Biochemistry and Biophysics Reports 9 (2017) 266–272

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Development of a primary microglia screening assay and its use to characterize inhibition of system x_c^- by erastin and its analogs

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ARTICLE INFO

Keywords:
Primary microglia
CCF-STTG-1 cells
System x_c^-
Erastin
Sulfasalazine
(*S*)-4-CPG

ABSTRACT

The inflammatory response in the central nervous system involves activated microglia. Under normal conditions they remove damaged neurons by phagocytosis. On the other hand, neurodegenerative diseases are thought to involve chronic microglia activation resulting in release of excess glutamate, proinflammatory cytokines and reactive oxygen species, leading to neuronal death. System x_C^- cystine/glutamate antiporter (SXC), a sodium independent heterodimeric transporter found in microglia and astrocytes in the CNS, imports cystine into the cell and exports glutamate. SXC has been shown to be upregulated in neurodegenerative diseases including multiple sclerosis, ALS, neuroAIDS Parkinson's disease and Alzheimer's disease. Consequently, SXC inhibitors could be of use in the treatment of diseases characterized by neuroinflammation and glutamate excitotoxicity. We report on the optimization of a primary microglia-based assay to screen for SXC inhibitors. Rat primary microglia were activated using lipopolysaccharides (LPS) and glutamate release and cystine uptake were monitored by fluorescence and radioactivity respectively. LPS-induced glutamate release increased with increasing cell density, time of incubation and LPS concentration. Conditions to screen for SXC inhibitors were optimized in 96-well format and subsequently used to evaluate SXC inhibitors. Known SXC inhibitors sulfasalazine, S-4CPG and erastin blocked glutamate release and cystine uptake while R-4CPG, the inactive enantiomer of S-4CPG, failed to inhibit glutamate release or cystine transport. In addition, several erastin analogs were evaluated using primary microglia and found to have EC_{50} values in agreement with previous studies using established cell lines.

1. Introduction

Inflammation in the central nervous system (CNS) is thought to play an important role in the pathogenesis of neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, HIV-1 associated neurocognitive disorders, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and stroke. The inflammatory response is mediated by activated microglia, the resident immune cells of the CNS. Microglial cells represent 10–20% of the cellular population in the CNS and similar to their peripheral counterparts, the macrophages, microglia behavior and morphology are highly dependent on their microenvironment [1,2]. Under normal conditions microglial cells are partly responsible for innate immunity in the CNS; microglia respond to neuronal damage and remove damaged cells by phagocytosis. On the other hand, chronic activation of microglia may cause neuronal damage involving excess glutamate release. Excess glutamate release is thought to induce excitotoxicity through increased activation of neuronal glutamate receptors, increased calcium intake and neuronal apoptosis [3,4].

One glutamate transporter that contributes to nonvesicular release

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http://dx.doi.org/10.1016/j.bbrep.2016.12.009
Received 28 September 2016; Received in revised form 21 December 2016; Accepted 29 December 2016
Available online 17 January 2017

