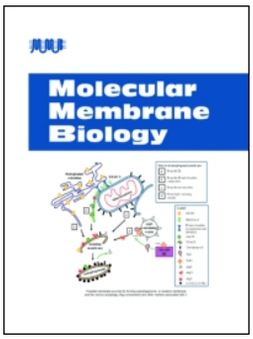

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REVIEW ARTICLE

Emerging roles of system x_c^- antiporter and its inhibition in CNS disorders

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Abstract

System x_c^- is an antiporter belonging to the hetero(di)meric amino acid transporter family. It is located on astrocytes as well as on blood-brain barrier within the CNS. It plays a pivotal role in free radical neutralization as well as neuronal signalling by regulating the glutathione production which occurs via the exchange of intracellular glutamate with extracellular cystine at 1:1 molar ratio. Understandably, it is a vital component responsible for the maintenance of neuronal homeostasis (e.g. redox state). Hence, it could be postulated that any perturbation in system x_c^- function may contribute, directly or indirectly, to the pathophysiology of a variety of CNS disorders like Alzheimer's disease, schizophrenia, drug addiction, depression, multiple sclerosis, hypoglycemic neuronal cell death, glioma, and excitotoxicity, making system x_c^- a promising target for treating CNS disorders. In recent times, recognizing the potential of this target, variety of inhibitors has been synthesized by modifying commercially available potent inhibitors including sulfasalazine, erastin, and sorafenib. Although, they have demonstrated efficacy, the in-depth data is still lacking to warrant their use for the treatment of aforementioned CNS disorders. In this review, we discuss the in-depth role of system x_c^- transporter in maintaining normal physiology as well as in the pathophysiology of CNS diseases. Additionally, we have also listed some of the potent inhibitors of system x_c^-. In conclusion, the critical role of system x_c^- in multiple CNS disorders and advanced research on its inhibitors have promising future prospects for better management of the CNS ailments.

Keywords

CNS disorders, excitotoxicity, glutamate, glutathione, sulfasalazine, transporter

History

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Introduction

The origin of neuronal diseases is very challenging as it involves understanding of the complex networks of receptors, membrane-expressed enzymes, transporters, and molecular mechanisms of neurotransmitters (Bridges et al., 2012a; DiNunzio & Williams, 2008). The central nervous system (CNS) is the most complicated system which controls almost all of the complex activities including sensory inputs, their interpretation and appropriate response in human body (Alazne et al., 2013). Hence, any disturbances in this regulatory system can result in multiple complications and disorders. Although difficult, it can be envisaged that, if possible, modification of one target could serve as a suitable treatment strategy for amelioration of several related CNS disorders.

System x_c^- transporter belongs to the family of heteromeric amino acid transporters (HAT) (Bridges et al., 2012b) that plays a role in regulating intracellular cystine level and thus intracellular glutathione (GSH) production in mouse and human brain (Burdo et al., 2006). It was identified three decades ago as a Na^+-independent exchanger (Bannai & Kitamura, 1980) that plays a role as a cystine-glutamate antiporter or exchanger. The exact role was to promote the uptake of one molecule of cystine with release of one molecule of glutamate dictated by the relative concentration gradients of the substrates across the cell membrane (Bannai et al., 1986; Bridges et al., 2012a; Lo et al., 2008). The expression of system x_c^- was found to be higher in astrocytes as compared to other body tissues known to release non-vesicular glutamate for the regulation of synaptic activity (Baker et al., 2002b; Kupchik et al., 2012; Moran et al., 2005; Pow, 2001). Owing to the pivotal role of system x_c^- in the maintenance of neuronal plasticity as well as glutathione production, any aberration in this system could contribute to pathophysiological changes in cystine and glutamate related functions in brain (Moussawi et al., 2011). This assumption has been corroborated with the research findings documented in various investigations carried out on epileptic seizures and glioma (De Bundel et al., 2011; Lewerenz et al., 2014; Takeuchi et al., 2013), neurodegenerative diseases like Alzheimer's disease (AD) (Barger, 2004; Qin et al., 2006; Schallier et al., 2011), Parkinson's disease (PD) (Massie et al., 2011), and multiple sclerosis (MS) (Pampliega et al., 2011). Besides this, there are reports which suggest indirect involvement of system x_c^- in the psychological disorders

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such as schizophrenia, mania, depression, and drug addiction (Lutgen, 2012). Based on these observations, it can be proposed that system x_c^- could serve as a potential common therapeutic target for numerous neurological disorders.

Recently, in the inhibition studies conducted on system x_c^-, sulfasalazine (Table 1) was identified as a potent inhibitor. Although, it was a potent molecule, it lacked the desired pharmacokinetic profile. There had been several attempts to modify sulfasalazine to improve its pharmacokinetic profile (Shukla et al., 2011). These resulted in no further improvement in the parent structure. In recent time two small molecules, Erastin and Sorafenib have been studied for their *in vitro* system x_c^- inhibition activity (Dixon et al., 2014; Singer et al., 2015). Amongst these two potent inhibitors, Erastin and its analogues are still under their *in vitro* pharmacological evaluation (Dixon et al., 2014; Singer et al., 2015) while Sorafenib is currently being investigated for its clinical efficacy in glioblastoma (National Cancer Institute, 2015). Considering the relevance of this target, this paper reviews the current understanding of the target, system x_c^- antiporter, in regulating neuronal physiology and pathophysiology. In addition to this, the paper also provides insights about the research outcomes on the novel inhibitors of system x_c^-.

Structure of system x_c^-

In contrast to other known endogenous amino acid transporters studied until now, system x_c^- (Kilberg & Hausingger, 1992), which belongs to the family heterodimeric amino acid transporters, comprises of two subunits: a light chain and a heavy chain (Table 1) (Verrey et al., 2004; Wagner et al., 2001). This heterodimer consists of the 4F2 heavy chain (4F2hc), covalently linked via a disulphide bond to one of the several hydrophobic, non-glycosylated 'light chain xCT' (Bridges et al., 2001).

4F2 heavy chain

The human heavy chain of 4F2 is made up of 529 amino acids (AAs) (94 kDa, glycosylated; 72 kDa, unglycosylated) (Mosckovitz et al., 1993; Wells et al., 1992). The amino acid sequence indicates that the 4F2hc have only one transmembrane helix where intracellular domain holds N-terminus while C-terminus is located extracellularly (Mosckovitz et al., 1993). This chain has been classified as type II membrane proteins. Until now, there is dearth of complete information that clearly elucidates the structure of 4F2hc. Nevertheless, there is information about the glycosylation pattern in 4F2hc which evidently shows that a bulky portion of the C-terminus is positioned extracellularly. Besides this, a part of 4F2 is structurally related to glycosidases; however, no glycosidase activity has been demonstrated (Wells & Hediger, 1992).

xCT light chain

The xCT light chain (502 Aas, ~40 kDa) belongs to the family of solute carrier family 7 (SLC7), which consists of two subgroups. One subgroup comprises cationic amino acid transporters (CAT) while other subgroup the light chains of HATs (Verrey et al., 2004). These subunits are hydrophobic in

Table 1. Amino acid transport systems in different mammalian cells.

		Mammalian Cell types							
				Exocrine		Placenta microvillous membrane vesicles		Enterocyte membrane vesicles	
	Substrates	Fibroblasts	Hepatocytes	Pancreas cells	Blastocyst	Maternal	Fetal	Brush border	Basolateral
Neutral	Pro, Gly, Ala, Ser, Met, Gln	A	A	A	A	A	A	A	A
	Ala, Se, Cys, Thr, Gln	ASC	ASC	ASC			ASC		ASC
	Small Zwitterionic AA			asc		asc			
	Leu, Phe, Met, Cys, Gln	L	L_1, L_2	L		L	L	L	L
	Gln, Asn, His		N					N	
	Gly, Sarcosine		*Gly*	*Gly*	*Gly*				
	Pro, MeAIB							IMINO	
	Most Zwitterionic AA							NBB	
	Phe, Met							PHE	
Anionic	Asp, Glu	X_{AG}^-	X_{AG}^-			X_{AG}^-	X_{AG}^-	X_{AG}^-	
	Glu, Cys	x_c^-							
Cationic	Lys, Arg	y^+	y^+	y^+				y^+	
	Cationic AA				b_1^+, b_2^+				
Cationic and neutral aa	Most cationic and Zwitterionic AA				$B^{0,+}$				
	Bulky cationic and α-β-unbranched Zwitterionic AA				$b^{o,+}$				

MeAIB, 2-(methyl amino)isobutyric acid; NBB, Neutral Brush Border.

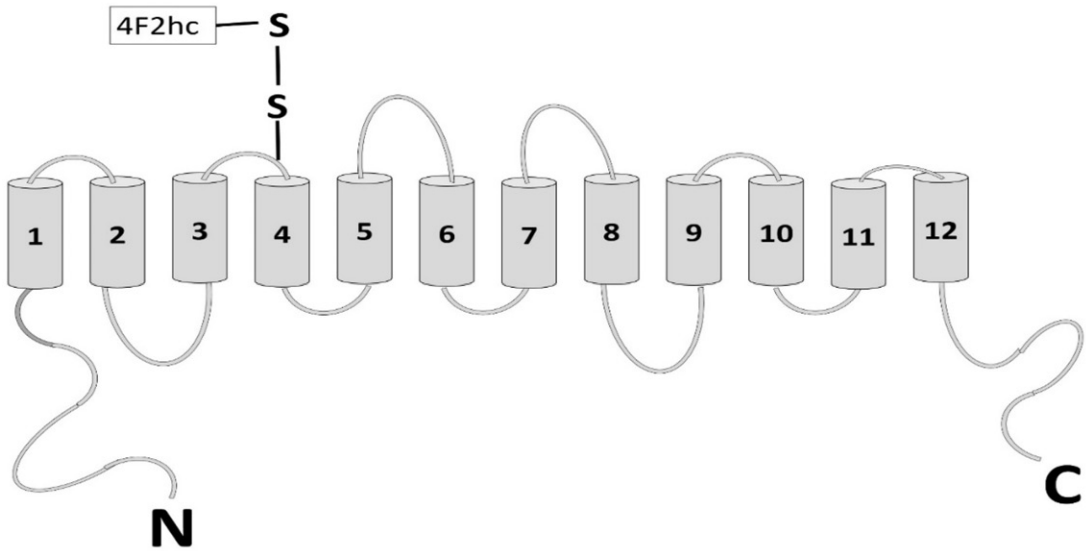


Figure 1. Structure of the functional chain-xCT with 12 TM domains and both the N-and C-termini are located intracellularly. Light chain is attached at TM4 by forming disulphide bond. There is a re-entrant loop in between TM2 and 3. On addition to this the binding and transporter activity is associated to TM8 (adapted from Gasol et al., 2004).

nature and constitute non-glycosylated 12 transmembrane (TM) domains where both N- and C-termini are located intracellularly (Gasol et al., 2004) (Figure 1). For surface expression of these subunits, they require one of the two disulphide-linked heavy chain type-II N glycoprotein. Even though the main transporting activity is carried out through xCT subunit, the presence of 4F2hc subunit is mandatory for functional characteristics and for trafficking of xCT to the plasma membrane (Nakamura et al., 1999). Interestingly, in one of the experiments, Sato et al. (1999) showed that L-Cys/L-Glu exchange is only functional when both the xCT and 4F2hc proteins are expressed together in *Xenopus* oocytes.

Localization

Although there have been many reports stating the presence of system x_c^- at different sites, its functional character is still unclear (Lim et al., 2007). Sato et al. (2002) used *in situ* hybridization to localize the expression of xCT and 4F2hc in the brain. A relatively high expression of system x_c^- was found in choroid plexus, leptomeninges, ependymal cells, vascular endothelial cells and on blood brain barrier (Burdo et al., 2006). It is also located in the astrocytes, microglia, retinal muller cells, and immature cortical neurons (Allen et al., 2001; Cho & Bannai, 1990; Gochenauer & Robinson, 2001; Piani & Fontana, 1994). Besides the CNS, it is also expressed on non-CNS cells like fibroblasts, macrophages, hepatocytes, endothelial cells and pancreatic cells (Bannai et al., 1986; Hosoya et al., 2002; Sato et al., 1999; Sasaki et al., 2002). Furthermore, the expression of subunits xCT and 4F2hc was detected immunohistochemically at borderline membrane of kidney and duodenum.

