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Glutamate production by HIV-1 infected human macrophage is blocked by the inhibition of glutaminase

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Abstract

Mononuclear phagocyte (macrophages and microglia) dysfunction plays a significant role in the pathogenesis of human immunodeficiency virus (HIV) associated dementia (HAD) through the production and release of soluble neurotoxic factors including glutamate. The mechanism of glutamate regulation by HIV-1 infection remains unclear. In this report, we investigated whether the enzyme glutaminase is responsible for glutamate generation by HIV-1 infected monocyte-derived macrophages. We tested the functionality of novel small molecule inhibitors designed to specifically block the activity of glutaminase. Glutaminase inhibitors were first characterized in a kinetic assay with crude glutaminase from rat brain revealing an uncompetitive mechanism of inhibition. The inhibitors were then tested *in vitro* for their ability to prevent glutamate generation by HIV-infected macrophages, their effect upon macrophage viability, and HIV infection. To validate these findings, glutaminase specific siRNA was tested for its ability to prevent glutamate increase during infection. Our results show that both glutaminase specific small molecule inhibitors and glutaminase specific siRNA were effective at preventing increases in glutamate by HIV-1 infected macrophage. These findings support glutaminase as a potential component of the HAD pathogenic process and identify a possible therapeutic avenue for the treatment of neuroinflammatory states such as HAD.

Keywords

glutamate; glutaminase; HIV-1-associated dementia; macrophages

HIV-1 associated dementia (HAD) is a significant consequence of HIV infection resulting in a chronic, progressive dementia. The dementia is a consequence of neuronal damage that results from prolonged inflammation in the CNS. Mononuclear phagocytes (MP) are critical to HAD pathogenesis and have been hypothesized to induce neuronal injury through the production and release of various soluble neurotoxic factors including glutamate (Giulian *et al*.

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1993;Pulliam *et al.* 1994;Zink *et al.* 1999;Belmadani *et al.* 2001;Jiang *et al.* 2001). Glutamate mediates numerous physiological functions through activation of multiple receptors (Cutler and Dudzinski 1974;Fonnum 1984;Orrego and Villanueva 1993); however, high concentrations of extracellular glutamate induce neuronal damage (Olney 1971;McCall *et al*. 1979;Choi 1988;Newcomb *et al*. 1997). HIV-1 infected macrophages are an important cellular source of extracellular glutamate (Jiang *et al*. 2001), and HIV-1-infected patients have significantly higher concentrations of glutamate in plasma as compared with uninfected controls (Droge *et al*. 1987;Ollenschlager *et al*. 1988). The cerebrospinal fluid of HAD patients has also been found to have increased amounts of glutamate (Ferrarese *et al*. 2001). The production of excess glutamate by MP in HAD may exacerbate the excitotoxic environment of HAD leading to neuronal damage and death, but the mechanism of glutamate regulation by HIV-1 infection remains unclear.

Phosphate-activated glutaminase (PAG, EC 3.5.1.2) is the primary enzyme for the production of glutamate (Ward *et al*. 1983;Nicklas *et al*. 1987;Wurdig and Kugler 1991;Curthoys and Watford 1995) and is also the predominant glutamine-utilizing enzyme of the brain (Kvamme *et al*. 1982;Holcomb *et al*. 2000). Although compartmentalization of glutamate is critical to proper neurotransmission, glutamine is widely abundant in the extracellular space, the highest concentration of any amino acid in brain extracellular fluid (Tossman *et al.* 1986). Glutamine is freely passed between cells, and does not pose the excitotoxic threat of excitatory amino acids, but provides an abundant substrate for PAG *in vivo* (Newcomb *et al*. 1997). Glutaminase is generally localized in the inner membrane of the mitochondria and catalyzes the deamination of glutamine to glutamate, a hydrolysis resulting in stoichiometric amounts of glutamate and ammonia (Shapiro *et al*. 1985,1991;Laake *et al*. 1999). Previously, we identified glutamine dependent generation of glutamate by HIV-1 infected human monocyte-derived macrophage (MDM), an effect that was blocked with antiviral treatment and by 6-diazo-5-oxo-l-norleucine, a glutaminase inhibitor applied at millimolar concentrations (Zhao *et al*. 2004). Increase in amount, activity or release of glutaminase mediated by the infective process of HIV-1 may facilitate uncontrolled generation of glutamate in the CNS. In this report, we propose that glutaminase is responsible for the generation of glutamate by infected macrophage. We tested novel water-soluble, small-molecule inhibitors designed to specifically block the activity of glutaminase. Glutaminase specific siRNA was tested for its ability to prevent glutamate increase during infection. The results presented below support the hypothesis that glutaminase mediates glutamate generation in HIV-infected human macrophages, and demonstrate the potential of new glutaminase inhibitors to efficiently block this increase in glutamate.