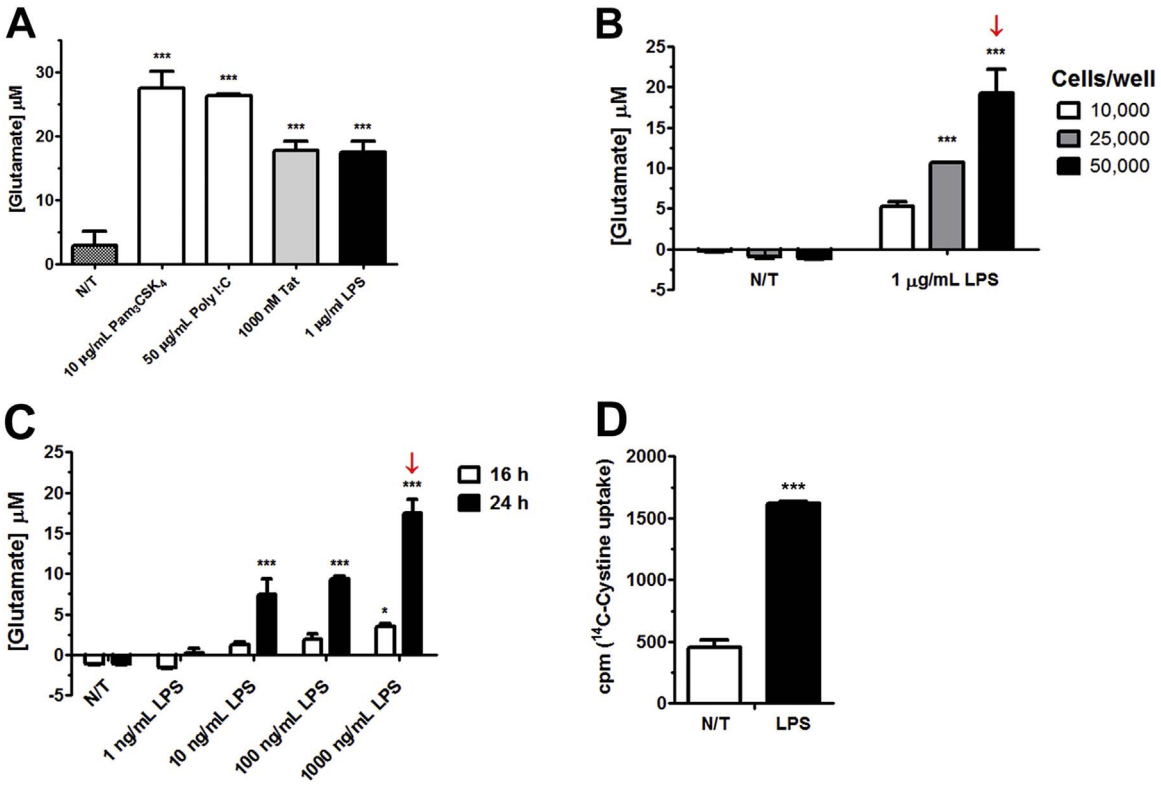


Fig. 1. Characterization of microglia activation assay in 96-well format - (A) Induction of glutamate release from rat primary microglia after 24 h stimulation with Pam_3CSK_4 (10 µg/ml), poly I: C (50 µg/ml), Tat (1 µM) or LPS (1 µg/ml). **(B)** Dependence of glutamate release on cell density after 24 h incubation with LPS (1 µg/ml). **(C)** Dependence of glutamate release on LPS-concentration and time of incubation. Arrows indicate the number of cells (50,000 cells), concentration of LPS (1 µg/ml) and incubation time (24 h) chosen for the 96-well plate microglia activation assays. **(D)** Cystine uptake by primary microglia after 1 µg/ml LPS treatment for 24 h. N/T: no treatment. Specific rate of cystine uptake in microglial cells in the absence of inhibitors from three independent experiments conducted in duplicate was 0.015 ± 0.002 nmol/mg/min. Statistically significant differences (*$p < 0.05$ and ***$p < 0.001$) correspond to a comparison to no treatment (N/T) group.

of glutamate is the cystine/glutamate antiporter SXC [5–7]. This is a Na^+-independent, Cl^--dependent antiporter [5] expressed in a wide variety of cells in the CNS where it plays a key role in glutamatergic neurotransmission as a result of its ability to release glutamate [8–10]. Most recently it has been shown that SXC is a key factor in maintenance of cell homeostasis in oligodendrocytes [11]. SXC is also the transporter largely responsible for glutamate release in activated microglia associated with certain brain pathologies [12–14] and it is a potential drug target for neuroinflammatory diseases associated with excess extracellular glutamate [15]. SXC imports cystine and exports glutamate in a 1:1 ratio [5,16]. However, a recent study shows that in astrocytoma cancer cells (CCF-STTG1) the ratio of cystine uptake to glutamate release could be 10:1 [15]. SXC has been associated with neurological diseases such as AD [17], MS [18,19], ALS [8], neuroAIDS [20] and PD [21,22] and its expression can be enhanced in cell culture by LPS [19] and cytokines like IL-1β [23]. Once cystine is transported inside the cell by SXC, it is reduced to cysteine, which is then utilized for synthesis of the antioxidant tripeptide glutathione (GSH) [24]. GSH is essential for restoring intracellular redox balance when ROS is produced [12]. Inhibition of SXC with small molecules such as (*S*)-4-carboxyphenyl glycine ((*S*)-4-CPG), L-α-aminoadipic acid, sulfasalazine and erastin cause both protective and toxic effects in a variety of cells and *in vivo* systems [16,25–28]. On the positive side, therapeutic inhibition of SXC in cells such as microglia and macrophages protect against neuronal cell death due to exposure to high extracellular glutamate levels and excessive stimulation of glutamate receptors [12,21]. SXC activity has also been associated with various aspects of cancer, including growth and metastasis, glutathione dependent drug resistance and excitotoxicity. Inhibition or deficiency of the transporter leads to cystine starvation and cancer cell growth arrest, cell death and/or enhanced sensitivity to chemotherapy [29]. Although promising as a therapeutic target, there has been a concern that cystine starvation and reduced GSH levels could have detrimental effects in non-cancerous cells exposed to oxidative stress [30]. However, SXC knockout (KO) animals develop normally and are fertile [31]. Further, studies with SXC inhibitors and siRNA have shown good tolerability [32,33]. Given this, SXC has become a target of interest so that monitoring glutamate release during microglia activation and evaluating the effect of potential drugs on blocking this release is of major interest in drug discovery.

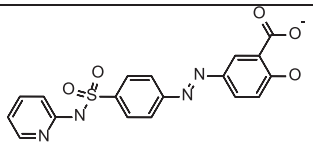
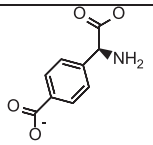
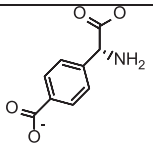
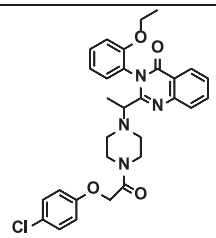
Even though primary microglia have previously been used to evaluate SXC inhibitors [18,34–36], the assay has not been thoroughly characterized or implemented to enable moderate to high throughput screening for SXC inhibitors. Herein, we report for the first time, on the characterization of a 96-well format primary microglia-based assay to screen for SXC inhibitors. The assay was systematically characterized for activation of primary microglia as measured by LPS-induced glutamate release with respect to different activating agents, cell density and time of incubation. Subsequently, we used the optimized assay to assess a correlation between glutamate release and cystine uptake using erastin analogs.

2. Materials and methods

2.1. Reagents

Lipopolysaccharides (LPS) from *Escherichia coli* strain O111:B4, erastin, polyinosinic: polycytidylic acid (poly I:C), Pam_3CSK_4, apigenin, buthionine sulphoximine (BSO) and cystine were purchased from Sigma-Aldrich. Cell culture reagents and supplies were purchased from Life Technologies and Greiner Bio One respectively. HIV1- Tat_{1-86} protein was obtained from Diatheva.