Regulation

The expression of system x_c^- antiporter is regulated by a number of mechanisms including oxidative stress, xenobiotic exposure, amino acid deprivation, and pathological conditions.

Oxidative stress

In astrocytes glutathione synthesis depends on cysteine derived from cystine imported via sytem x_c^- (Shih et al., 2006). This transportation of cystine through system x_c^- is a rate-limiting step in the synthesis of GSH after its entry inside the cultured cells (Sagara et al., 1993). More precisely, astrocytes present in the CNS may play a role in the intracellular transport of cystine which gets converted to cysteine and ultimately to glutathione as shown in vitro (Dringen et al., 1999).

Free radicals produced through mitochondrial electron transport, paroxysmal fatty acid, cytochrome P450 and phagocytic cells have deleterious effects on cell components and connective tissues and thus it is major cause of oxidative stress (Gemma et al., 2007). Treatment of ethanol to mouse hepatic stellate cells was found to significantly increase system x_c^- mRNA and protein levels *in vitro* (Lin et al., 2013). During oxidative stress, there is an increased demand of GSH which might be one of the noticeable reasons for the up-regulation (through activation of Nrf-2: ARE signalling pathway (Figure 2) of the system x_c^- antiporter. However, De Bundel et al. (2011) reported that there is no need of system x_c^- for basic GSH production in mouse brains indicating other alternative pathways for GSH production *in vivo*. In few studies it is reported that system x_{AG}^- or the high-affinity glutamate transporters in cultured astrocytes are also capable of importing cysteine (Kranich et al., 1998; Shanker & Aschner, 2001) which might be one of the alternatives for GSH production.

Xenobiotic exposure

In an *in vivo* experiment, Ramos-Chávez et al. (2015) reported that inorganic arsenic when orally administered to female rats during gestation period resulted in increased expression of system x_c^- in brain tissues of the offspring. Ceftriaxone, a third-generation cephalosporin, was also found to induce system x_c^- expression and increases its mRNA

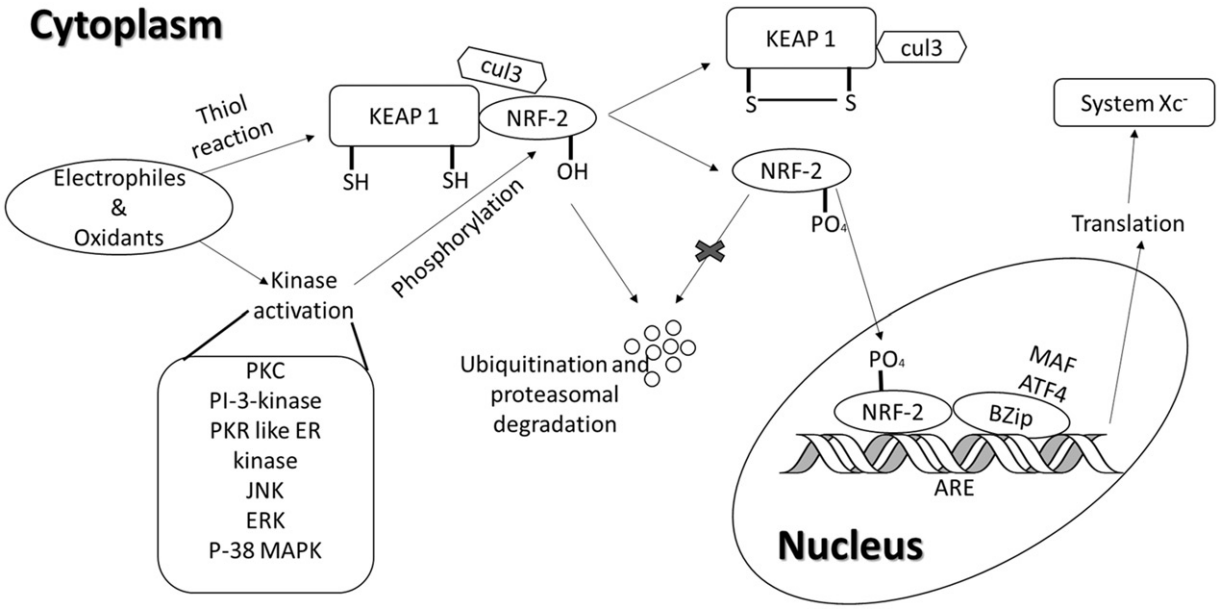


Figure 2. Nrf-2/ARE activation pathway. Under normal conditions Nrf-2 dimerizes with KEAP1 followed by its degradation due to ubiquitination by Cul3. Stimuli like free radicals and oxidizing agents can interrupt with dimerization of Nrf2 and KEAP by altering cysteine residues in KEAP1 or by phosphorylation of Nrf2 at Ser40 with the help of protein kinases. This phosphorylated Nrf2 is then translocated to the nucleus followed by its binding to the adapter proteins which increase ARE-driven transcription (adapted from Bridges et al., 2012b).

expression *in vitro* (Lewerenz et al., 2009). In addition, there is an evidence of the up-regulation of the system x_c^- antiporter in protoplasmic astrocytes when treated with electrophile, tert-butylhydroquinone (tBHQ). However, the same phenomenon was not observed in differentiated culture signifying a distinct regulatory mechanism. Many experiments have failed to mimic this up-regulation by adding H_2O_2 to the astrocytes and thus, it could be interpreted that it is not the xenobiotics but GSH concentration that triggers the induction (Bridges et al., 2012a). Contrary to these above observations, self-administration of nicotine in rats showed decreased expression of system x_c^- (Knackstedt et al., 2009).

Amino acid deprivation

Recent studies have confirmed that depletion of the AAs results in the stimulation of several genes through the induction of genomic cis-element termed amino acid response element (AARE) (Sato et al., 2004). System x_c^- is very specific for cystine and glutamate, and its activity is known to be induced by cystine deprivation. In one of the studies conducted using mouse NIH3T3 cells, up-regulation of system x_c^- was observed *in vitro*. However, this induction was not only because of cystine deprivation but also due to some other amino acids (Sato et al., 2004).

Pathophysiological conditions

System x_c^- is known to maintain the cell redox system by releasing one molecule of glutamate and simultaneous uptake of one molecule of cystine to maintain neuronal plasticity. Thus, any pathological changes may alter behaviour of system x_c^- resulting in increased or decreased expression (Bridges et al., 2012a; Lewerenz et al., 2013).

Cystine, a vital component of GSH biosynthetic pathway, is required by cells to protect themselves from endogenously-produced reactive oxygen species (ROS). Glioma cells depend upon system x_c^- to fulfil their GSH requirement. Compromised system x_c^- function may cause a rapid depletion of GSH, and the resulting loss of ROS defence causes caspase-mediated apoptosis. Thus, to address the deficit in the glutathione concentration, system x_c^- is overexpressed in glioma cells (Chung et al., 2005). System x_c^- up-regulation has also been demonstrated *in vitro* at the mRNA or protein level in lymphomas (Gout et al., 1997) and pancreatic cancers (Lo et al., 2008). Moreover, Qin et al. (2006) demonstrated significantly increased expression of *xCT* gene in the activated microglia in the cerebral cortex of Thy1-APP751 transgenic mice and adult mouse hippocampus after treatment with amyloid-β protein. Additionally, the injection of 6-hydroxydopamine showed increase in striatal xCT protein levels *in vivo* after three weeks of injection (Massie et al., 2008). However, this study did not include functional significance, the follow-up studies using system x_c^--deficient mice reported no deleterious effects of 6-hydroxydopamine *in vivo*. (Massie et al., 2011). Altered expression of *xCT* was also found in CNS of patients suffering from MS as well in acute and chronic animal models of MS (Pampliega et al., 2011). Furthermore, alterations in *xCT* expression levels in the hippocampus were also observed in animal models of epilepsy (Takaki et al., 2008). Few reports mention the altered *xCT* expression in the nucleus accumbens in addiction *in vivo* (Knackstedt et al., 2009).

Pathway of regulation

xCT is a functional unit of system x_c^- antiporter and therefore its regulation has been focused mainly by several studies (Sato et al., 1999). The leading pathways for xCT regulation mainly includes: (i) Nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant responsive element (ARE), and

(ii) eukaryotic initiation factor-2 (eIF2)-activating transcription factor (ATF) 4-amino acid response element (AARE). Along with these two key regulation pathways, *xCT* expression is also regulated through activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), activator protein (AP-1), hypoxia-inducible factor (HIF), cAMP, and growth factors.

(i) *Nuclear factor erythroid 2-related factor 2* (*Nrf2*)-*antioxidant responsive element* (*ARE*) *pathway* (Figure 2). The 5′-flanking region of the mouse *xCT* gene possesses four ARE-like sequences (also known as electrophile response element) (Sasaki et al., 2002) and amongst these four sequences two are completely conserved in 5′-flanking region of the human *xCT* gene (Sasaki et al., 2002; Sato et al., 2000). As proposed by Venugopal and Jaiswal (1996), Nrf2 is present in cytosol in bound form with keap-1 (Itoh et al., 1999). Oxidative burden releases the Nrf2b from keap-1 through PKC-δ-mediated phosphorylation of Nrf2 which translocates to the nucleus and binds to ARE to initialize specific protein transcription (Niture et al., 2009). ARE, a cis-acting regulatory element found in the promoter regions of multiple genes encodes antioxidant proteins including heme oxygenase, γ-glutamylcyteine synthatase, glutamate-cystine ligase, glutathione synthatase, glutathione S-transferase, glutathione reductase, multidrug resistance protein and xCT as well (Bannai, 1984; Erickson et al., 2002; Lee & Johnson, 2004; Sasaki et al., 2002).

(ii) *Eukaryotic initiation factor-2* (*eIF2*)-*activating transcription factor* (*ATF*) *4-amino acid response element* (*AARE*) *pathway*. The 5′-flanking regions of *xCT* also contains two amino acids response element (AARE)-like sequence (Sato et al., 2004; Lewerenz & Maher, 2009). Compromised amino acids levels initiate the signalling cascade followed by the increased translation of activating transcription factor (ATF) 4 and initiation of translational and transcription pathways. Ultimately, levels of proteins like membrane transporters, growth factors and metabolizing enzymes are increased (Kilberg et al., 2005). While there are compromised levels of AA in cells, the general control non-repressible proteins 2 (GCN2) kinase is activated through free excessive tRNAs. Activation of GCN2 phosphorylates the eIF2 (Zhang et al., 2002). This phosphorylated eIF2 inhibits eIF2B (factor responsible for formation of initiation complex for translation) (Kimball, 1999; Rowlands et al., 1988).

Another mechanism associated with the activation of AARE regulated gene transcription is phosphorylation of eIF2B (Proud, 2005) by Glycogen synthase 3β (GSK3β). Due to eIF2B phosphorylation, it loses its guanine nucleotide exchange factor property and ultimately prevention of formation of initiation complex for translation (Welsh & Proud, 1993). Phosphoinositide-3 kinase (PI3K) (Welsh et al., 1998) and MEK/ERK (Kleijn & Proud, 2000; Quevedo et al., 2000) activated by hormones, mitogens, and growth factors, inactivates GSK3β by its phosphorylations and thus protein translation is up-regulated. However, it is has been recently reported that PI3K signalling by inhibiting GSK3beta and thereby activating GCN2 induces eIF2alpha phosphorylation, increases ATF4 protein levels and xCT expression (Lewerenz et al., 2014). Thus, phosphorylation of eIF2 and eIF2B reduces total protein translation but at the same time there is increased the translation of certain mRNAs including ATF4. ATF4 in turn activates the transcription of AARE-regulated genes including xCT, the specific subunit of system x_c^-. Deficiency of cystine was found to activate ATF4-AARE pathway to induce the uptake of cystine in cultured human fibroblast cells after 24 hours (Bannai, 1984) suggesting the role of ATF4-AARE pathway in system x_c^- regulation.