Materials and Methods

Glutaminase activity assay

Assays were performed using rat-brain mitochondria solubilized with 1% Triton X-100 as a source of native rat glutaminase (Shapiro *et al*. 1985). Protein concentrations were determined by the Lowry method using bovine serum albumin as standard (Lowry *et al*. 1951). Glutaminase activity was measured using a two-step assay (Curthoys *et al*. 1974). Glutamine is first hydrolyzed to glutamate and then glutamate dehydrogenase is used to catalyze oxidative deamination of glutamate to form α-ketoglutarate and NADH. Generated α-ketoglutarate is removed by its reaction with hydrazine to form a hydrazide. Fifty milligrams of diluted glutaminase was added to 100 μL of initial assay mix (10 mmol/L glutamine, 0.15 mol/L phosphate, 0.2 mmol/L EDTA, and 50 mmol/L Tris-acetate, pH 8.6) and incubated at 37°C for 20 min. The reaction was stopped by the addition of 10 μL of 3 N HCl to inactivate the glutaminase. Then, 1 mL of the second reaction mixture containing 0.4 mg of purified bovine liver glutamate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA), 0.08 mol/L Tris-acetate, pH 9.4, 0.2 mol/L hydrazine, 0.25 mmol/L ADP, and 2 mmol/L NAD was added. The samples

were mixed and incubated for 30 min at 25°C. Measuring absorbance at 340 nm, glutamate concentration was determined using a standard curve of 10, 5, 2.5, 1.25, 0.625, and 0.0 mmol/L glutamate run simultaneously with unknowns.

Inhibitors 14256, 19560, 20767, 20638, and 5000 were added in increasing concentrations (3-300 μmol/L) to the initial assay mix. IC_{50} values were estimated using non-linear regression analysis through GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A substrate saturation curve was generated using increasing concentrations of glutamine (0-40 mmol/L) in the presence of the representative inhibitors 14256 or 19560 at concentrations of 0, 30 and 100 μmol/L. Constants K_M and V_{MAX} were derived from Michaelis-Menten plots using the non-linear regression GraphPad Prism software. The K_I for a representative inhibitors, 14256 and 19560 was determined with non-linear regression by competitive inhibitor analysis: $K_M^{app} = K_M(1 + I / K_I)$ and $Y = V_{MAX}X / (K_M^{app} + X)$ uncompetitive inhibitor analysis $V_{MAX}^{App} = V_{MAX} / (1 + I / K_I)$, $K_M^{app} = K_M / (1 + I / K_I)$, and $Y = V_{MAX}^{App}X / (K_M^{app} + X)$ where V_{MAX}^{App} and K_M^{app} are the apparent maximal velocity and Michaelis constants at each inhibitor concentration, I is the inhibitor concentration and K_I is the inhibition constant.

Isolation and culture of MDM

Human monocytes were recovered from peripheral blood mononuclear cells of HIV-1, -2 and hepatitis B seronegative donors after leukophoresis, and then purified by counter current centrifugal elutriation. Monocytes were cultured as adherent monolayers (1.1×10^6 cells/well in 24-well plates) for 7 days in Dulbecco's Modified Eagle's Medium (GIBCO, Invitrogen Corp, Carlsbad, CA, USA) supplemented with 10% heat-inactivated pooled human serum, 50 g/mL gentamicin (Sigma), 10 g/mL ciprofloxacin (Sigma) and macrophage colony stimulating factor (M-CSF) (1000 U/mL highly purified recombinant human M-CSF; a generous gift from Wyeth Institute, Inc., Cambridge, MA, USA). After 7 days of culture in the presence of M-CSF monocytes were considered MDM. All tissue reagents were screened and found negative for endotoxin (<10 pg/mL; Associates of Cape Cod, Inc., Woods Hole, MA, USA) and mycoplasma contamination (Gen-probe II; Gen-probe Inc., San Diego, CA, USA).

Infection and treatment of MDM

Seven days after plating, MDM were infected with HIV-1_{ADA}, HIV-$1_{JR\text{-}FL}$, HIV-1_{BAL} or HIV-$1_{89.6}$, at a multiplicity of infection of 0.1 virus/target cell. Viral stocks were screened for mycoplasma and endotoxin using hybridization and limulus amebocyte lysate assays, respectively. Culture medium was half-exchanged every 2 days.

Inhibitor studies with human MDM

Inhibitor studies were carried out 1, 3, 5, 7, or 8 days after infection, HIV-1-infected and uninfected MDM were washed two times with Dulbecco's modified eagle's medium and incubated for a 24-h period in 0.5 mL 0.01% bovine serum albumin serum-free neurobasal media with 5 mmol/L glutamine and 20767 (10 μmol/L), 14256 (10 μmol/L), 5000 (10 μmol/L), 20638 (10 μmol/L) or 19560 (10 μmol/L). The concentration of glutamate in cell-supernatants was determined by reverse-phase high performance liquid chromatography (RP-HPLC). Reverse-transcriptase (RT) activity and p24 levels were determined in triplicate samples of the culture fluids as described below. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay as described below.