Table 1
Glutamate release and cystine uptake inhibition by SXC inhibitors in CCF-STTG-1 cells and LPS-treated microglial cells. [a] SXC activity was evaluated in CCF-STTG-1 human astrocytoma cell line and primary microglia by determining glutamate release using the fluorometric assay (methods). EC_{50} ± S.D. for the compounds shown were estimated from 8-point dose response curves. [b] ^{14}C-cystine transport by LPS-activated rat primary microglia was determined at concentrations ranging from 1 to 1000 μM.

Compound	Structure	Glu release[a] inhibition STTG1 EC_{50} (μM)	Glu release[a] inhibition microglia EC_{50} (μM)	Cys uptake[b] inhibition microglia EC_{50} (μM)
Sulfasalazine		20 ± 4	16 ± 4	38 ± 14
S-4CPG		1.9 ± 0.5	4.4 ± 2.5	3.9 ± 0.7
R-4CPG		> 1000	> 1000	> 500
Erastin		0.17 ± 0.05	0.13 ± 0.04	0.82 ± 0.17

2.2. Primary microglia cultures

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under protocol number MO15M485 issued to Barbara S. Slusher. Rat primary microglia cells cultures (≥95% purity) were generated and isolated following standard protocols with few modifications [37]. Briefly, brains of 1–2 day old pups euthanized by CO_2 asphyxiation were dissected in ice cold L15 solution (Thermo Fisher Scientific). Meninges and cerebellum were removed and cell dissociation was carried out by pipetting up and down cells in L15 solution containing Accutase (Innovative Cell Technologies Inc.) diluted 1:4. Cells were diluted two-fold with DMEM/F12 with 10% fetal bovine serum (FBS) and ampicillin/amphotericin antibiotic (complete culture medium). Cells were then pelleted at RT for 10 min at 200×g, and resuspended in L15 solution using 1/4 of the original volume. The cell suspension was passed through a 40 μm nylon cell strainer (BD), and cells were washed with complete culture medium once and seeded at a density of 1 rat brain per 75 cm^2 flask. After cells were in culture for 7 days, half the culture medium was replaced with fresh complete medium. Subsequently, similar media changes were conducted every 2 – 3 days until time of harvest.

2.3. Microglia activation assay

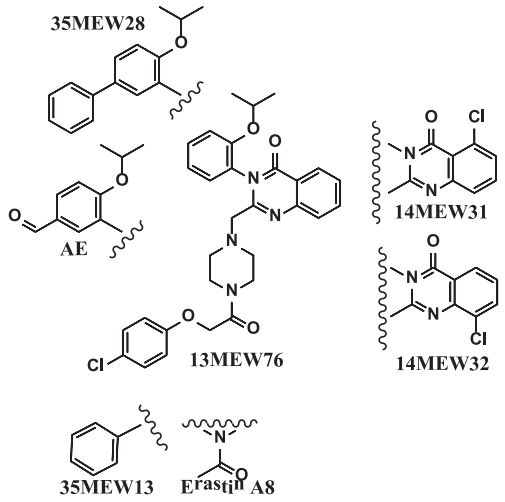
Primary microglia cells were harvested from 14 to 21 days *in vitro* mixed glial cultures. Fresh complete media were added to the flasks prior to harvest and the flasks placed on a shaker at 200 rpm for 2 h at 37 °C. While the detached microglia were plated at 50,000 cells per well in poly-D-Lys coated 96-well plates in complete culture medium, the undetached, remaining microglia were allowed to grow in flasks for another 7 days prior to a second microglia harvest. Poly-D-Lys coated plates enhanced cell binding, therefore minimizing losses during washing steps prior to LPS activation. After 24 h, the culture medium was replaced with serum-free and phenol red-free DMEM containing 2 mM glutamine (assay medium). Activation was done by treating the cells with 1 μg/ml LPS for 24 h. Test compounds were added to cells 15 min prior to activation in assay medium containing a final concentration of 0.1% DMSO. Experiments were carried out in duplicate or triplicate using 8 concentrations in the 10 pM −100 μM range.

2.4. Glutamate release measurements

Glutamate release experiments were conducted in 96-well format in phenol-free media (DMEM, ThermoFisher Scientific Catalog No. 31053-028) containing cystine (0.2 mM). Microglia cells were exposed to LPS (or other activators) for 24 h (±) inhibitors. Extracellular glutamate levels were determined using an enzyme-coupled, Amplex UltraRed fluorescence-based assay as previously reported [38]. Parallel determinations (counter screens) were always carried out with all components of the assay except microglia (SXC source) to insure potential inhibitors were not inhibiting the coupling enzymes in the detection system and appearing as false positives. Concentration of glutamate release was obtained from a standard curve of fluorescence readings vs. glutamate concentration. The standard curve was generated by incubating known glutamate concentrations with the Amplex UltraRed detection system and measuring the corresponding fluores-

Table 2
Effect of erastin analogs on SXC transporter activity in LPS-treated rat primary microglial cells. (A) Chemical structures of erastin analogs used in SAR study (B) Inhibition of SXC transporter activity in microglia - EC_{50} ± S.D. for both glutamate release and cystine uptake are shown. Erastin A8 structure has an ethyl instead of an isopropyl substitution in the northern part of the molecule.