Also, the 5′-flanking regions of *xCT* contains binding sites for both NF-κB and AP 1 (Sato et al., 2001). However, there is no direct involvement of NF-κB and AP 1 in regulating system x_c^- reported until now. Additionally, hypoxic preconditioning increased xCT levels in hippocampus (*in vivo*) and mouse neuronal stem cells (*in vitro*), through both transcription and translation (Ogunrinu & Sontheimer, 2010; Sims et al., 2012). However, direct mechanism of system x_c^- regulation by hypoxia is yet to be studied.

Growth factors like insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), and fibroblast growth factor-2 (FGF-2) have been reported to up-regulate the system x_c^- function *in vitro* (Liu et al., 2012; Pauly et al., 2011; Yang & Yee, 2014). Moreover, cAMP also increases the level of xCT mRNA in cultured cells (Gochenauer & Robinson, 2001; Seib et al., 2011). However, the exact mechanism for the same is yet to be studied.

Functions

Transporters like system x_c^- present on glial cells take part in maintaining both intra and extracellular environment within CNS (Sato et al., 2002). They do so by up taking amino acid (Cystine) required for production of intracellular compounds like GSH with the release of neurotransmitter (Glutamate) in the synaptic cleft to maintain neuronal plasticity (Bannai & Kitamura, 1980; Bannai et al., 1986). This exchange occurs in 1:1 molar ratio per carrier with the participation of Cl^- component (Waniewski & Martin, 1984). However, these processes are independent of Na^+ availability (Bannai & Kitamura, 1981). Additionally, this phenomenon is electroneutral and pH-dependent where each substrate having a net negative charge is exchanged via the transporter (Bannai et al., 1986). Overall, system x_c^- plays a vital role on GSH, an antioxidant, production in cells and neurotransmission through glutamate release.

System x_c^- and glutathione production

The CNS requires the highest amount of energy compared to other body systems to function properly. To satisfy this energy requirement, the brain is supplied with enough blood which maintains sufficient glucose and oxygen levels (Sibson et al., 1998). During the process, there is ample generation of ROS, which must be neutralized in order to prevent brain damage. Additionally, metabolisms of xenobiotics as well as oxidative stress equally participate in generating ROS (Halliwell, 2006). To deactivate ROS, the body requires a counter mechanism that can protect brain from oxidative damage. With regard to this, system x_c^- antiporter plays a vital role in generating GSH

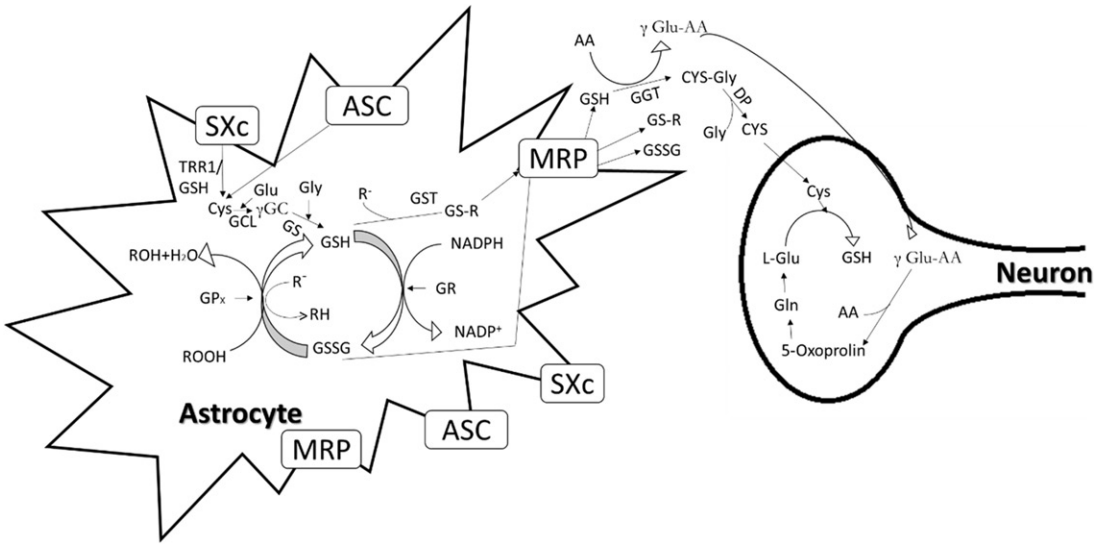


Figure 3. Production of glutathione in astrocytes and neurons via system x_c^- transporter. SXc, System x_c^-, ASC, alanine-serinecysteine; MRP, multi-drug resistance proteins; GCL, glutamate cysteine ligase; GS, glutathione synthase; GSSG-GSH disulphide; TRR1, thioredoxin reductase; GGL, γ-glutamyl transferase (adapted from Lewerenz et al., 2013).

by providing cystine as a synthetic precursor (Bridges et al., 2012a; Shih et al., 2006).

As mentioned above, system x_c^- exchanges glutamate present in the astrocytes with cystine available in nanomolar concentration in CSF (Baker et al., 2003). The expression of system x_c^- is much more on astrocytes cultures as compared to the mature neuron culture and therefore, neurons are dependent on astrocytes for the production of GSH. After the entry of cystine inside the cells, it is reduced to cysteine and then to GSH (Sagara et al., 1993). The synthesized GSH is then released in the extracellular space followed by its enzymatic catabolism by γ-glutamyltranspeptidase to form γ-glutamyl-GSH or L-glutamate and L-cysteine-Gly. The released GSH is also converted non-enzymatically to cystine to form cysteine and cysteine-GSH. Thus, cysteine produced by both the pathways enters into the neurons and generate GSH (Dringen et al., 1999; Wang & Cynader, 2000) (Figure 3).

Extrasynaptic excitatory neurotransmission

System x_c^- is involved not only in the uptake of cystine and subsequent production of the GSH, but also in the release of glutamate which is critically important for signalling pathway. Baker et al. (2002b) reported that blockade of system x_c^- in striatum results in 60% reduced glutamate levels in extra-synaptic space *in vivo*, thus nonvesicular release of glutamate plays an important role in extrasynaptic neurotransmission in striatum. This released glutamate through system x_c^- plays a physiological role in the tonal regulation of extrasynaptic group II mGluRs and thereby controls neurotransmitter release throughout the CNS (Baker et al., 2002a; Mohan et al., 2011; Xi et al., 2003). The aptitude of system x_c^- to contribute in neuronal signalling by releasing glutamate in extrasynaptic environment is still unclear. Recent studies reported that K_m for glutamate uptake is 2- to 5-fold higher in glioma cell lines compared to primary cultures of differentiated astrocytes (Seib et al., 2011). In another study, cystine in the concentration range of 0.1–0.3 mM was able to produce system x_c^--mediated release of glutamate. This efflux of glutamate decreased the synaptic release of glutamate as a consequence of presynaptic group II mGluRs activation (Moran et al., 2005). Based on these research findings it can be concluded that the ability of glutamate released from system x_c^- to activate the EAA receptors follows different pathways. Moreover, glutamate released through vesicular and non-vesicular routes is primarily cleared by diffusion through a family of Na^+-dependent excitatory amino acid transporter (EAATs) (Bridges et al., 2012a). This high capacity of EAATs for clearing glutamate is attributed to their surface density which is almost equivalent to number of glutamate molecules released during action potential (Lehre & Danbolt, 1998). Additionally, EAATs have an uptake capacity around 10 $\mu M.ms^{-1}$ which is capable of preventing the excitotoxicity-like effects of glutamate released through respective transporters (Asztely et al., 1997; Diamond & Jahr, 2000; Zheng et al., 2008). While this interaction or binding of glutamate occurs very rapidly, the translocation of glutamate may take bit longer (approx. 70 ms, i.e. time required for 90% of glutamate to diffuse out of the synaptic cleft) (Savtchenko & Rusakov, 2004; Wadiche et al., 1995).

In contrast, Augustin et al. (2007) in their study on *Drosophila melanogaster* reported, system x_c^- as a source of glutamate along with its contribution to the signalling and plasticity. The activity of system x_c^- transporter was also confirmed by performing studies on *xCT* homologue gender blind *D. melanogaster* (Grosjean et al., 2008). The damage of this associated gene decreases extracellular L-glutamate levels by about half. Moreover, alterations in the ambient L-glutamate levels arising from altered system x_c^- activity were shown to affect both the function (i.e. desensitization) and localization (i.e. clustering) of the ionotropic glutamate receptors (iGluRs) in the *Drosophila* CNS (Augustin et al., 2007; Featherstone, 2011).

Although the study conducted by Augustin et al. (2007) states that system x_c^--like activity was seen in insects, the real exchange activity by system x_c^- through radiolabelled amino

acid uptake assay was not been reported. Additionally, phylogenetic analysis performed by Augustin et al. did not include system L and ASC light chains. These were included in another phylogenetic analysis along with xCT, which demonstrated that gender blind is closely related to system L and ASC but not xCT (Lewerenz et al., 2013). Moreover phylogenetic analysis for insect SLC7 light chains using the genome of the mosquito *Aedes aegypti* also demonstrated the absence of an xCT homologue in insects (Carpenter et al., 2012).

System x_c^- and CNS disorders

Drug addiction

Drug addiction is a disorder where neurotransmission is manipulated by administration of some drugs resulting in increased craving for the respective drug. These drug-induced effects on neuronal activity accounts for the addiction which is followed by chronic changes in functioning of corticostriatal pathways initiated in the prefrontal cortex and angled to the nucleus accumbens (Kalivas, 2009; Kalivas et al., 2005; Uys & LaLumiere, 2008; Wolf, 2010).

It has been observed that system x_c^--mediated transport of cystine and glutamate is effectively reduced in nucleus accumbens by the administration of cocaine and other drugs of abuse (Figure 4). During this process, glutamate signalling pathway starting from its release to its binding to its receptors is affected, resulting in drug seeking behaviour or addiction (Baker et al., 2003; Kau et al., 2008; Knackstedt et al., 2009; Madayag et al., 2007). More precisely, repeated cocaine exposure to animals significantly increased K_m values for the cystine uptake in tissue slices of nucleus accumbens (Baker et al., 2003; Kau et al., 2008; Madayag et al., 2007). In one of the pre-clinical studies, it was observed that there was significant decrease in glutamate concentration (in non-habituated rats) followed by system x_c^- blockade while there was no change in glutamate levels in cocaine withdrawn rats suggesting decreased expression of system x_c^- antiporter (Baker et al., 2003). Thus, as a result of reduced activity of system x_c^-, there is probably decreased extracellular concentration of glutamate and diminished functional activity in cocaine withdrawn rats. Corroborating the above-mentioned findings, Baker et al. (2003) observed that on infusing cystine into nucleus accumbens of cocaine withdrawn rats, the glutamate levels were elevated to the levels almost equivalent to normal levels. Thus, this increased glutamate levels in nucleus accumbens prevents the cocaine-induced synaptic release of glutamate resulting in reduced cravings for cocaine *in vivo* (Madayag et al., 2007). Furthermore, Baker et al. (2003) also reported that acute administration of N-acetylcysteine (Cysteine prodrug) blocked cocaine-induced addictive behavior *in vivo*. Moreover, repeated administration of N-acetylcysteine was capable of blocking not only drug-induced cocaine-seeking behavior but also effect of drug related stimuli (Madayag et al., 2007; Reichel et al., 2011). Though N-acetylcysteine helps in managing addiction, it is not directly transported through system x_c^-; in fact this translocation of N-acetylcysteine and its metabolite cysteine is achieved by

