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Small molecule glutaminase inhibitors block glutamate release from stimulated microglia

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ABSTRACT

Glutaminase plays a critical role in the generation of glutamate, a key excitatory neurotransmitter in the CNS. Excess glutamate release from activated macrophages and microglia correlates with upregulated glutaminase suggesting a pathogenic role for glutaminase. Both glutaminase siRNA and small molecule inhibitors have been shown to decrease excess glutamate and provide neuroprotection in multiple models of disease, including HIV-associated dementia (HAD), multiple sclerosis and ischemia. Consequently, inhibition of glutaminase could be of interest for treatment of these diseases. Bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) and 6-diazo-5-oxo-L-norleucine (DON), two most commonly used glutaminase inhibitors, are either poorly soluble or non-specific. Recently, several new BPTES analogs with improved physicochemical properties were reported. To evaluate these new inhibitors, we established a cell-based microglial activation assay measuring glutamate release. Microglia-mediated glutamate levels were significantly augmented by tumor necrosis factor (TNF)-α, phorbol 12-myristate 13-acetate (PMA) and Toll-like receptor (TLR) ligands coincident with increased glutaminase activity. While several potent glutaminase inhibitors abrogated the increase in glutamate, a structurally related analog devoid of glutaminase activity was unable to block the increase. In the absence of glutamine, glutamate levels were significantly attenuated. These data suggest that the *in vitro* microglia assay may be a useful tool in developing glutaminase inhibitors of therapeutic interest.

1. Introduction

Microglia play a dual role in the neuroprotection and neurotoxicity associated with various neurodegenerative diseases in the central nervous system (CNS) [1–3]. As a neuroprotectant, microglia serve as resident sentinels that provide the necessary innate immune response against injury, infection and other adverse stimuli. As a source of neurotoxicity, uncontrolled and excessively activated microglia contribute to neuroinflammation, a hallmark of several neurodegenerative diseases [1]. In response to stimuli, activated microglia produce pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, IL-18, IP-10, PGE_2, TNF-α), reactive oxygen species (NO, O_2^-, H_2O_2, OH^-, NOO^-) and excess glutamate that have been shown to injure CNS cells, both *in vitro* and *in vivo* [4]. Much attention has been paid to therapeutic strategies aimed at eliminating neurotoxic microglial activation, including the use of enzyme inhibitors, receptor antagonists, natural products and neutralizing antibodies to cytokines [5–11]. Here, we suggest modulation of excitotoxic glutamate via the inhibition of microglial glutaminase as an alternative therapeutic strategy.

Glutaminase is an enzyme that catalyzes the hydrolysis of glutamine to glutamate and is thought to play a central role in the generation of excitotoxic glutamate in neuroinflammatory CNS disorders [12–14]. Recent studies have shown that the excess extracellular glutamate is released from CNS-resident activated microglia through gap junctions, after its conversion from glutamine via glutaminase [12,14,15]. In fact, in work using

Abbreviations: BPTES, bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl)ethyl sulfide; CNS, central nervous system; DON, 6-diazo-5-oxo-L-norleucine; GC, CpG oligodeoxynucleotide; GLS, glutaminase; HAD, HIV-associated dementia; HIV, human immunodeficiency virus; HRP, horse radish peroxidase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; Pam3SK4, tripalmitoyl-S-glyceryl-cysteine; PMA, phorbol 12-myristate 13-acetate; poly I:C, polyinosinic–polycytidylic; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.

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HIV-infected human macrophages, prototype glutaminase small molecule inhibitors and glutaminase specific siRNA were able to abrogate the glutamine-dependent increases in glutamate [12]. Glutaminase-mediated glutamate release from microglia was also shown to occur in a model of multiple sclerosis [13]. Thus glutaminase inhibition could be of broad therapeutic interest for neuroinflammatory disorders.

However, to date, there are no known potent and selective glutaminase inhibitors available. The two prototype inhibitors often employed, 6-Diazo-5-oxo-L-norleucine (DON) and bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), are non-specific and insoluble, respectively [16,17]. Recently, analogs of BPTES were made in an effort to improve on its drug-like properties, including size and solubility while retaining potency [17]. To evaluate these new glutaminase inhibitors, we established a microglial-based assay quantifying glutamate release in response to diverse agents including tumor necrosis factor (TNF)-α, pattern recognition Toll-like receptor (TLR) agonists and phorbol 12-myristate 13-acetate (PMA). We report that glutamate released from microglia is blocked by glutaminase inhibitors, is dependent on glutamine levels and is correlated with glutaminase activity.