(A)



(B)	Glu release inhibition EC_{50} (μM)	Cys uptake inhibition EC_{50} (μM)
13MEW76	0.16 ± 0.02	4.78 ± 1.74
35MEW28	0.04 ± 0.01	0.38 ± 0.04
AE	0.14 ± 0.01	0.56 ± 0.06
14MEW31	0.12 ± 0.02	0.68 ± 0.10
14MEW32	0.2 ± 0.03	1.75 ± 0.39
35MEW13	> 10	> 100
Erastin A8	> 10	> 100

cence units. Correlation of fluorescence with glutamate concentration was linear in the 0.5–64 μM range of glutamate concentrations. Concentrations of glutamate release from microglia in the absence of inhibitors were in the 14.5–18.0 μM range.

2.5. ^{14}C-Cystine uptake measurements

Cystine uptake experiments were conducted in 96-well format following previously published methods [39]. Microglia cells were first exposed to either HBSS or LPS (in HBSS) for 24 h (in DMEM medium containing 0.2 mM cystine as in glutamate release experiments). Medium was then removed and cystine uptake was measured after incubation with [^{14}C]-cystine as a tracer (45 μCi/μmol, 10 μM) in the presence of LPS ± inhibitors for 15 min in Earle's Balanced Salt Solution (EBSS, Sigma Catalog No. E3024). An aliquot of the DMEM Medium from the initial 24 h incubation with LPS was used to measure glutamate release to insure microglia had been activated. Specific uptake of [^{14}C]-cystine corrected for radioactive contaminants like leakage and binding was determined by subtracting background control from each [^{14}C]-cystine measurement. Background controls were obtained by incubating non-stimulated microglia (HBSS only) with [^{14}C]-cystine tracer in the absence of LPS but in the presence of 1 mM sulfasalazine, known to completely inhibit SXC activity.

2.5.1. Biological data collection and statistical analyses

Results are average of at least four determinations. Curve fitting and statistical analyses (one-way ANOVA with Tukey's post hoc test) were done in Prism 5 (GraphPad). Results were considered statistically significant when p values were ≤0.05 (indicated with *). P values < 0.01 and < 0.001 were indicated with (**) and (***) respectively.

3. Results and discussion

3.1. Screening for inhibitors of SXC can be performed using primary microglia in 96-well format

Studies on pharmacological SXC inhibition highlight its importance in the maintenance of physiological processes like glutamatergic neurotransmission [8–10], and cell homeostasis in oligodendrocytes [11]. In addition, pharmacological studies are suggestive of the potential of SXC as a drug target in a variety of diseases including Alzheimer"s disease [36], autoimmune inflammatory demyelination [34] and malignant glioma [40]. Unfortunately, inhibitors that have been used to study the role of SXC are not very potent (μM and mM affinities), exhibit off-target effects and /or are metabolically unstable [12]. Currently there are no potent, selective SXC inhibitors that can be advanced to the clinic for the treatment of diseases where SXC is involved. One way to find new SXC inhibitors is through the screening of a large number of compounds for SXC inhibition using well characterized screening assays. The use of primary microglia in a screening assay is of interest given its relevance in SXC mediated effects. Even though primary microglial cells have been used to evaluate SXC inhibitors [34–36], to our knowledge the assay has not been systematically characterized. In the present work we implemented an SXC screening assay in 96-well format using primary microglia after optimizing it for glutamate release with respect to different activating