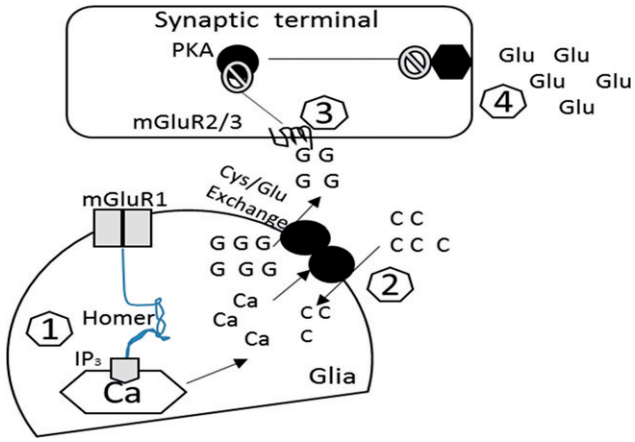


Figure 4. The possible pathways of glutamatergic transmission in the nucleus accumbens that may be responsible for drug-seeking behavior. Cocaine treatment increases the extrasynaptic release of glutamate which promotes the synaptic release of glutamate and ultimately drug-seeking behavior (adapted from Kalivas, 2004). G, intracellular glutamate; C, cystine; Glu, released glutamate; Ca, calcium; PKA, protein kinase A. (1) There is a reduction of homer protein in the nucleus accumbens, creating a decrease in signalling through mGluR1 receptors via inositol trisphosphate (IP3) receptor regulation of inner calcium (Ca^{2+}) stocks. (2) The glutamate released through system x_c^- activates mGlutR1. However, down-regulation of this receptor in addiction may be due to the altered activity of cystine/glutamate transporter. (3) The altered activity of system x_c^- results in the decreased release of glutamate and thus reduced activation of the presynaptic mGlu2/3 autoreceptors. (4) This decreased activation of mGlu2/3 results in a prevention of its inhibitory regulation for the glutamate release.

cysteine transporters (Kupchik et al., 2012). Confirming the therapeutic efficacy of N-acetylcysteine in preclinical studies, clinical studies had demonstrated the improvement in drug-seeking behavior when treated with N-acetylcysteine (Knackstedt et al., 2009; LaRowe et al., 2007; Mardikian et al., 2007). Consistent with these studies one more clinical study proved the aforementioned observations stating that N-acetylcysteine was able to improve cocaine induced addiction in humans without affecting the euphoric properties of drugs (Amen et al., 2011).

Thus, the above-mentioned findings indicate the potential role of system x_c^- in drug-seeking behavior and therefore increased urge of cocaine administration with reduced system x_c^- activity which might be associated with the pathophysiology of addiction (Breiter et al., 1997; Pierce et al., 1996; Reid & Berger, 1996).

Under normal conditions, released glutamate from astrocytes transports to synaptic cleft and binds to glutamate receptors, i.e. group I and II metabotropic and NMDA receptors (Angulo et al., 2004; D'Ascenzo et al., 2007; Jabaudon et al., 1999; Kupchik et al., 2012; Moussawi et al., 2011). However, reduced activation of group II mGluRs in cocaine withdrawn rats was observed (Xi et al., 2002). This diminished activation of group II mGluRs resulted in increased vesicular release of glutamate in nucleus accumbens after repeated administration of cocaine. N-acetylcyteine was found to increase the glutamate levels and thus activates the group II mGluRs helping in better management of addictive conditions *in vivo* (Madayag et al., 2010).

Schizophrenia

Schizophrenia is a CNS disorder characterized by an interruption in thinking and poor emotional response. Various studies demonstrated the involvement of a variety of factors contributing to schizophrenia including but not limited to genetic predisposition and pre- or postnatal environmental stressors leading to abnormal neurodevelopment (Brown, 2011; Chua & Murray, 1996; Weinberger, 1987). Consequently, there is an abnormal pyramidal neuron activation followed by altered network oscillatory activity in the dorsolateral prefrontal cortex (Cho et al., 2006; Gonzalez-Burgos & Lewis, 2008; Gonzalez-Burgos et al., 2010). The central mechanism for such kind of abnormality includes alteration in glutamatergic activity and changes in N-methyl-D-aspartate (NMDA) as well as other glutamate receptor activation (Coyle et al., 2003; Marino & Conn, 2002; Rung et al., 2005; Verma & Moghaddam, 1996).

As already discussed, system x_c^- also takes part in glutamatergic neurotransmission through non-vesicular release of glutamate which interacts with glutamate receptors (particularly mGluR2/3 and NMDA receptors) along with cystine uptake for GSH production (Baker et al., 2002a; Mohan et al., 2011). The intracellularly synthesized glutathione is transported outside the cell and then facilitate NMDA receptor activation through reducing extracellular redox sites with release of co-agonist glycine (Metabolite of GSH) (Abbott & Meister, 1986; Kohr et al., 1994; Lipton et al., 2002). However, this hypothesis still requires extensive research in terms of preclinical and clinical studies. Thus, altered or diminished activity of system x_c^- transporter may possibly cause neuronal network desynchronization either through decrease in extrasynaptic glutamate signalling or decrease in glutathione concentration. As a consequence of this, there is a diminished receptor activation followed by diminished oscillatory activity. Additionally, reduction in intracellular concentration of GSH may cause compromised management of oxidative stress. Moreover, diminished group II mGluR activation cannot negatively modulate vesicular release which eventually results in abnormal pyramidal neuron activation (Conn & Pin, 1997; Xi et al., 2002). Hypoglutamatergic condition through reduced non-vesicular release of glutamate and hyperglutamatergic condition at synapse due to reduced inhibition of group II mGluRs contributes to schizophrenia symptoms.

In support of the above discussion, some studies have set an evidence of altered GSH metabolism in schizophrenics (Do et al., 2000; Raffa et al., 2009), possibly associated with altered activity of system x_c^- as moderate cystine is available for uptake by system x_c^- upon altered metabolism of GSH (Lutgen, 2012; Kim et al., 2001; Shih et al., 2006). Another study also reported slightly increased expression of xCT (active subunit of system x_c^-), in the post-mortem brain tissues of schizophrenic patients (Baker et al., 2008) probably for acute management of oxidative stress. Additionally, increase in group II mGluRs was observed in schizophrenic patients (Gupta et al., 2005), possibly due to chronic understimulation of receptor due to compromised non-vesicular release of glutamate.

Recent research work describing involvement of system x_c^- in schizophrenia has revealed that the cystine prodrug N-acetylcysteine improved learning ability in rats (Lutgen, 2012). Additionally, studies conducted by Berk et al. (2008) and Bulut et al. (2009) showed significant improvement in schizophrenic symptoms upon repeated administration of N-acetylcysteine. Thus, it is possible that targeting system x_c^- antiporter may help in attenuating the schizophrenic condition.

Alzheimer's disease

Amyloid-β (Aβ) peptide is one of the major causes associated with the pathogenesis of AD (Hardy & Selkoe, 2002). Aβ is known to stimulate microglia that promotes death of neurons by producing toxins such as glutamate, cytokines and free radicals (Giulian et al., 1995; Ishii et al., 2000; McDonald et al., 1997; Meda et al., 1995; Weldon et al., 1998). As already discussed, system x_c^- is located on glial cells and transports glutamate to the extrasynaptic space. Therefore, the release of glutamate may be associated with Aβ's interaction with system x_c^- present on glial cells. Using co-cultures of microglia and neurons, Qin et al. (2006) demonstrated that system x_c^- facilitated release of glutamate from Aβ-stimulated microglia is neurotoxic by activating NMDA receptors. In the same study, it was shown that in transgenic mice expressing mutant human amyloid precursor protein in vicinity of amyloid plaques or after injecting Aβ into the brain of C57BL/6 mice stimulated microglia express high levels of xCT. Earlier studies have revealed that exposure of the neurons to Aβ augments neuronal responses to glutamate, along with the rise of intracellular Ca^{2+} and subsequent cell death (Koh et al., 1990; Mattson et al., 1992). The up-regulation of system x_c^- in Alzheimer's patients is believed to be due to abundant phosphorylation of α subunit of the eukaryotic initiation factor 2 and activating transcriptional factor 4 (Lewerenz & Maher, 2009).

Even though such studies suggest the involvement of system x_c^- in AD, the impact of such changes is not clear. The reason being the dual function of system x_c^- wherein it releases glutamate (activation of extrasynaptic NMDA receptors, may contribute to Aβ production and toxicity) with the uptake of cystine required for the production of glutathione to neutralize ROS in AD (Bordji et al., 2010; Olivieri et al., 2001; Qin et al., 2006). Though such findings is not making the clear role of system x_c^-, many Alzheimer's patients treated with N-acetylcysteine have shown significant improvement in some of the cognitive tasks (Adair et al., 2001). Improved GSH production may reduce oxidative stress induced through Aβ followed by diminished abnormal protein expression normalizing the system x_c^- transporter activity (Begni et al., 2004; Olivieri et al., 2001; Onyango et al., 2005; Tanel & Averill-Bates, 2007).

It is very important to meet the antioxidant demand with the reduced release of glutamate for better management of AD. The system x_c^- inhibitors may serve the purpose with compromised GSH synthesis. In such conditions, compounds having both system x_c^- inhibition and ROS-neutralizing capability may be of great advantage in establishing a good drug candidate for treatment of AD.

Parkinson's disease

Two prime factors involved in pathogenesis of neurodegenerative disease are excitotoxicity and oxidative stress. More particularly, in PD neurodegeneration is a result of increased extracellular striatal glutamate levels along with hyperactivity of the subthalamic nucleus (STN) (Blandini et al., 2000). Moreover, GSH is the key antioxidant in the brain, having significant and early role in the pathogenesis of PD. Thus, loss of GSH production may worsen the condition in PD (Schulz et al., 2000). The involvement of glutamate and cystine (Precursor for GSH) both at the same time may contribute towards the vital role of system x_c^- antiporter in the pathogenesis of PD.

In an interesting study, Massie et al. (2011) showed that in *xCT*-knockout mice, there is a decreased glutamate level in the extrasynaptic space. However, there was no change in the GSH production suggesting a different pathway for the intracellular movement of cystine. Recently, a study conducted on hemi-Parkinson's rats described over expression of *xCT* in their striatum. (Massie et al., 2008). Taken together, it supports the role of system x_c^- in the pathophysiology of PD.

In contrast to the above mentioned studies, a number of animal studies exhibited that N-acetylcysteine treatment showed significant improvement in PD-related symptoms indicating the involvement of system x_c^--mediated cystine transport. In support, N-acetylcysteine was capable of showing positive outcomes in animals treated with 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (Aoyama et al., 2008; Park et al., 2004), or in α-synuclein overexpression and EAA carrier 1-null mice (Berman et al., 2011; Clark et al., 2010). A clinical trial of a sulphonamide, i.e. zonisamide showed the reduction in disease severity (Murata et al., 2001), and the mechanism responsible for such a therapeutic effect may involve increased system x_c^- activity. Moreover, frequent zonisamide treatment produced improved GSH synthesis in hemiparkinsonian mice, as a result of zonisamide mediated overexpression of system x_c^- (Asanuma et al., 2010).

In PD, there is a cascade of functional changes as a result of degeneration of dopaminergic neurons in *substantia nigra pars compacta* which affects the whole basal ganglia network. The utmost significant changes affect the output nuclei of the circuit, the *medial globus pallidus* and *substantia nigra pars reticulata*, which become hyperactive. Enhanced glutamatergic inputs that the output nuclei receive from the subthalamic nucleus sustains such hyperactivity. However, mechanisms leading to the subthalamic disinhibition are still poorly understood (Blandini et al., 2000). It is also known that GSH is decreased in *substantia nigra* in PD patients (Martin & Teismann, 2009). While it is necessary to have enough GSH levels in brain, it is also important to decrease glutamate levels for the management of PD. So while inhibiting system x_c^- antiporter, glutamate release may be reduced but at the same time GSH production is also probably dampened. However, several studies have quoted the alternatives for GSH production during increased generation of ROS (Massie et al., 2011) and thus perfect combination of dopaminergic receptor modulator and system x_c^- inhibitor may form the decent treatment for PD.