Cell viability assay

Cell cytotoxicity was assessed by MTT (Sigma) mitochondrial dehydrogenase assay. Cells were incubated with a 1 : 10 dilution of MTT solution to cell media for 20 min at 37°C. The extent of MTT conversion to formazan by mitochondrial dehydrogenase was determined by measuring optical density (OD) at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The ratio of OD from treated cells to OD from control cells reflected the percentage of surviving cells and was used to standardize glutamate production as determined by RP-HPLC.

Measurement of RT activity

Reverse-transcriptase activity was determined in triplicate samples of cell culture fluids. For this assay 10 L of supernatant was added to a reaction mixture of 0.05% Nonidet P-40, 10 g/mL poly (A), 0.25 g/mL oligo (dT), 5 mmol/L dithiothreitol, 150 mmol/L KCl, 15 mmol/L $MgCl_2$, and [H^3]-2′-deoxythymidine 5′-triphosphate (NEN, Boston, MA, USA) in Tris-HCl buffer (pH 7.9) for 24 h at 37°C. Radiolabeled nucleotides were precipitated with cold 10% trichloroacetic acid and 95% ethanol in an automatic Filtermate™ Cell Harvester (Packard, Stamford, CT, USA) on 96-well unifilter GF/C plates (Packard). MicroScint-20 liquid scintillation cocktail (Packard) was added and radioactivity determined in a TopCount NXT liquid scintillation counter (Packard).

Analyses of glutamate and glutamine by RP-HPLC

HPLC analysis was performed using an HP Series II 1090 liquid chromatograph and HP1046A fluorescence detector (Hewlett Packard; Packard) as previously described (Zhao *et al*. 2004).

siRNA knockdown of Glutaminase

siRNA knockdown in MDM was performed as previously described (Peng *et al*. 2006). Briefly, smart-pool pre-designed siRNA duplexes targeted against glutaminase mRNA were synthesized by Dharmacon (Lafayette, CO, USA). Human monocytes were plated at a density of 1.1×10^6 cells/well in 24-well plates (BD Biosciences, San Diego, CA, USA). Seven days after plating, MDM were infected with HIV-1_{ADA} at a multiplicity of infection of 0.1 virus/target cell. Two days post-infection cells were transfected with 100 nmol/L siRNA duplex for 24 h in the presence of siIMPORTER (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) according to the manufacturer's instructions. A non-specific control siRNA (Dharmacon) was also transfected at the same concentration as control. To evaluate transfection efficiency, control and HIV-1-infected MDM were transfected with control siRNA siGLO (red fluorescence tagged siRNA) (Dharmacon). At 24 h post-transfection, cells were incubated with Hoechst 33342 (1 : 5000, Sigma) for nuclear staining, transfected and total cells were counted.

Western blot analysis of Glutaminase

Proteins from lysates were separated on a 10% Tris-HCl gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoretic transfer to polyvinyldifluoridene membranes (Millipore, Bedford, MA, USA and Bio-Rad, Hercules, CA, USA), proteins were treated with purified primary antibody to phosphate-activated mitochondrial glutaminase overnight at 4°C followed by a horseradish peroxidase-linked secondary anti-rabbit antibody (1 : 5000 dilution; Cell Signaling Technologies, Danvers, MA, USA). Antigen-antibody complexes were visualized by enhanced chemiluminescence western blotting on Hyperfilm ECL (GE Healthcare/Amersham Bioscience, Piscataway, NJ, USA). For data quantification the films were scanned with a CanonScan 9950F scanner (Canon USA, Lake Success, NY, USA); the acquired images were then analyzed on a Macintosh computer (Apple Inc.,

Sunnyvale, CA, USA) using the public domain NIH image program (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Data were analyzed as means ± SD. The data were evaluated statistically by the analysis of variance, followed by the student's *t*-test for paired observations. Significance was determined as $p < 0.05$ and $p < 0.01$.