2. Materials and methods

2.1. Materials

Tumor necrosis factor (TNF)-α, tripalmitoyl-S-glyceryl-cysteine (Pam3SK4 – TLR 1/2 agonist), polyinosinic-polycytidylic (poly I:C – TLR 3 agonist), lipopolysaccharide (LPS – TLR 4 agonist), CpG oligodeoxynucleotide (GC – TLR 9 agonist) and phorbol 12-myristate 13-acetate (PMA) were all obtained from Invivogen (San Diego, CA). Amplex UltraRed, Dulbecco's Minimum Essential Media (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY), Horse Radish Peroxidase (HRP) from Worthington Biochemical Corporation (Lakewood, NJ), TRIS from Sigma (St. Louis, MO), Complete Protease Inhibitor Cocktail from Roche (Indianapolis, IN), 96-Well spin columns from Harvard Apparatus (Holliston, MA) and the strong anion ion-exchange resin from BioRad (Hercules, CA). Glutamate oxidase was acquired from either US Biological Life Sciences (Swampscott, MA) or from Sigma (St. Louis, MO). L-[2,3,4-^{3}H]-Glutamine and 96-well LumaPlates were purchased from American Radiolabeled Chemicals (Saint Louis, MO) and Perkin Elmer (Waltham, MA), respectively. Finally, BPTES and its analogs were synthesized in-house [17].

2.2. Microglia assay

Single suspension cells were prepared from whole brains of 1–2 d old mice, as described previously [18]. Cells were cultured in flasks in high glucose DMEM with 15% FBS. After 7–10 days, microglia were dislodged from adherent cells by shaking the flasks for 1 h at 200 rpm. Cells were re-plated at 100,000 cells per well in a 48-well plate and the effects of stimulants and glutaminase inhibitors evaluated in an acute paradigm. 1–2 days after plating, microglia were stimulated with either TNF-α (100 ng/ml), TLR ligands (Pam3SK4, 1 μg/ml; poly I:C, 10 μg/ml; LPS, 1 μg/ml and GC, 5 μM) or PMA (100 ng/ml). Glutaminase inhibitors (10 μM) were added 10 min prior to the addition of the stimulant. Supernatants were collected 16–18 h after stimulation and assayed for glutamate.

2.3. Glutamate analysis

Glutamate levels were determined using a two-enzyme Amplex UltraRed assay system [19]. The assay was carried out at room temperature in TRIS buffer (pH 7.4), in black, medium bind, Greiner 96-well plates with microclear bottom using glutamate oxidase (0.04 U/ml) and HRP (0.125 U/ml) coupled with fluorogenic Amplex UltraRed (50 μM). The rate of change of fluorescence intensity (at ex 530, em 590) was measured over a 20 min period and correlated to the glutamate levels in the supernatants. Enzyme stocks were constituted in 100 mM TRIS–HCl buffer (pH 7.4) containing 20% glycerol and stored at −80 °C until the day of the experiment. Amplex UltraRed stock was made up in 100% anhydrous DMSO.

2.4. Glutaminase activity analysis

Glutaminase activity in microglial cells was determined using radiolabeled glutamine (0.076 μM, specific activity 60 Ci/mmol) as substrate [20]. The cells were suspended in ice-cold phosphate buffer (KH_2PO_4, 45 mM, pH 8.2) containing protease inhibitors (Roche's Complete Inhibitor Cocktail, 1 tablet in 50 ml) and the cells disrupted using Kontes' MicroUltrasonic Cell Disrupter (output control set at 60; 3 pulses each of 10 s duration and on ice). The cell lysate was added to the substrate and the reaction conducted at room temperature (90 min incubation). The assay was terminated upon the addition of 20 mM imidazole buffer (pH 7). 96-Well microplate spin columns packed with strong anion ion-exchange resin were used to separate the unhydrolyzed substrate and the reaction product. Unreacted [^{3}H]-glutamine was removed by washing with imidazole buffer. [^{3}H]-Glutamate, the reaction product, was then eluted with 0.1 M HCl and analyzed for radioactivity.

3. Results

3.1. Glutamate release in microglia is induced by TNF-α, TLR agonists and PMA

Glutamate levels in unstimulated (control) microglia in the presence and absence of glutamine were 37 ± 5 μM (Fig. 1A) and 32 ± 3 μM (Fig. 1B), respectively. Following stimulation in the presence of glutamine, glutamate levels were significantly increased by TNF-α (115 ± 4 μM), Pam3SK4 (170 ± 12 μM), poly I:C (91 ± 10 μM), LPS (102 ± 16 μM), GC (171 ± 4 μM) and PMA (164 ± 2 μM) (Fig. 1A). Following stimulation in the absence of glutamine, glutamate levels increased to a much lesser degree with TNF-α (47 ± 2 μM), Pam3SK4 (81 ± 2 μM), poly I:C (37 ± 5 μM), LPS (63 ± 3 μM), GC (80 ± 4 μM) and PMA (95 ± 1 μM) (Fig. 1B).

3.2. Glutamate release in activated microglia is inhibited by glutaminase inhibitors

BPTES and its analogs (Table 1) were evaluated for their ability to block TLR-mediated glutamate release. While the active glutaminase inhibitors (BPTES, JHU-198 and JHU-212) abrogated the glutamate release induced by the various TLR agonists, a structurally related analog devoid of glutaminase activity (JHU-329) was unable to block the release (Fig. 2).

3.3. Glutamate release in activated microglia correlates with glutaminase activity

To evaluate if the increased glutamate levels correlated with increased active glutaminase, microglial cells were activated with the potent TLR agonist, Pam3SK4, in the presence and absence of a soluble glutaminase inhibitor, JHU-212. Subsequent to the activation, glutaminase activity was monitored using radiolabeled glutamine. Relative to unactivated microglial cells, glutaminase activity in Pam3SK4-activated microglial cells was increased by