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the CNS characterized by infiltration of immune cells, demyelination, oligodendroglial death and axonal degeneration (Barnett & Prineas, 2004; Lucchinetti et al., 1999; Trapp et al., 1998). Amongst the etiological characteristics, oligodendroglial death and demyelination are believed to be due to glutamate excitotoxicity through activation of ionotropic glutamate receptors (iGluRs) (Matute et al., 1997). Several observations supporting the excitotoxic hypothesis in MS comprise the evidence of elevated glutamate levels in MS, both at CNS and peripheral blood (Pampliega et al., 2011). Glutamate is increased in cerebrospinal fluid (CSF) in acute MS conditions as well (Stover et al., 1997). Thus, the role of system x_c^- in transporting glutamate outside the glial cell may contribute to the pathophysiology of MS.

Recent studies have shown evidence of increased xCT expression in activated macrophages-microglia in the CNS in MS as well as in leukocytes from MS patients (Pampliega et al., 2011). There is also evidence that the simultaneous glutamate release from activated microglia by the system x_c^- antiporter and the inhibition of Na^+-dependent glutamate uptake by the activated microglia results in local increase in extracellular glutamate that leads to excitotoxic oligodendrocyte death (Domercq et al., 2007).

The inflammatory process in MS is one of the reasons for neurodegeneration. Besides this, glutamate is also believed to be one of the major culprits for neurodegeneration. The activation of glutamate receptors through system x_c^--mediated glutamate release may be targeted for better efficacy. Either glutamate receptor modulators or system x_c^- inhibitors could help in such conditions. However, management of inflammation (as a result of ROS) still remains unanswered. In such circumstances, a known anti-inflammatory drug sulfasalazine which showed system x_c^- inhibition capability may become a worthy candidate for treatment of MS. However, few studies reported adverse effects associated with sulfasalazine in animal models of MS (Correale et al., 1991), while in case of clinical trials conducted on MS patients showed no significant outcome with sulfasalazine treatment (Noseworthy et al., 1998). These further require specific studies to understand the exact reasons why sulfasalazine is not able to improve the MS condition.

Depression

Depression is a psychological condition where individuals thought, feelings and sense of well-being are affected thus leads to low mood and displeasure to activity. Current studies have suggested the involvement of glutamatergic neurotransmission interconnected with GABAergic and monoaminergic pathways in the pathophysiology of neuropsychiatric disorder like depression (McCarthy et al., 2012; Sanacora et al., 2012). Moreover, many clinical and preclinical studies verified that maladaptive changes in excitatory/inhibitory circuitry, particularly in glutamate homeostasis and neurotransmission, have a main part in the mood and anxiety disorders (Musazzi et al., 2012). Hence, the variation in glutamate release, clearance and metabolism within certain brain region may

contribute to mood disorders like depression. System x_c^- transporter being an important medium in managing glutamate homeostasis, may have critical involvement in the pathophysiology of depression and thus, could become a novel target in treating the same.

Fibroblast growth factor-2 (FGF-2) has a vital role in the neurogenesis and normal brain development (Zechel et al., 2010). Additionally, recent *in vitro* studies have also suggested the contribution of FGF-2 in the pathophysiology of depression through system x_c^--mediated glutamate release. In support of this, studies have proved that there is an up-regulation of system x_c^- as a result of FGF 2 treatment *in vitro* (Liu et al., 2012). The report obtained from the post-mortem of depressed patients' brains showed reduced mRNA for FGF-2 (Evans et al., 2004) which may be, given the up-regulation of system x_c^- by FGF2 *in vitro*, associated with decreased expression of system x_c^-. Thus, disruption of FGF-2 signalling pathway followed by dysfunctioning of system x_c^- may be associated with depression.

Furthermore, there are evidences of diminished GSH levels in patients suffering from depression (Kodydková et al., 2009). Since GSH diminution has been associated in depression along with disturbance of the FGF-2 system, it is likely that the pathological effects of FGF-2 disruption are likely to be associated to reduced system x_c^- activity but still we require studies proving the role of system x_c^-.

It will be of great interest to evaluate whether the inhibition or activation of system x_c^- can benefit in management of depression. However, glutamate is required for managing depression-like conditions and thus it might be advantageous to explore the role of system x_c^- in depression with extensive research.

Glioma and excitotoxicity

Gliomas are tumors of glial cells that spread into the healthy brain parenchyma with the release of glutamate being one factor that might facilitate this mechanism. Elevated glutamate interacts with postsynaptic receptors and generates hyperexcitation of the neurons which ultimately results in neuronal cell death and thus ease the way for cancer cells to distribute (de Groot & Sontheimer, 2011). The elevated concentration of glutamate in the synapse is attributed to the over activity of the system x_c^- antiporter along with down-regulation of other EAATs (Chung et al., 2005; Kim et al., 2001; Rothstein, 2002; Takano et al., 2001; Ye & Sontheimer, 1999; Ye et al., 1999). Furthermore, due to high activity of system x_c^- antiporter in glioma cells, the impact of chemotherapy and radiation reduces (Chung et al., 2005). Taken together, the increased expression of system x_c^- in glioma releases high levels of glutamate that can lead to seizures and neuronal death. At the same time, system x_c^- uptakes cystine to generate glutathione and thus protect cancer cells from ROS.

GSH requirement is mandatory for cancer cells to survive. The inhibition of system x_c^- will not only decrease GSH production but also compromise glutamate release. Thus, targeting system x_c^- could help in finding novel compounds for both glioma as well as epileptic seizures. It has been shown that sulfasalazine is able to inhibit system x_c^- effectively (Bridges et al., 2012b). Moreover, Chung et al. (2005) reported that inhibition of cystine uptake by sulfasalazine halts the progress of primary brain tumours *in vivo*. However, due to poor pharmacokinetic properties of sulfasalazine, the bioavailability is very low at the site of action (CNS) when administered orally (Kusuhara et al., 2012). Additionally, clinical trial of sulfasalazine in recurrent glioma had to be terminated prematurely due to deleterious effects of the compound (Robe et al., 2009). Thus, with suitable chemical modification of sulfasalazine moiety, it is possible to synthesize molecules with inhibitory activity like parent moiety with improved pharmacokinetic characteristics. Other than sulfasalazine, erastin and its analogues also demonstrated their system x_c^- inhibition potential *in vitro*. However, erastin is still under preliminary evaluation (Dixon et al., 2014; Singer et al., 2015). Moreover, an already approved anti-cancer drug for kidney and liver cancer, Sorafenib is under clinical trials for its efficacy in glioblastoma (NCI, 2015).

Hypoglycaemic neuronal death

As already mentioned above, the brain's energy requirement is highest compared to all body systems thus, if glucose concentration falls below desired level, it may cause neuronal injury. It has been shown that when glucose concentrations in the blood are below 2 mM, brain glucose level touches zero leading to neuronal injury (Choi et al., 2001; Langan et al., 1991; Ryan et al., 1990; Suh et al., 2007; Xu et al., 2011). Neurons, particularly those present in the hippocampus and cortex, are extremely sensitive to glucose deprivation (GD) (Auer et al., 1984; Goldberg & Choi, 1993; Monyer & Choi, 1988; Monyer et al., 1989). To balance glucose deprivation, astrocytes contribute to work energy generation via glycogen metabolism. Additionally, glutamate released from the astrocytes undergoes metabolic pathway (TCA cycle) and gets converted to pyruvate for more energy generation. (Bakken et al., 1998; Butterworth, 1999; Dennis & Clark, 1978). In spite of this, neuronal cell death does not seem to be a straight consequence of the energy failure. Numerous studies exhibited that hypoglycaemic neuronal injury happens subsequently to glutamate excitotoxicity, as insulin-induced hypoglycaemia effects increase glutamate concentration in the rat hippocampus and striatum (Sandberg et al., 1986; Silverstein et al., 1990) and in the cerebrum of the pig (Ichord et al., 1999). Jackman et al. (2012) also confirmed the involvement of high levels of glutamate in the hypoglycaemic neuronal cell death.

As already known, system x_c^- antiporter located on the astrocytes releases glutamate in the extracellular space. The higher concentration of glutamate in hippocampus and striatum may be responsible for hypoglycaemic cell death. Interestingly, it has also been shown that system x_c^- contributes to hypoglycaemic neuronal cell *in vitro* by glutamate release and subsequent activation of neuronal glutamate receptors (Jackman et al., 2012).

System x_c^- inhibition

The involvement of system x_c^- in the pathophysiology of a variety of CNS disorders makes it a promising target for treatment. Until now, a lot of work has been done in this area.

Almost all the conducted experiments were based on competitive assays that screened structurally similar analogues for their ability to block the system x_c^--mediated exchange of radiolabeled L-glutamate for L-cystine. From the preliminary studies, two structurally dissimilar molecules, sulfasalazine and (S)-4-carboxyphenylglycine 2 (S-4CPG) were found to inhibit system x_c^- antiporter (Gout et al., 2001; Ye et al., 1999). Along with system x_c^- inhibition, sulfasalazine is also a well-known NF-kappa B (NF-kB) inhibitor indicating its possible role in ailments like cancer, inflammation, infection and autoimmune diseases (Chávez et al., 2012).

Although these molecules exhibited inhibitory properties, further research was limited because of their secondary effects and poor pharmacokinetic profiles (Chung et al., 2005; Savaskan et al., 2008). Compound **2** also antagonizes group I metabotropic receptors (mGluRs) (Hayashi et al., 1994) along with system x_c^- inhibition while **3** is metabolized by CYP enzymes to produce inactive metabolites (Azad et al., 1983).

To overcome such problems, many researchers have modified existing inhibitors like **3** to get metabolically stable and effective analogues (Table 2). Apart from the modifications in **3** that exhibited inhibitory activity, a lot of other modifications can be done to increase the pool of inhibitors (Table 3).

Recently, an antitumor agent erastin **28** has been found as a selective inhibitor of system x_c^-. Additionally, analogues for **28** have also been generated to improve potency (Dixon et al., 2014; Singer et al., 2015). Erastin and its analogues represent a novel scaffold for system x_c^- inhibitors. Currently, such novel scaffolds are needed to shift the gears in this field. Other than these two molecules, sorafenib, a protein kinase inhibitor, also inhibits the transporter. Dixon et al. (2014) reported that sorafenib could triggered ER stress and ferroptosis through system x_c^- inhibition.

Molecular modelling

The X-ray crystal structure of system x_c^- transporter is not available in the Protein Data Bank (PDB). Few groups of researchers have tried to build the homology model of the transporter based on a related amino acid, polyamine and organocation transporter (ApcT) from a thermophile *Methanocaldococcus jannaschii* (PDB ID 3GIA) (Matti et al., 2013). The developed and validated homology model was then used for docking studies of the novel system x_c^- inhibitors belonging to isoxazole hydrazide class (Table 1). The binding mode clearly showed the importance of –COOH group located on the isoxazole ring along with the hydrazide –C=O which were found to interact with Arg135 (electrostatic interaction) and Ser330 (H-bonding), respectively. In addition, the O group of isoxazole ring was found to interact with Tyr251 via H-bonding. Overall, the homology model assisted the authors in rationalizing the observed potency. Due to lack of significant number of inhibitors, ligand-based approaches such as 3D pharmacophore and quantitative structure-activity relationship (QSAR) studies are limited in the literature. Substantial efforts in this direction will help in discovering novel classes of system x_c^- inhibitors.