Results

HIV-1 infection leads to glutamine dependent glutamate production in human monocyte derived macrophages

To evaluate the production of glutamate following HIV-1 infection of MDM, elutriated human monocytes were differentiated for 7 days into MDM and were then infected with HIV-1_{ADA}. After 7 days of infection, culture media was removed and fresh neurobasal media containing either 5 mmol/L glutamine or no glutamine was added back to culture for overnight incubation. Media supernatants were then collected for glutamate analysis by HPLC for glutamate concentration (Fig. 1). Macrophage-conditioned media (MCM) collected from infected cell cultures contained significantly higher amounts of glutamate as compared with MCM from uninfected cells. In all donors tested, glutamate concentration was significantly higher in infected cultures; however, glutamate increase was almost completely blocked by the removal of glutamine. In the representative donor presented, glutamate concentrations were measured as 175 μmol/L, but MCM lacking glutamine contained only 4 μmol/L glutamate. These findings indicate that glutamine is the primary precursor for the production of glutamate from HIV-1 infected MDM. *in vivo*, glutamate is primarily generated from glutamine by the enzyme PAG. The dependence of glutamate production on the presence of glutamine indicated that glutaminase may be responsible for excess glutamate production.

Glutaminase inhibitors block glutamate generation by rat glutaminase

After identifying glutaminase as a likely source of excess glutamate production, a panel of small-molecule glutaminase inhibitors was characterized to establish an approach for efficiently and specifically blocking glutaminase. To evaluate the performance of the glutaminase inhibitors, we used rat glutaminase in an optimized kinetic assay with or without the addition of inhibitors. The water-soluble, small-molecule inhibitors designed to specifically block glutaminase are named: 14256, 19560 and 20767. Two additional agents were also tested, 20638, a structurally similar but inactive control, and 5000, an inhibitor designed to target NAALA-Dase (N-acetylated alpha-linked acid-dipeptidase), another enzyme known to generate glutamate (Ghadge *et al*. 2003).

First, increasing concentrations of inhibitors (1 nmol/L to 300 μmol/L) were added to standard and optimized enzymatic assay conditions with 10 mmol/L glutamine, 150 mmol/L phosphate and 50 μg rat glutaminase (Fig. 2). Inhibitors 14256, 19560 and 20767 each reduced overall glutamate production in this system. IC_{50} values as determined by GraphPad were 38 μmol/L for 14256, 48 μmol/L for 19560 and 84 μmol/L for 20767, indicating that 14256 and 19560 were the most potent of the related inhibitors tested in the optimized conditions of the enzyme assay. Representative inhibitors 14256 and 19560 were applied to the enzymatic assay with glutamine from 0 to 40 mmol/L at concentrations of 0, 30, and 100 μmol/L to evaluate the type of inhibition (Figs 3a and 4a). The V_{MAX} and K_M values were determined (Table 1). Linear double reciprocal plots of glutamine saturation data from inhibitors 14256 and 19560 indicate changes in both K_M and V_{MAX} supporting uncompetitive inhibition (Figs 2b and 3b). Data were analyzed by competitive and uncompetitive models of inhibition using GraphPad non-linear analysis. Data from glutamine saturation plots of both inhibitors 14256 and 19560 best

fit the uncompetitive inhibition model indicating inhibitors 14256 and 19560 inhibit glutaminase at a site distinct from the active site.

Glutaminase-specific inhibitors block the generation of excess glutamate from HIV-1 infected human macrophages

We next tested whether the panel of glutaminase-specific inhibitors could block glutamate generation at various stages of HIV-1 infection in human MDM. Individual inhibitors were added at a concentration of 10 μmol/L before supernatants were collected and analyzed by HPLC for glutamate. The amount of glutamate generated by infected control macrophage increased as the stage of infection progressed. At 3 days post-infection, glutamate levels from infected macrophages without inhibitor treatment approached 100 μmol/L, while glutamate measured at 7 days post-infection was nearly 150 μmol/L. However, in the presence of inhibitors 14256, 19560 and 20767 glutamate production was significantly reduced in each case (Fig. 5). Negative control 20638 and NAALADase inhibitor 5000 were unable to block glutamate production to the same extent at 10 μmol/L. Thus, inhibitors reduced glutamate to nearly control levels at all stages of infection despite increasing glutamate levels and changes in macrophage culture including cell function and survival. These findings support the role of glutaminase in glutamate generation based upon the effectivity of specific inhibitors to block HIV-1 mediated glutamate production.

We next tested each inhibitor at different concentrations to assess potency. Inhibitors were applied at concentrations of 0.1, 1.0, and 10 μmol/L. Inhibitors 14256, 19560, and 20767 were each able to significantly reduce glutamate at the 10 μmol/L dose (Fig. 6). Inhibitor 19560 was found to significantly reduce glutamate at a dose of 0.1 μmol/L from 92 μmol/L glutamate to 17 μmol/L, with further reductions at 1.0 and 10 μmol/L. Inhibitor 20767 was less potent with significant reduction only at the 10 μmol/L dose, and the effect of 14256 was intermediate. Negative controls 20638 and 5000 had no measurable effect upon glutamate levels at any dose.