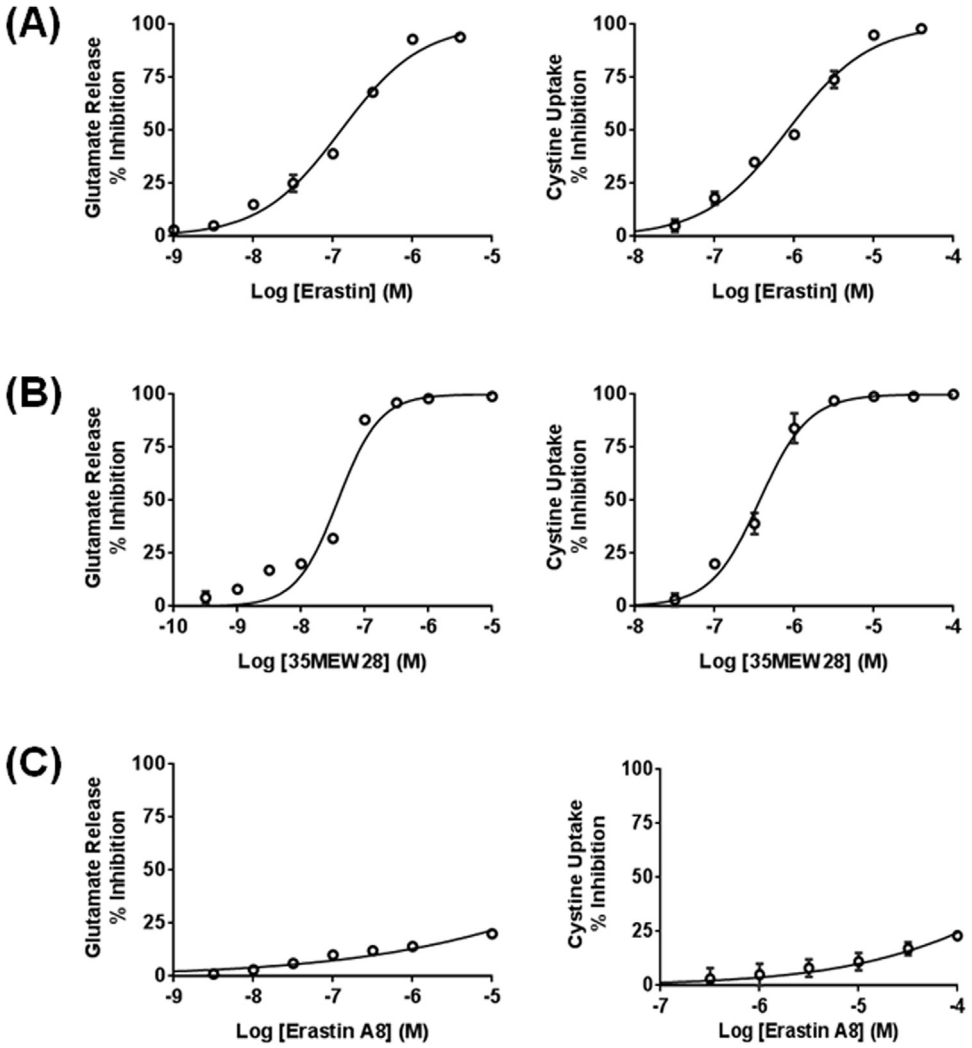


Fig. 2. Inhibition of SXC activity by erastin and analogs - Representative dose response curves illustrating inhibition of primary microglia glutamate release (left panels) and cystine uptake (right panels) by (A) erastin, (B) the most potent inhibitor 35MEW28 and (C) an inactive analog Erastin A8. Each data point corresponds to the average of three replicates. Specific rate of cystine uptake in microglial cells in the absence of inhibitors from three independent experiments conducted in duplicate was 0.015 ± 0.002 nmol/mg/min. Error bars are S.E.M. When error bars are not seen it is because their magnitude is smaller than the symbol depicting the average.

agents, cell density and time of incubation. First, we characterized microglia stimulation with various known microglia activators. We used rat rather than mice cell cultures as SXC source because rat microglia exhibited larger and more reproducible increases in glutamate release upon treatment with various microglia activators than primary microglia isolated from mice. Significant glutamate release occurred after 24 h treatment with various toll-like receptor ligands such as Pam_3CSK_4, poly I:C and LPS and with the HIV-1 Tat_{1-86} protein (Fig. 1A). LPS was the stimulant of choice because even though it did not exhibit the most activation, it still exhibited a robust response (~ 4-fold increase in glutamate over untreated microglia), it is commercially available, it is reasonably priced, and there is an abundance of literature on its uses as a microglial or macrophage activator [41–43]. Second, we characterized the measurement of glutamate release with respect to cell density (Fig. 1B), LPS concentration and time of incubation (Fig. 1C). We chose 50,000 cells per well and 1 μg/ml LPS treatment for 24 h to monitor glutamate release. Signal to background ratio for glutamate induction under these conditions was 4 to 5-fold for activated microglia vs. untreated cells. Since cystine concentration was 0.2 mM for all cells when measuring glutamate release (methods), the only parameter that was different was the presence or absence of microglia activators. As a result, increases in glutamate release when using untreated microglia (N/T) vs. microglia treated with LPS or other activators (Fig. 1A, B, C) must have been due to the presence of activators. In addition to LPS-induced glutamate release, cystine transport was approximately 3-fold higher in LPS-treated microglia vs. untreated microglia (Fig. 1D).

In the next set of experiments we used prototype SXC inhibitors to demonstrate that under these conditions inhibition of both glutamate release and cystine uptake could be monitored (Table 1). As controls, we measured LPS-induced glutamate release ± known inhibitors of SXC [15,44]. Inhibition of glutamate release by sulfasalazine, S-4CPG and erastin was the same within error when using CCF-STTG-1 cells or primary microglia (Table 1**).** Inhibition of cystine uptake was also observed with the three prototype inhibitors (Table 1). In contrast, (*R*)-4-CPG, the inactive enantiomer of (*S*)-4-CPG, did not inhibit glutamate release or cystine uptake. Even though some of these prototype inhibitors have been shown to inhibit additional targets [45–47] they are all SXC inhibitors and could be used for their inhibitory activity in the SXC assay characterization.

3.2. Erastin analogs attenuate LPS-induced glutamate release and cystine uptake by primary microglia

A recent study showed that erastin and some of its analogs were very potent inhibitors of SXC function in cancer cells; SXC inhibition

was shown to interfere with protein folding and to cause endoplasmic reticulum stress [44]. Given that these compounds are the most potent SXC inhibitors known, they were used to help appraise the validity of our assays. We evaluated the ability of seven erastin analogs to inhibit LPS-induced glutamate release or cystine uptake in primary microglia (Table 2). In agreement with previous studies using established cell lines [44,48], replacement of the ethoxy group on erastin with an isoproxy group and generation of an achiral analog by deletion of the methyl group at the chiral center did not affect inhibitory activity (13MEW76); EC_{50} values for blockade of microglia glutamate release by erastin and 13MEW76 were 0.13 ± 0.04 and 0.16 ± 0.02 μM respectively (Tables 1 and 2). Elimination of the para-chloro phenoxy moiety (Erastin A8) or the para-chloro substituent (35MEW13) abolished inhibitory activity (Table 2). Chlorine substitutions on the benzene ring of the quinazolinone scaffold (14MEW31 and 14MEW32) did not affect inhibitory activity (Table 2). Introduction of benzene on the meta position (35MEW28) increased activity 3-fold compared to erastin while substitution with an aldehyde functional group in the same position (AE) did not affect activity when compared to erastin (Tables 1 and 2). Active erastin analogs inhibited glutamate release in microglia at nM concentrations (Fig. 2 and Table 2) and exhibited similar rank potencies as in previous studies where the compounds had been used to evaluate their ability to inhibit glutamate release in CCF-STTG-1 cells [44,48]. Similar to previous studies, replacement of the ethoxy group on erastin with an isoproxy group and generation of an achiral analog by deletion of the methyl group at the chiral center did not affect inhibitory activity (13MEW76). Also, elimination of the para-chloro substituent (35MEW13) abolished inhibitory activity (Table 2). This latter result has been used to suggest that the chlorine atom could be making a key bonding interaction with SXC essential for binding [44].