Structure-activity relationship

Sulfasalazine and analogues

Shukla et al. (2011) synthesized sulfasalazine analogues and determined the concentration required by newly synthesized molecules to reduce the cellular cystine uptake by 50% compared to the solvent control in U87 cell line. Sulfasalazine (**3**, $IC_{50} = 30\,\mu M$), a prodrug of sulfapyridine (**4**, $IC_{50} > 1\,mM$) and 5-aminosalicylic acid (**5**, $IC_{50} > 1\,mM$) is a well-known system x_c^- inhibitor. Both sulfapyridine (**4**) and 5-aminosalicylic acid (**5**) exhibited complete loss of system x_c^- activity. Modification of the central planar linker with trans geometry (N = N) in **3**, with planar ethyne linker led to conservation of activity (**7**, $IC_{50} = 30\,\mu M$) demonstrating that the azo linkage was not absolute necessity. The corresponding ester of **7** did not show any activity (**6**, Table 2). On the similar lines, the replacement of azo linkage with ethylene linker (**9**, Table 2) led to 2-fold reduction in activity. Disruption of the conjugation in **3**, resulted in over 10-fold reduction in activity (**11**, Table 2). All other modifications in the parent structure led to loss of activity (Table 2).

Isooxazole analogues

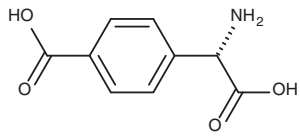
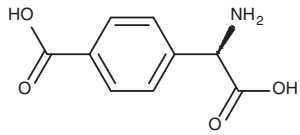
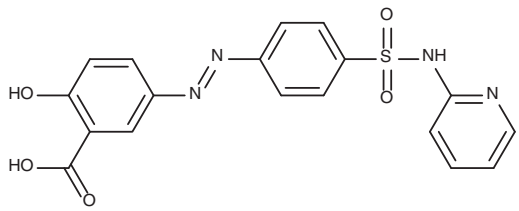
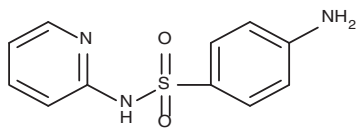
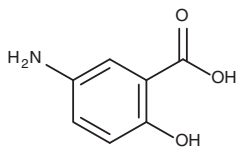
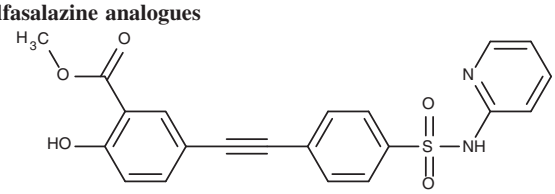
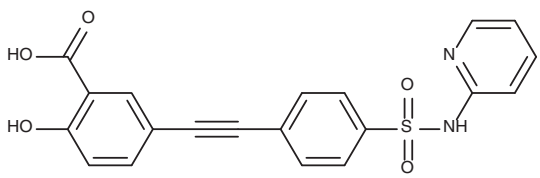
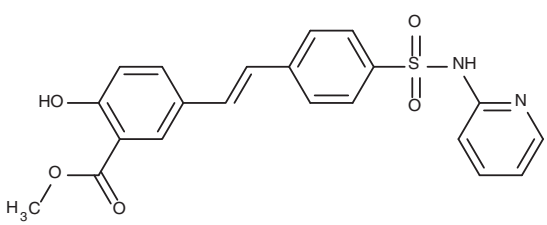
Matti et al. (2013) reported a set of isooxazole derivatives which were evaluated for their system x_c^--inhibiting potential using SNB-19 cell lines. Cells were tested for L-[^{3}H]Glu uptake under Cl^- conditions in the presence of isoxazole-hydrazide derivatives. A novel series of isooxazole 3-carboxylic acids exhibited less sensitivity to the structured modification ([^{3}H]Glu uptake). The reported series explored effect of substituents on the distant aryl ring (Table 2). The aryl ring was found to tolerate a variety of substituents such as $–OCH_3$, –OH, –F, –H etc. without appreciable change in activity.

In a similar attempt, Newell et al. (2014) further explored the SAR of **22** reported earlier to be a competitive inhibitor. Extension of 5-methyl group as (un)substituted arylmethyl group also resulted in potent analogues (22a–22c, Table 2) as non-competitive inhibitors. This demonstrated the tight structural requirements of inhibitors with respect to the kinetics of inhibition (competitive vs. non-competitive). Nonetheless, the isoxazole scaffold is a novel scaffold, quite different from the sulfasalazine and analogs. This may be further explored synthetically or computationally to fill the void in the availability of novel, orally bioavailable system x_c^- inhibitors.

Erastin and analogues

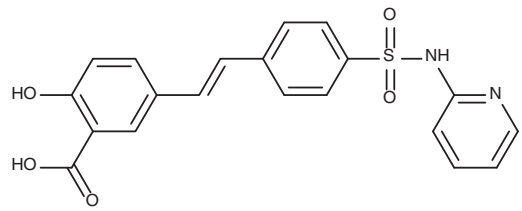
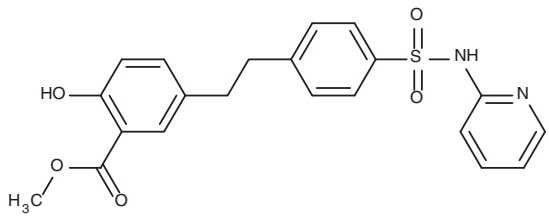
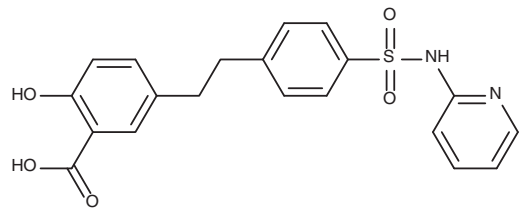
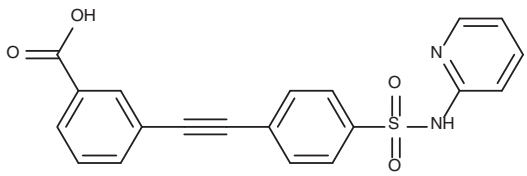
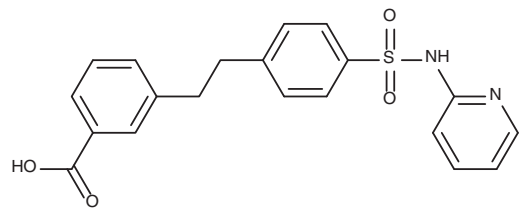
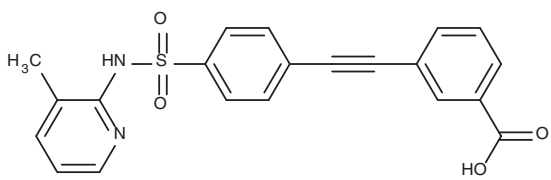
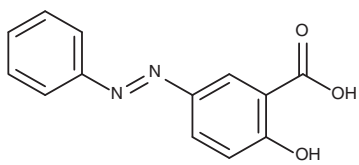
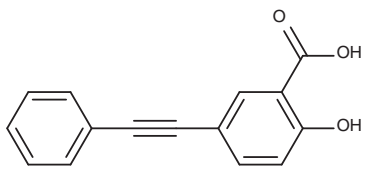
Lethal effective concentration (EC) for all synthesized erastin analogues was determined in HT-1080 cells in a 10-point, two-fold dilution assay, starting at a high concentration of 20 μM, ±β-ME (18 μM). IC_{50} values were also determined for the synthesized analogues in CCF-STTG1 cells using glutamate uptake assay by Dixon et al. (2014). Erastin (**32**, Table 2, $IC_{50} = 0.20\,\mu M$) a quinolone-4-one, provided another lead structure for the design and development of potent system x_c^- inhibitors. Systematic structural modifications resulted in quite potent analogues with IC_{50} as low as 3.5 nM (**49**, Table 2). The majority of the analogues retained the

Table 2. List of system x_c^- inhibitors along with their IC_{50} value and potency comparison with sulfasalazine (Dixon et al., 2014; Matti et al., 2013; Shukla et al., 2011). a: [^{3}H]Glu uptake (% control), b: EC_{50}.

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
1	(S)-4-carboxyphenylglycine		15	–
2	(R)-4-carboxyphenylglycine		>1000	–
3	Sulfasalazine		30	–
4	Sulfapyridine		>1000	Less
5	5-Amino salicylic acid		>1000	Less
	Sulfasalazine analogues			
6	Methyl 2-hydroxy-5-(2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethynyl)benzoate		>1000	Less
7	2-Hydroxy-5-(2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethynyl)benzoic acid		30	Same
8	2-Hydroxy-5-[(E)-2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethenyl]benzoate		900	Less

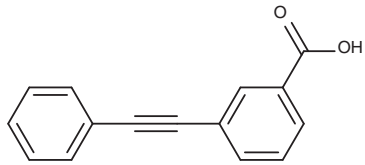
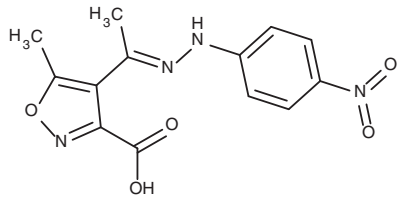
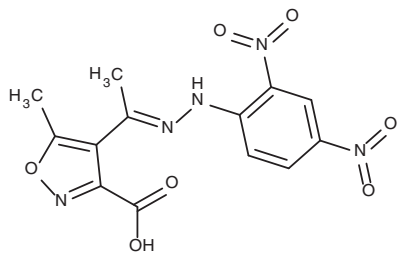
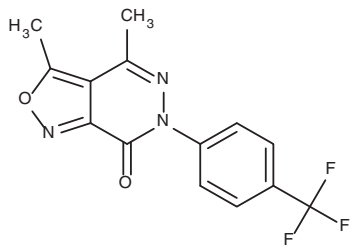
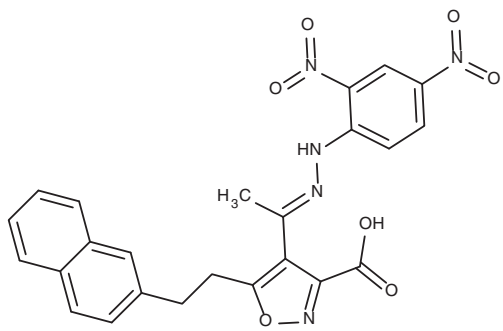
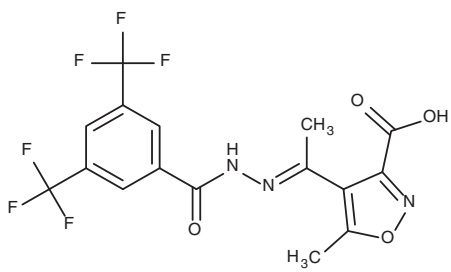
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
9	2-Hydroxy-5-[(E)-2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethenyl]benzoic acid		70	Slight Less
10	Methyl 2-hydroxy-5-(2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethyl)benzoate		>1000	Less
11	2-Hydroxy-5-(2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}ethyl)benzoic acid		700	Less
12	3-(2-{4-[(Pyridin-2-yl)sulfamoyl]phenyl}ethynyl)benzoic acid		75	Slight Less
13	3-(2-{4-[(Pyridin-2-yl)sulfamoyl]phenyl}ethyl)benzoic acid		920	Less
14	3-(2-{4-[(3-Methylpyridin-2-yl)sulfamoyl]phenyl}ethynyl)benzoic acid		70	Slight Less
15	2-Hydroxy-5-[(E)-2-phenyldiazen-1-yl]benzoic acid		200	Less
16	2-Hydroxy-5-(2-phenylethynyl)benzoic acid		150	Less