Glutamate generation from various HIV strains is blocked with glutaminase inhibitors

After demonstrating the ability of inhibitors 14256, 19560, and 20767 to block HIV-1_{ADA} mediated glutamate production, inhibitors were tested in MDM infected by various HIV-1 viral strains to evaluate whether inhibitor function was influenced by viral variance. Following 7 days culture with M-CSF, human MDM were infected with HIV-1_{ADA}, HIV-1_{BAL}, HIV-$1_{JR\text{-}FL}$ and HIV-$1_{89.6}$ for 8 days. HIV-1_{ADA}, HIV-1_{BAL}, and HIV-$1_{JR\text{-}FL}$ are each macrophage-tropic strains and rely primarily on the chemokine receptor CCR5 as co-receptor, whereas HIV-$1_{89.6}$ is a dual tropic strain and can use both CCR5 and CXCR4 as co-receptors (Zheng *et al.* 1999). The glutamate generation observed in previous experiments is dependent upon the stage of infection; because each viral isolate has a unique rate of progression influenced by variation between human donors, measured glutamate concentrations varied between tested strains. While glutamate concentrations varied, in each tested strain significant levels of glutamate were generated in infected as compared with control MDM supernatants. ADA infection generated the highest glutamate level of 173 μmol/L. Inhibitors were applied to infected cultures of each viral strain and then incubated overnight similar to above the experiments. For each viral strain tested, inhibitors 14256, 19560, and 20767 significantly reduced glutamate production (Fig. 7). These results indicate that the mechanism of each inhibitor is independent of variations in HIV-1 strain.

Inhibitors do not alter cell viability or HIV infectivity

To assess whether cell death caused the observed reduction in glutamate production by MDM treated with glutaminase inhibitors, viability of MDM with or without inhibitor treatment was assessed. Although HIV infection consistently decreased the viability of macrophage in culture as measured by MTT assay, there were no observed changes in the viability of infected or

uninfected macrophages treated with inhibitors (Fig. 8). Cultured MDM were treated as before with each inhibitor at concentrations of 0.1, 1.0, and 10 μmol/L. These data suggest that the inhibitors did not induce significant cell toxicity to macrophage cultures. Furthermore, changes in glutamate regulation did not greatly affect macrophage viability.

Because antiviral treatment with zidovudine has been shown to prevent glutamate generation (Zhao *et al*. 2004), the effect of inhibitors on viral replication was addressed using RT assay. Inhibitors had no effect on ADA infection as determined by RT assay (Fig. 9). Similarly, viral strains BAL, JR-FL, and 89.6 were not affected by inhibitor treatment. Similar results were obtained through measurement of viral protein, p24, released from HIV-1 infected MDM (data not shown).

Glutaminase specific siRNA decreases glutamate production from HIV-infected macrophages

To further validate the contribution of glutaminase to excess glutamate production by infected macrophage, we used siRNA knockdown-targeting glutaminase to test whether glutamate production was reduced. To evaluate the transfection efficiency of primary human macrophage, cells were first transfected with control siRNA siGLO and analyzed by microscopy. The transfection efficiency of siGLO was near 70% as measured 24 h post-transfection (data not shown). HIV-infection induced no significant effect on siRNA transfection compared with non-infected cells. Two days post-HIV infection, macrophage were transfected with either non-specific siRNA or siRNA targeting glutaminase. We used siRNA smart pool from Dharmacon, a combination of three unique siRNAs targeting the glutaminase enzyme. Three days post-siRNA transfection, MCM and protein were collected from control and infected macrophage. Western analysis demonstrated a decrease in glutaminase protein by glutaminase siRNA (Fig. 10). As measured by RP-HPLC, MCM glutamate levels were found to be significantly decreased by glutaminase siRNA in infected macrophage cultures as compared with non-specific siRNA and controls. Although the reduction in glutamate did not quite decrease to control levels, this decrease was significant with a p-value < 0.01.

Discussion

Macrophages have previously been reported to generate glutamate (Jiang *et al*. 2001;Zhao *et al*. 2004). In this report we demonstrate through three distinct approaches the contribution of glutaminase to the generation of glutamate by HIV-1 infected macrophages. *First*, by removing glutamine, the substrate of glutaminase, we were able to block the generation of glutamate (Fig. 1). *Second*, using small molecule inhibitors specific for glutaminase (Figs 5-7). *Third*, using glutaminase specific siRNA, we were able to prevent the generation of glutamate (Fig. 10). These findings provide insight into a possible excitotoxic mechanism of HIV-1 pathogenesis.