Inhibition of cystine uptake resulted in inhibitor EC_{50} values in the range 0.5–5 μM that were 4 to 30-fold less potent than those for inhibition of glutamate release. The results are consistent with a previous finding showing that even though the two processes are SXC-mediated, cystine uptake could be 10-fold higher than glutamate release [15]. Higher cystine concentrations going inside the cell would require higher concentrations of compound to prevent it. Differences in EC_{50}'s could also be due to multiple transporters. However, there are reports in the literature that indicate increases of SXC expression in microglia upon LPS treatment suggesting that SXC is at least one of the transporters involved [19,43]. Higher EC_{50} values for cystine uptake vs. glutamate release when using erastin analogs could also be the result of time dependent inhibition; inhibitors in the glutamate release experiment are exposed to the cells longer than in the cystine uptake experiment (Materials and Methods) thus giving an opportunity to a time-dependent inhibitor to appear more potent. Rank order potency as a result of structural changes remained the same for erastin, 35MEW28, 14MEW31 and AE. Interestingly, 13MEW76 and 14MEW32 exhibited EC_{50} values for inhibition of cystine uptake of 4.78 ± 1.74 and 1.75 ± 0.39 μM respectively. Lower potency inhibition of cystine uptake vs. glutamate release in all compounds as well as differential responses in inhibitory potency for the two processes (13MEW76 and 14MEW32 vs. erastin, 35MEW28, 14MEW31 and AE) suggest that even though the two processes have been shown to be SXC-mediated [15], they are not necessarily synchronized for 1:1 uptake/release ratio.

Erastin is the most potent SXC inhibitor (Table 1) and it is also known to induce ferroptosis with an EC_{50}=1.2 μM [28,44,49]. A possible explanation for cell death induction by erastin at concentrations well above that needed for SXC inhibition could be that downstream factors in microglia suppress cell death, such as those needed for lethal lipid peroxidation [50]. Results suggest that erastin can effectively inhibit glutamate release and cystine uptake from LPS-activated microglia at concentrations far lower than toxic levels thus preserving microglia, an important innate immunity cell population.

In summary, we report for the first time, on the characterization of a 96-well format primary microglia-based assay to perform moderate to high throughput screening for SXC inhibitors. The assay will help identify novel scaffolds since all current inhibitors are weak or non-selective. Advantages of the assay include the use of primary microglial cells, which resemble more closely the behavior of cells *in vivo* [51] as compared to immortalized cell lines and the use of 96-well format which makes it amenable to screen a large number of compounds. One limitation of this assay is the length of time required for microglial cell processing and isolation from mixed glial cultures, which must be maintained *in vitro* for at least 14 days before microglia isolation.

Funding

This research was supported by NIH Grants (R21NS074062 and P30 MH075673) to Barbara S. Slusher and by NCI (R01CA097061) to Brent R. Stockwell.