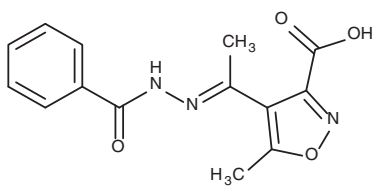
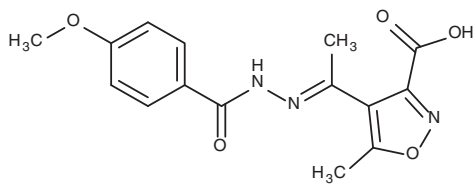
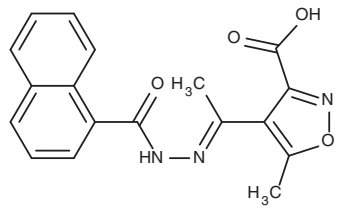
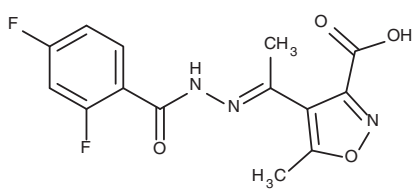
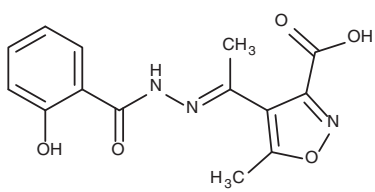
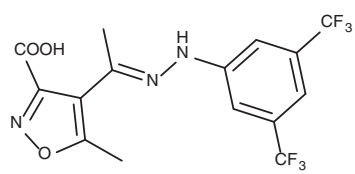
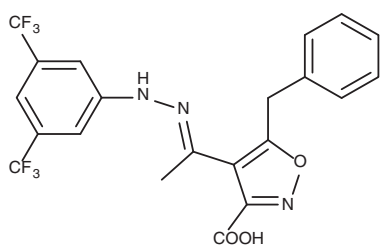
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
17	3-(2-Phenylethynyl)benzoic acid		270	Less
	Iso-oxazole derivatives			
18	5-Methyl-4-[(1E)-1-[2- (4-nitrophenyl)hydrazin-1-ylidene]ethyl]-1,2-oxazole-3-carboxylic acid		127	Less
19	4-[(1E)-1-[2-(2,4-Dinitrophenyl)hydrazin-1-ylidene]ethyl]-5-methyl-1,2-oxazole-3-carboxylic acid		290	Less
20	3,4-Dimethyl-6-[4-(trifluoromethyl)phenyl]-6H,7H-[1,2]oxazolo[3,4-d]pyridazin-7-one		129	Less
21	4-[(1E)-1-[2-(2,4-Dinitrophenyl)hydrazin-1-ylidene]ethyl]-5-[2-(naphthalen-2-yl)ethyl]-1,2-oxazole-3-carboxylic acid		225	Less
22	4-{1-[(3,5-Bis-trifluoromethyl-cyclohexa-1,3-dienecarbonyl)-hydrazono]-ethyl}-5-methyl-isoxazole-3-carboxylic acid		90[a]	

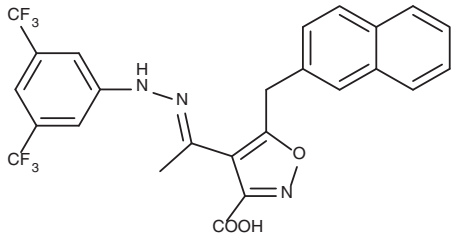
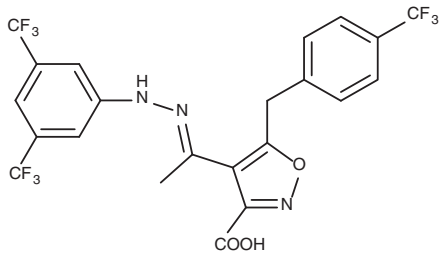
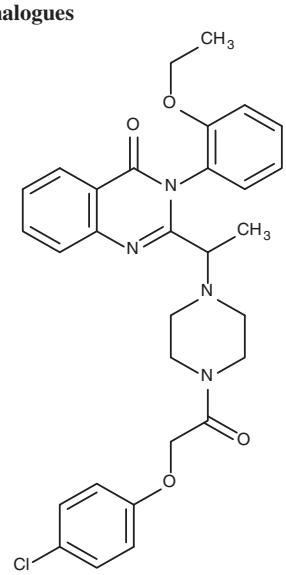
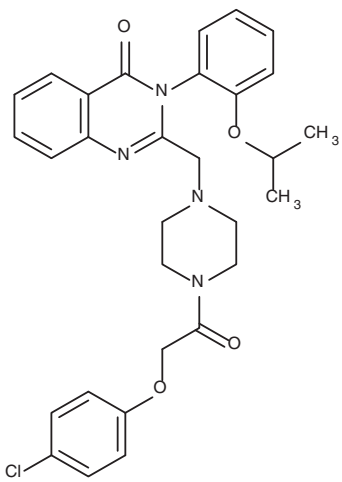
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
23	4-{1-[(Cyclohexa-1,3-dienecarbonyl)-hydrazono]-ethyl}-5-methyl-isoxazole-3-carboxylic acid		83[a]	
24	4-{1-[(4-Methoxy-cyclohexa-1,3-dienecarbonyl)-hydrazono]-ethyl}-5-methyl-isoxazole-3-carboxylic acid		68[a]	
25	5-Methyl-4-{1-[(naphthalene-1-carbonyl)-hydrazono]-ethyl}-isoxazole-3-carboxylic acid		68[a]	
26	4-{1-[(2,4-Difluoro-cyclohexa-1,3-dienecarbonyl)-hydrazono]-ethyl}-5-methyl-isoxazole-3-carboxylic acid		58[a]	
27	4-{1-[(2-Hydroxy-cyclohexa-1,3-dienecarbonyl)-hydrazono]-ethyl}-5-methyl-isoxazole-3-carboxylic acid		52[a]	
22	4-(1-(2-(3,5- bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5-methylisoxazole-3-carboxylic acid		14[a]	
22a	5-benzyl-4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)-hydrazono)ethyl)isox azole-3-carboxylic acid		6[a]	

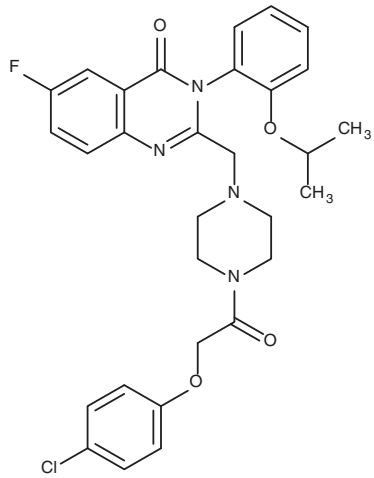
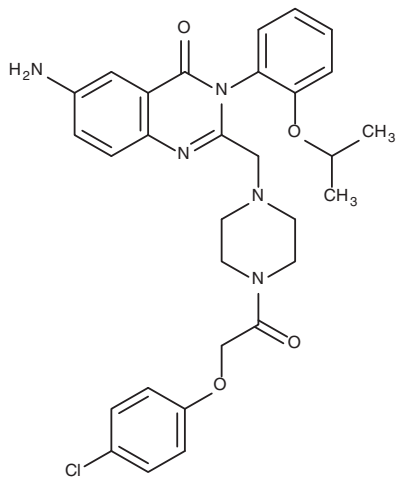
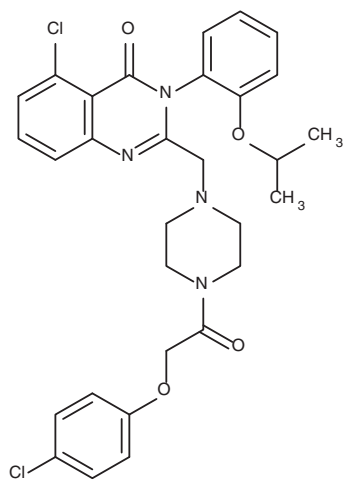
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
22b	4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5- (naphthalen-2-ylmethyl)isoxazole-3-carboxylic acid		14[a]	
22c	4-(1-(2-(3,5-bis(trifluoromethyl)phenyl)hydrazono)ethyl)-5- (4-(trifluoromethyl)benzyl)isoxazole-3-carboxylic acid		6[a]	
	Erastin and its analogues			
28	2-(1-{4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}ethyl)-3-(2-ethoxyphenyl)-3,4-dihydroquinazolin-4-one (Erastin)		0.20	High
29	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.09	High

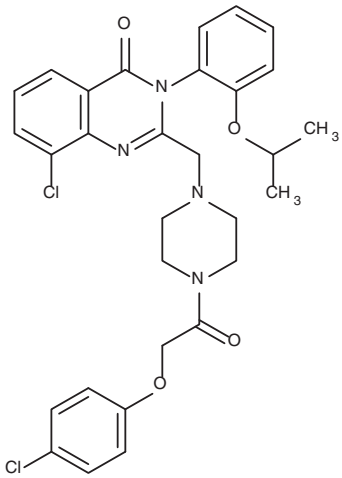
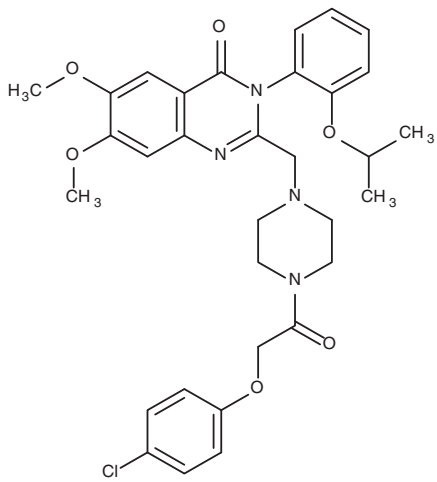
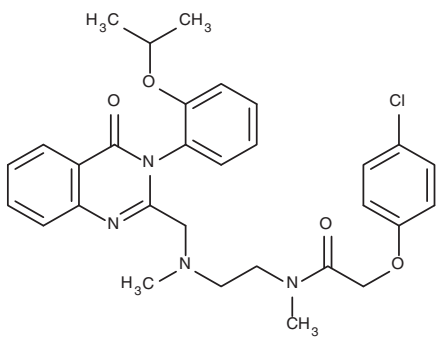
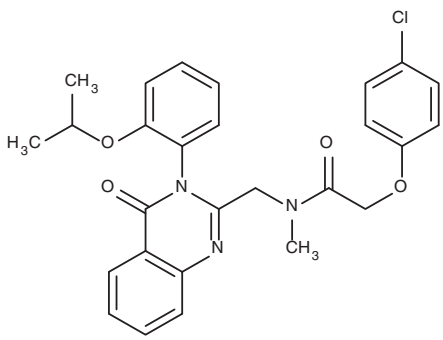
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
30	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-6-fluoro-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.14	High
31	6-Amino-2-({4-[2-(4-chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.54	High
32	5-Chloro-2-({4-[2-(4-chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.042	High

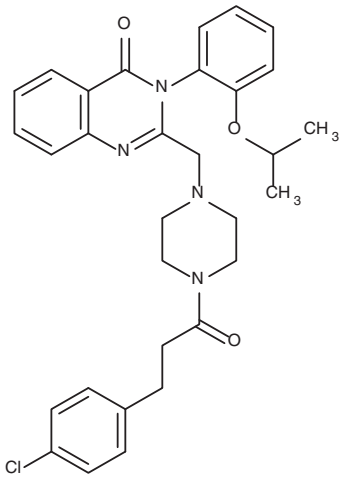
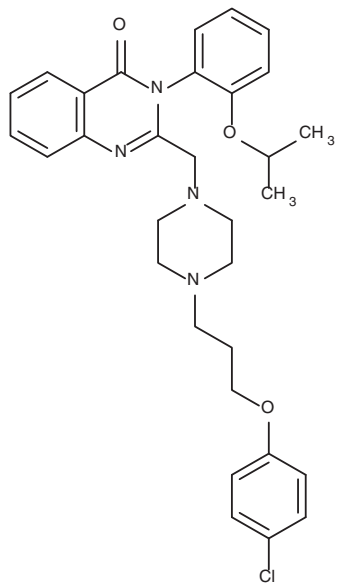
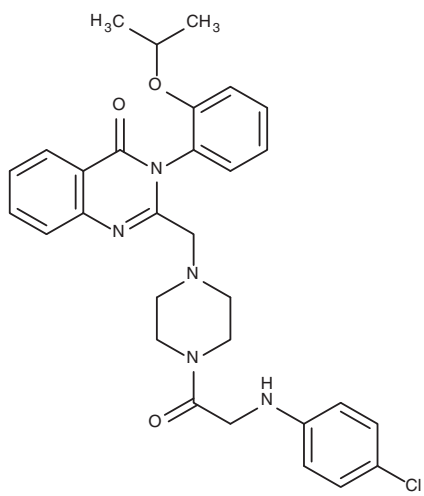
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
33	8-Chloro-2-({4-[2-(4-chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.074	High
34	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-6,7-dimethoxy-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.15	High
35	2-(4-Chlorophenoxy)-N-methyl-N-{2-[methyl({4-oxo-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-2-yl}methyl)amino]ethyl}acetamide		0.56	High
36	2-(4-Chlorophenoxy)-N-methyl-N-({4-oxo-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-2-yl}methyl)acetamide		>10	High