HIV-1 infection of human macrophages leads to a drastic and potentially pathogenic increase in glutamate. This increase in glutamate is dependent upon the presence of glutamine (Fig. 1). Glutamine is widely available in the CNS, typically in the millimolar range in cerebrospinal fluid. We hypothesized the observed glutamate increase was mediated by the enzyme glutaminase. A panel of glutaminase inhibitors were found to efficiently block excess glutamate production by HIV-infected macrophages (Fig. 5). Three inhibitors, 14256, 19560, and 20767 each were able to reduce glutamate to near control levels when applied at 10 μmol/L. Negative control inhibitor 20638 and NAALA-Dase inhibitor 5000 had little to no effect on glutamate production. The inhibitors effectively blocked glutamate increases at multiple time points post-infection with various glutamate levels achieved by infected macrophage. In human macrophage culture, inhibitor 19560 was found to be effective at concentrations as low as 0.10 μmol/L, whereas 14256 and 20767 were able to block glutaminase when applied at higher

concentrations (Fig. 6). This inhibitory effect on glutamate production was determined to be independent of viral strain by testing two additional macrophage-tropic viral strains (BAL and JR-FL) and a dual-tropic viral strain (89.6) (Fig. 7). In each viral strain tested, glutamate was produced by infected macrophages, but this glutamate production was blocked by inhibitor treatment. The effect of inhibitor treatment on cell viability and HIV infection was also tested, but revealed no significant increase in cell death (Fig. 8), nor any change in infectivity (Fig. 9). Additionally, siRNA targeting glutaminase was able to drastically reduce glutamate levels in macrophage culture supernatants (Fig. 10). These cumulative studies clearly establish the phenomenon of HIV mediated glutamate production by HIV-1 infected macrophages, and that this production is a consequence of enzymatic conversion of glutamine to glutamate by glutaminase.

The novel glutaminase inhibitors described in this work were found to be effective at micromolar concentrations. Inhibitors 14256 and 19560 were found to be the most potent as determined through an enzyme activity assay (Fig. 2). This inhibition appears to occur through an uncompetitve mechanism (Figs 3 and 4). In macrophage studies, inhibitor 19560 was found to have a higher potency than inhibitor 14256 (Fig. 6). The discrepancy between systems may be explained by the difference in conditions between the enzyme assay with crude brain enzyme and macrophage cell-culture systems where the amount of enzyme, substrate and activating phosphate is distinctly different; slight differences in chemical structure may also lead to variations in cell interaction and permeability, thus altering inhibitor efficiency.

Increase in amount, activity or release of glutaminase could facilitate uncontrolled generation of glutamate in the CNS extracellular space, promoting glutamate induced excitotoxicity. In stroke, significant regions of tissue can experience catastrophic damage and cell death. In this type of model, Newcomb *et al*. (1997) proposed widespread neuron death as a means of glutaminase release, resulting in unregulated glutamate generation, supplied by an abundant substrate of glutamine. Chronic neurodegenerative disorders typically lack rampant cell death, and are better characterized by prolonged inflammation with noticeable lymphocyte and monocyte infiltration and accumulation. In human multiple sclerosis patients, immunohistochemistry identified enhanced glutaminase staining in inflammatory lesions as compared with control specimens; this glutaminase correlated with axonal damage (Werner *et al*. 2001). In HAD, significant numbers of MP migrate into the CNS where they are productively infected as well as activated. Glutamate is secreted in large quantities by macrophage (Piani *et al*. 1991;Jiang *et al*. 2001;Zhao *et al*. 2004). The MP cell population expresses glutaminase at significant levels (Zhao *et al*. 2004). We have demonstrated a glutamine dependent increase of glutamate production by HIV-1 infected macrophage cultures, an effect nearly eliminated in the presence of antiviral treatment (Zhao *et al*. 2004). Our current work with glutaminase-specific inhibitors and siRNA provide further evidence for the dependence on glutaminase in the HIV-1 mediated increase of glutamate by macrophages. HIV-1 may lead to increased enzyme activity or release of enzyme into a glutamine rich environment, allowing excess glutamate generation from macrophage populations.

The data from Fig. 10 indicate an increase in glutamate production following non-specific siRNA treatment. This observation appears similar to findings from our previous work, where staurosporine (STS) induced cell death led to modest increases in glutamate production by macrophage cultures. However, STS induced cell death following HIV-1 infection caused large increases in glutamate production, exceeding that of STS treated or HIV-1 infected cultures. The observed glutamate increase is likely a consequence of siRNA treatment toxicity, indicating cell damage and/or death participates in the mechanism of glutamate generation by glutaminase. This mechanism may be passive release of glutaminase from dead or dying cells, or a regulatory consequence yet to be described. How glutaminase is regulated by HIV-1 infection and to what extent is still unclear and currently under investigation.

