRI structures. Nat. Struct. Mol. Biol. 16 (6), 652–657.

Zurcher, M., Diederich, F., 2008. Structure-based drug design: exploring the proper filling of apolar pockets at enzyme active sites. J. Org. Chem. 73 (12), 4345–4361.

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