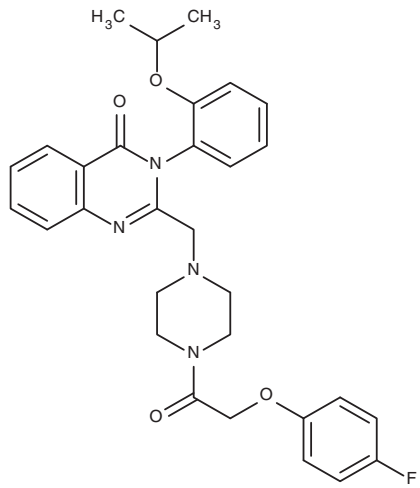
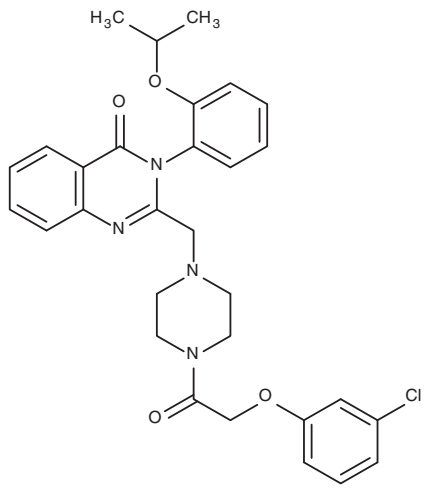
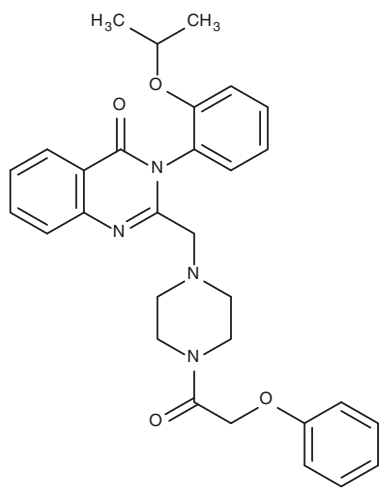
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
37	2-({4-[3-(4-Chlorophenyl)propanoyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.57	High
38	2-({4-[3-(4-Chlorophenoxy)propyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		3.7	High
39	2-[(4-{2-[(4-Chlorophenyl)amino]acetyl}piperazin-1-yl)methyl]-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.53	High

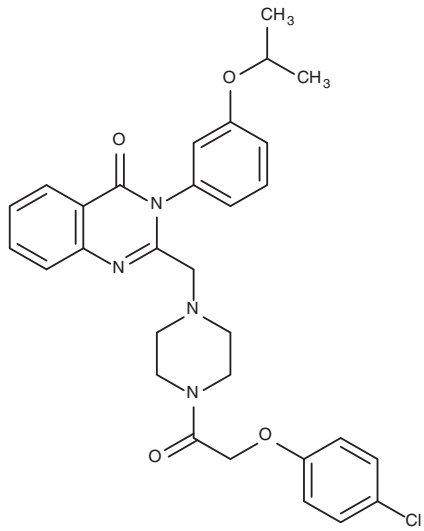
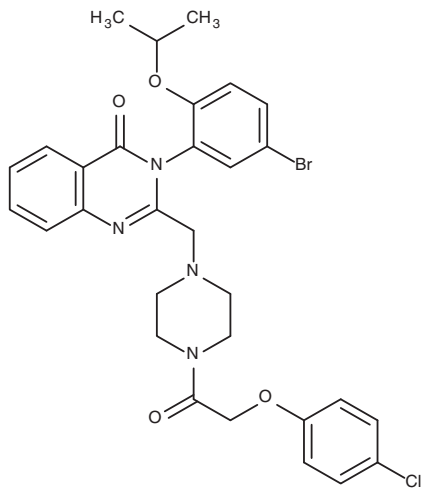
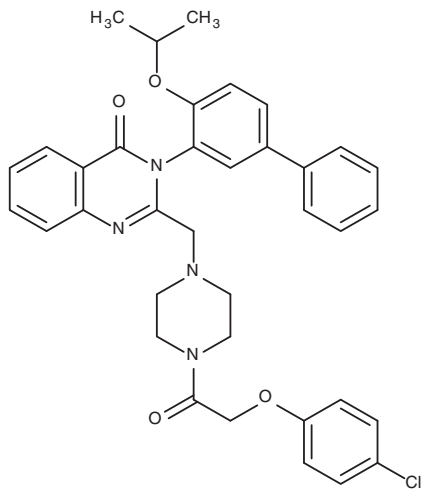
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
40	2-({4-[2-(4-Fluorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		1.2	High
41	2-({4-[2-(3-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		2.6	High
42	2-{[4-(2-Phenoxyacetyl)piperazin-1-yl]methyl}-3-[2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		>10	High

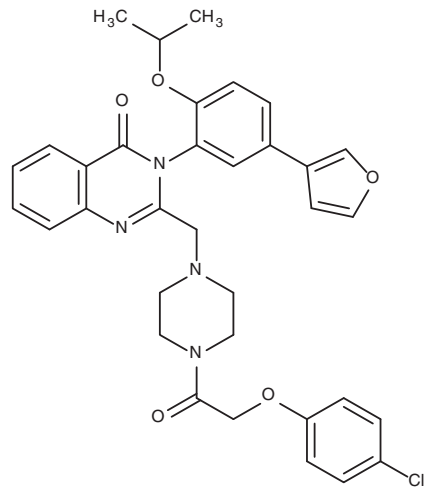
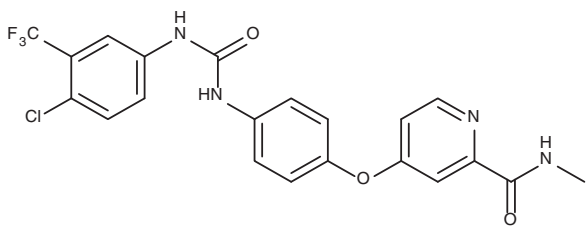
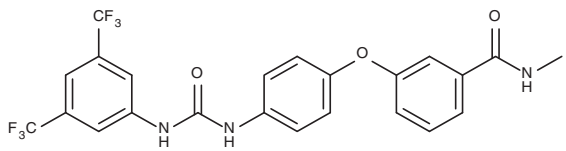
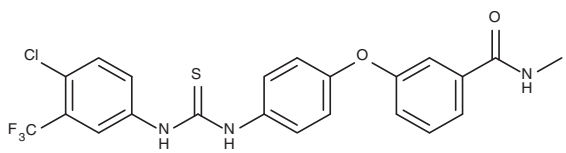
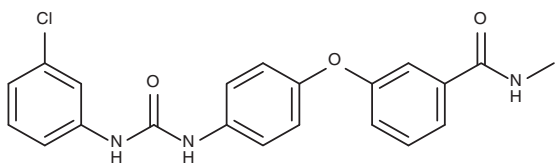
(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
43	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[3-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.042	High
44	3-[5-Bromo-2-(propan-2-yloxy)phenyl]-2-({4-[2-(4-chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3,4-dihydroquinazolin-4-one		0.0094	High
45	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[5-phenyl-2-(propan-2-yloxy)phenyl]-3,4-dihydroquinazolin-4-one		0.0035	High

(*continued*)

Table 2. Continued

Compound No.	Name	Structure	IC_{50} (μM)	Potency Comparison with Sulfasalazine
46	2-({4-[2-(4-Chlorophenoxy)acetyl]piperazin-1-yl}methyl)-3-[5-(furan-3-yl)-2-(propan-2-yloxy)-phenyl]-3,4-dihydroquinazolin-4-one		0.013	High
47	Sorafenib		18[b]	
48	3-{4-[3-(3,5-Bis-trifluoromethyl-phenyl)-ureido]-phenoxy}-N-methyl-benzamide			
49	3-{4-[3-(4-Chloro-3-trifluoromethyl-phenyl)-thioureido]-phenoxy}-N-methyl-benzamide			
50	3-{4-[3-(3-Chloro-phenyl)-ureido]-phenoxy}-N-methyl-benzamide			

original structural framework of **32**, with modification in the N^3- substituent which seem to require other bulky substitutes at C-5 on the aromatic ring such as –Br, –Ph etc. for improved potency. The 2-isopropoxy substituents also seemed necessary for the potency (**32** vs. **33**, Table 2). Addition of substituents on the core quinolone-4-one nucleus led to >1.5-fold reduction in potency (**33** vs. **34**, Table 2). Placement of 5-Cl substituent on the quinazoline-4-one nucleus, however increase potency 2-fold (**33** vs. **36**, Table 2). Conversion of the pendant piperazine ring in **32** to the open chain congener led to 3-fold drop in potency (**39**, Table 2). Also, replacing ether (–OPh) in distal part of **32** with –NH– led to 2.5-fold decrease in potency (**43**, Table 2). Other modifications led to reduction in potency.

Conclusions

The prevalence of CNS disorders is increasing day-by-day with a high mortality rate throughout the world. The adequate management of CNS disorders is becoming a tough

Table 3. Possible chemical modifications in sulfasalazine for its improved pharmacokinetic profile.

Replaceable functional group	Possible advantage
Diazo link	
–NH–CO–NH–	Metabolically stable
–NH–NH–	Conformationally flexible
–NH–CO–	Metabolically stable; maintains similar orientation of the two groups attached
$–CH_2–NH–$	Metabolically stable; conformationally flexible compared to sulfasalazine
Sulfonamide group	
–NH–CO–NH–	Possible improvement in aqueous solubility
–CO–NH–	
Salicylic acid moiety	
–COOEt	May act as a prodrug
	To emphasize the importance of –COOH group
–CH=CH–COOH	Increased lipophilicity
1-Naphthyl	
2-Naphthyl	
$–OCH_3$ for –OH	To emphasize the importance of –OH group
2-Nitrophenol	Bioisosteric replacement of –COOH; Increased lipophilicity
2-Aminophenol	To emphasize the importance of –COOH
$–CONH_2$ for –COOH	group
Benzoic acid	To emphasize the importance of –OH group
Pyridine moiety	
Thiazole	Increase the lipophilicity and/or reduce the
Benzene	topological polar surface area
$–COCH_3$	

task because of insufficient knowledge in such areas. Advances in understanding how system x_c^- contributes to the pathophysiology of CNS disorders might pave the way for future treatment strategies. The dual role of system x_c^- transporter, i.e. glutamate release and cysteine uptake, has participation in the pathophysiology of many CNS diseases. Thus, targeting system x_c^- could serve the purpose of treatment in CNS complaints. A number of research groups have generated small molecule hits/leads which are potent system x_c^- inhibitors but still their clinical efficacy will be a major concern. Hence, further research is needed to explore the system x_c^- target as a therapeutic option for CNS disorders.

Future prospects

This review describes various aspects of system x_c^- in variety of CNS disorders. Until now, only preliminary research or *in vitro* studies have been performed for modifying system x_c^- activity and there is very little documentation about preclinical and clinical studies. With regard to this, extensive research is required to draw a valid conclusion about how system x_c^- inhibition or activation will benefit in CNS disorders. Additionally, there are limited numbers of sulfasalazine analogues that have demonstrated therapeutic potential and therefore synthesis of additional active sulfasalazine analogues should also be pursued. Like sulfasalazine and its analogs, other drugs can also be repurposed and evaluated for their system x_c^- inhibition activity.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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