Because glutaminase may be contributing to neuronal damage through glutamate, the ability to block its function may provide a therapeutic avenue in a variety of diseases where excitotoxicity is prominent. In HAD, multiple pathways combine to sensitize neuronal populations and generate excitotoxic insults (Erdmann *et al*. 2006). Although N-methyl D-aspartatic acid receptor (NMDA) mediated Ca^{2+} influx is a fundamental component of neuronal excitotoxic damage, preventing NMDA receptor stimulation with agents such as MK-801 cause serious side effects and are not a viable therapeutic approach; however, partial blockade of NMDA receptors with the drug memantine has shown therapeutic benefit with limited complications (Anderson *et al*. 2004;Chen and Lipton 2006). Targeting all glutaminase is unacceptable due to its vital roles in not only generation of glutamate as a neurotransmitter, but also due to its contribution to cellular metabolism as was evidenced by recent knockout studies (Masson *et al*. 2006). Although glutaminase is critical to normal brain function, the phenomenon presented here indicates an HIV-1 mediated dysfunction of glutaminase facilitating uncontrolled glutamate generation. Through determination of the mechanisms involved in this process, the inhibitors tested may potentially be used in a manner effective in blocking inappropriate or excessive glutaminase activity.

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Abbreviations used

HAD, HIV-associated dementia; HIV, human immunodeficiency virus; MCM, macrophage conditioned media; MDM, monocyte-derived macrophages; MP, mononuclear phagocytes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; M-CSF, macrophage colony stimulating factor; NAALADase, N-acetylated-alpha-linked-acid-dipeptidase; OD, optical density; PAG, phosphate-activated glutaminase; RT, reverse-transcriptase; siGLO, red fluorescence tagged siRNA; STS, staurosporine.

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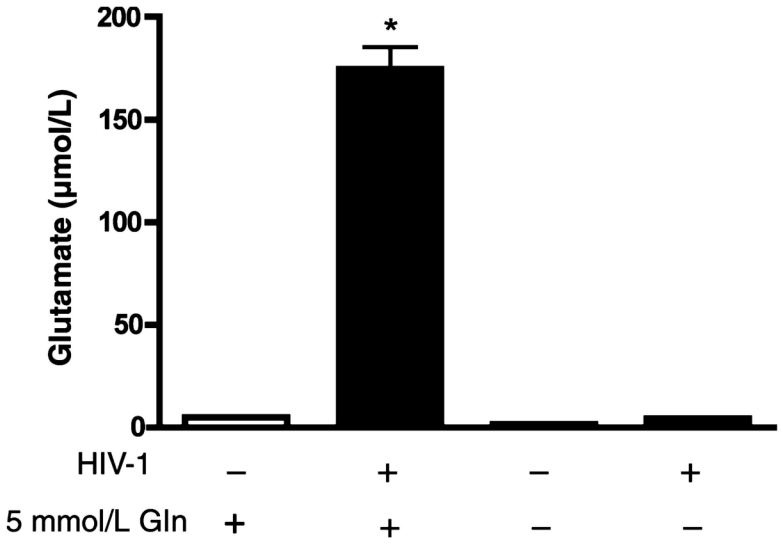

Fig 1.

HIV-1-mediated glutamate production is dependent on the presence of glutamine. Human monocyte-derived macrophages were infected with HIV-1_{ADA} for 7 days and then incubated in serum-free neurobasal media with or without 5 mmol/L glutamine. The concentration of glutamate in cell-free supernatants was determined by RP-HPLC. All data are expressed as absolute concentration of glutamate (µmol/L). Results are expressed as average ± SD of triplicate samples and are representative of three different donors. *Denotes $p < 0.01$ in comparison with control.

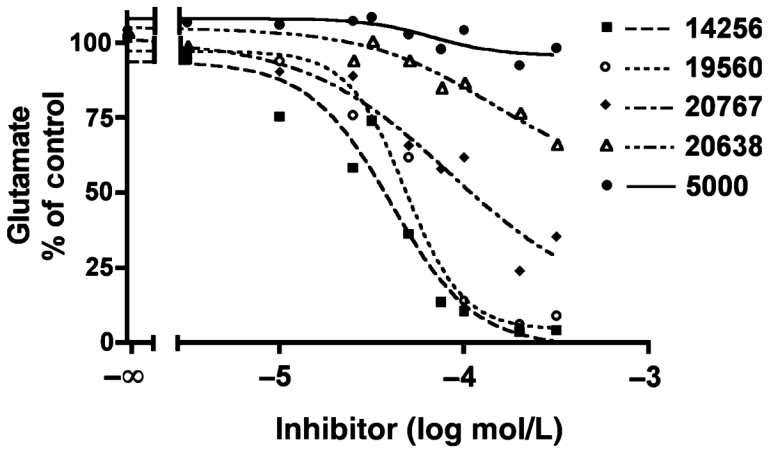

Fig 2.