References

[1] M.A. Lynch, The multifaceted profile of activated microglia, Mol. Neurobiol. 40 (2009) 139–156.
[2] R.M. Ransohoff, V.H. Perry, Microglial physiology: unique stimuli, specialized responses, Annu. Rev. Immunol. 27 (2009) 119–145.
[3] T. Chandra, W. Maier, H.G. Konig, K. Hirzel, D. Kogel, T. Schuler, A. Chandra, I. Demirhan, B. Laube, Molecular interactions of the type 1 human immunodeficiency virus transregulatory protein Tat with N-methyl-d-aspartate receptor subunits, Neuroscience 134 (2005) 145–153.
[4] Y. Wang, Z.H. Qin, Molecular and cellular mechanisms of excitotoxic neuronal death, Apoptosis: Int. J. Program. Cell Death 15 (2010) 1382–1402.
[5] S. Bannai, A. Takada, H. Kasuga, N. Tateishi, Induction of cystine transport activity in isolated rat hepatocytes by sulfobromophthalein and other electrophilic agents, Hepatology 6 (1986) 1361–1368.
[6] S. Broer, C.A. Wagner, Structure-function relationships of heterodimeric amino acid transporters, Cell Biochem Biophys. 36 (2002) 155–168.
[7] F. Verrey, E.I. Closs, C.A. Wagner, M. Palacin, H. Endou, Y. Kanai, CATs and HATs: the SLC7 family of amino acid transporters, Pflug. Arch. 447 (2004) 532–542.
[8] R. Bridges, V. Lutgen, D. Lobner, D.A. Baker, Thinking outside the cleft to understand synaptic activity: contribution of the cystine-glutamate antiporter (System xc-) to normal and pathological glutamatergic signaling, Pharm. Rev. 64 (2012) 780–802.
[9] R.J. Bridges, N.R. Natale, S.A. Patel, System xc(-) cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS, Br. J. Pharm. 165 (2012) 20–34.
[10] A. Massie, S. Boillee, S. Hewett, L. Knackstedt, J. Lewerenz, Main path and byways: non-vesicular glutamate release by system xc(-) as an important modifier of glutamatergic neurotransmission, J. Neurochem. 135 (2015) 1062–1079.
[11] F.N. Soria, A. Zabala, O. Pampliega, A. Palomino, C. Miguelez, L. Ugedo, H. Sato, C. Matute, M. Domercq, Cystine/glutamate antiporter blockage induces myelin degeneration, Glia 64 (2016) 1381–1395.
[12] J. Lewerenz, S.J. Hewett, Y. Huang, M. Lambros, P.W. Gout, P.W. Kalivas, A. Massie, I. Smolders, A. Methner, M. Pergande, S.B. Smith, V. Ganapathy, P. Maher, The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities, Antioxid. Redox Signal 18 (2013) 522–555.
[13] P. Mesci, S. Zaidi, C.S. Lobsiger, S. Millecamps, C. Escartin, D. Seilhean, H. Sato, M. Mallat, S. Boillee, System xC- is a mediator of microglial function and its deletion slows symptoms in amyotrophic lateral sclerosis mice, Brain 138 (2015) 53–68.
[14] D. Piani, A. Fontana, Involvement of the cystine transport system xc- in the macrophage-induced glutamate-dependent cytotoxicity to neurons, J. Immunol. 152 (1994) 3578–3585.
[15] A.G. Thomas, R. Sattler, K. Tendyke, K.A. Loiacono, H. Hansen, V. Sahni, Y. Hashizume, C. Rojas, B.S. Slusher, High-throughput assay development for cystine-glutamate antiporter (xc-) highlights faster cystine uptake than glutamate release in glioma cells, PLoS One 10 (2015) e0127785.
[16] P. Albrecht, J. Lewerenz, S. Dittmer, R. Noack, P. Maher, A. Methner, Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system xc- as a neuroprotective drug target, CNS Neurol. Disord. Drug Targets 9 (2010) 373–382.
[17] S.W. Barger, A.S. Basile, Activation of microglia by secreted amyloid precursor protein evokes release of glutamate by cystine exchange and attenuates synaptic function, J. Neurochem 76 (2001) 846–854.
[18] M. Domercq, M.V. Sanchez-Gomez, C. Sherwin, E. Etxebarria, R. Fern, C. Matute, System xc- and glutamate transporter inhibition mediates microglial toxicity to oligodendrocytes, J. Immunol. 178 (2007) 6549–6556.
[19] O. Pampliega, M. Domercq, F.N. Soria, P. Villoslada, A. Rodriguez-Antiguedad, C. Matute, Increased expression of cystine/glutamate antiporter in multiple sclerosis, J. Neuroinflamm. 8 (2011) 63.
[20] M.C. Potter, M. Figuera-Losada, C. Rojas, B.S. Slusher, Targeting the glutamatergic system for the treatment of HIV-associated neurocognitive disorders, J. Neuroimmune Pharm. 8 (2013) 594–607.
[21] A. Massie, A. Schallier, S.W. Kim, R. Fernando, S. Kobayashi, H. Beck, D. De

Bundel, K. Vermoesen, S. Bannai, I. Smolders, M. Conrad, N. Plesnila, H. Sato, Y. Michotte, Dopaminergic neurons of system x(c)(-)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity, FASEB J.: Off. Publ. Fed. Am. Soc. Exp. Biol. 25 (2011) 1359–1369.
[22] A. Massie, A. Schallier, B. Mertens, K. Vermoesen, S. Bannai, H. Sato, I. Smolders, Y. Michotte, Time-dependent changes in striatal xCT protein expression in hemi-Parkinson rats, Neuroreport 19 (2008) 1589–1592.
[23] B. Fogal, S.J. Hewett, Interleukin-1beta: a bridge between inflammation and excitotoxicity?, J. Neurochem. 106 (2008) 1–23.
[24] S.C. Lu, Regulation of glutathione synthesis, Mol. Asp. Med. 30 (2009) 42–59.
[25] P.W. Gout, A.R. Buckley, C.R. Simms, N. Bruchovsky, Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the x(c)- cystine transporter: a new action for an old drug, Leukemia 15 (2001) 1633–1640.
[26] Z.C. Ye, J.D. Rothstein, H. Sontheimer, Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange, J. Neurosci.: Off. J. Soc. Neurosci. 19 (1999) 10767–10777.
[27] S.A. Patel, B.A. Warren, J.F. Rhoderick, R.J. Bridges, Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine, Neuropharmacology 46 (2004) 273–284.
[28] S.J. Dixon, K.M. Lemberg, M.R. Lamprecht, R. Skouta, E.M. Zaitsev, C.E. Gleason, D.N. Patel, A.J. Bauer, A.M. Cantley, W.S. Yang, B. Morrison 3rd, B.R. Stockwell, Ferroptosis: an iron-dependent form of nonapoptotic cell death, Cell 149 (2012) 1060–1072.
[29] M. Lo, Y.Z. Wang, P.W. Gout, The x(c)- cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases, J. Cell. Physiol. 215 (2008) 593–602.
[30] M. Conrad, H. Sato, The oxidative stress-inducible cystine/glutamate antiporter, system x (c) (-): cystine supplier and beyond, Amino Acids 42 (2012) 231–246.
[31] H. Sato, A. Shiiya, M. Kimata, K. Maebara, M. Tamba, Y. Sakakura, N. Makino, F. Sugiyama, K. Yagami, T. Moriguchi, S. Takahashi, S. Bannai, Redox imbalance in cystine/glutamate transporter-deficient mice, J. Biol. Chem. 280 (2005) 37423–37429.
[32] N.E. Savaskan, A. Heckel, E. Hahnen, T. Engelhorn, A. Doerfler, O. Ganslandt, C. Nimsky, M. Buchfelder, I.Y. Eyupoglu, Small interfering RNA-mediated xCT silencing in gliomas inhibits neurodegeneration and alleviates brain edema, Nat. Med. 14 (2008) 629–632.
[33] L. Sleire, B.S. Skeie, I.A. Netland, H.E. Forde, E. Dodoo, F. Selheim, L. Leiss, J.I. Heggdal, P.H. Pedersen, J. Wang, P.O. Enger, Drug repurposing: sulfasalazine sensitizes gliomas to gamma knife radiosurgery by blocking cystine uptake through system Xc-, leading to glutathione depletion, Oncogene 34 (2015) 5951–5959.
[34] K.S. Evonuk, B.J. Baker, R.E. Doyle, C.E. Moseley, C.M. Sestero, B.P. Johnston, P. De Sarno, A. Tang, I. Gembitsky, S.J. Hewett, C.T. Weaver, C. Raman, T.M. DeSilva, Inhibition of system Xc(-) transporter attenuates autoimmune inflammatory demyelination, J. Immunol. 195 (2015) 450–463.
[35] M. Figuera-Losada, C. Rojas, B.S. Slusher, Inhibition of microglia activation as a phenotypic assay in early drug discovery, J. Biomol. Screen 19 (2014) 17–31.
[36] S. Qin, C. Colin, I. Hinners, A. Gervais, C. Cheret, M. Mallat, System Xc- and apolipoprotein E expressed by microglia have opposite effects on the neurotoxicity of amyloid-beta peptide 1-40, J. Neurosci. 26 (2006) 3345–3356.
[37] D. Giulian, T.J. Baker, Characterization of ameboid microglia isolated from developing mammalian brain, J. Neurosci.: Off. J. Soc. Neurosci. 6 (1986) 2163–2178.
[38] A.G. Thomas, C.M. O'Driscoll, J. Bressler, W. Kaufmann, C.J. Rojas, B.S. Slusher, Small molecule glutaminase inhibitors block glutamate release from stimulated microglia, Biochem. Biophys. Res. Commun. 443 (2014) 32–36.
[39] K. Shukla, A.G. Thomas, D.V. Ferraris, N. Hin, R. Sattler, J. Alt, C. Rojas, B.S. Slusher, T. Tsukamoto, Inhibition of xc(-) transporter-mediated cystine uptake by sulfasalazine analogs, Bioorg. Med. Chem. Lett. 21 (2011) 6184–6187.
[40] S.M. Robert, S.C. Buckingham, S.L. Campbell, S. Robel, K.T. Holt, T. Ogunrinu-Babarinde, P.P. Warren, D.M. White, M.A. Reid, J.M. Eschbacher, M.E. Berens, A.C. Lahti, L.B. Nabors, H. Sontheimer, SLC7A11 expression is associated with seizures and predicts poor survival in patients with malignant glioma, Sci. Transl. Med. 7 (2015) 289ra286.
[41] M. Patrizio, G. Levi, Glutamate production by cultured microglia: differences between rat and mouse, enhancement by lipopolysaccharide and lack effect of HIV coat protein gp120 and depolarizing agents, Neurosci. Lett. 178 (1994) 184–189.
[42] H. Sato, K. Fujiwara, J. Sagara, S. Bannai, Induction of cystine transport activity in mouse peritoneal macrophages by bacterial lipopolysaccharide, Biochem. J. 310 (1995) 547–551.
[43] K. Taguchi, M. Tamba, S. Bannai, H. Sato, Induction of cystine/glutamate transporter in bacterial lipopolysaccharide induced endotoxemia in mice, J. Inflamm. 4 (2007) 20.
[44] S.J. Dixon, D.N. Patel, M. Welsch, R. Skouta, E.D. Lee, M. Hayano, A.G. Thomas, C.E. Gleason, N.P. Tatonetti, B.S. Slusher, B.R. Stockwell, Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis, Elife 3 (2014) e02523.
[45] H.N. David, J.H. Abraini, The group I metabotropic glutamate receptor antagonist S-4-CPG modulates the locomotor response produced by the activation of D1-like, but not D2-like, dopamine receptors in the rat nucleus accumbens, Eur. J. Neurosci. 13 (2001) 2157–2164.
[46] Y. Kang, S. Tiziani, G. Park, M. Kaul, G. Paternostro, Cellular protection using Flt3 and PI3Kalpha inhibitors demonstrates multiple mechanisms of oxidative glutamate toxicity, Nat. Commun. 5 (2014) 3672.
[47] C. Wahl, S. Liptay, G. Adler, R.M. Schmid, Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B, J. Clin. Investig. 101 (1998) 1163–1174.
[48] M.H. Larraufie, W.S. Yang, E. Jiang, A.G. Thomas, B.S. Slusher, B.R. Stockwell, Incorporation of metabolically stable ketones into a small molecule probe to increase potency and water solubility, Bioorg. Med. Chem. Lett. 25 (2015) 4787–4792.
[49] N. Yagoda, M. von Rechenberg, E. Zaganjor, A.J. Bauer, W.S. Yang, D.J. Fridman, A.J. Wolpaw, I. Smukste, J.M. Peltier, J.J. Boniface, R. Smith, S.L. Lessnick, S. Sahasrabudhe, B.R. Stockwell, RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels, Nature 447 (2007) 864–868.
[50] W.S. Yang, B.R. Stockwell, Ferroptosis: death by lipid peroxidation, Trends Cell Biol. 26 (2016) 165–176.
[51] B. Stansley, J. Post, K. Hensley, A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease, J. Neuroinflamm. 9 (2012) 115.