Profile of glutaminase inhibitors in enzyme activity assay with rat brain glutaminase. The glutaminase inhibitors are designated as 14256, 19560, 20767, and negative controls 20638 and 5000. Inhibitors applied at concentrations of 0, 1, 3, 10, 25, 30, 50, 75, 100, 200, 300 μmol/L to reaction mix containing 10 mmol/L glutamine, 50 μg rat brain glutaminase and 150 mmol/L phosphate. Results are expressed as average ± SD of triplicate samples (% of inhibition as compared with control) and are representative of three independent experiments.

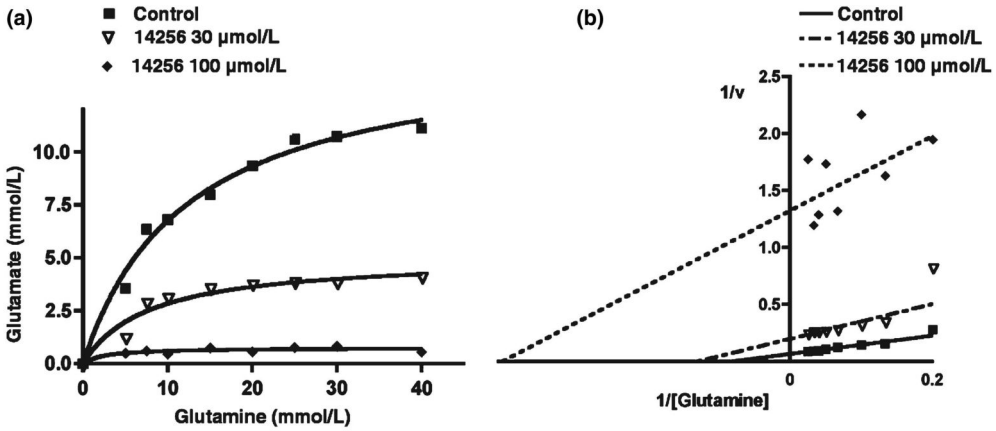

Fig 3.

Glutamine saturation profile of inhibitor 14256. Glutaminase added to assay containing various concentrations of substrate glutamine (0-40 mmol/L) in the presence of 0, 30 and 100 µmol/L inhibitor 14256 with reaction mix containing 50 µg rat brain glutaminase and 150 mmol/L phosphate. In panel (a), enzyme activity is plotted as glutamate versus glutamine concentration. The V_{MAX} as determined by GraphPad (GraphPad Software) was 15.0 ± 0.47, 5.0 ± 0.25, and 0.75 ± 0.05 and the K_M values were determined to be 12.3 ± 0.99, 7.7 ± 1.27, 2.5 ± 1.03 for 14256 treatments of 0, 30, and 100 µmol/L. In panel (b), a Lineweaver-Burk plot of the substrate-velocity curve is shown. Values measured in triplicate, error bars represent SD for each glutamine concentration and are representative of three independent experiments.

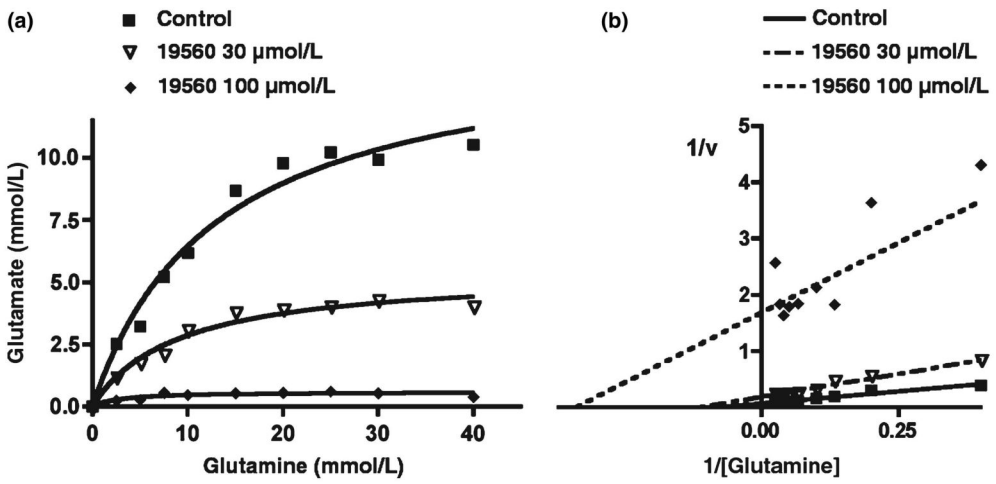

Fig 4.

Glutamine saturation profile of inhibitor 19560. Glutaminase added to assay containing various concentrations of substrate glutamine (0-40 mmol/L) in the presence of 0, 30, and 100 µmol/L inhibitor 14256 with reaction mix containing 50 µg rat brain glutaminase and 150 mmol/L phosphate. In panel (a), enzyme activity is plotted as glutamate versus glutamine concentration. The V_{MAX} as determined by GraphPad was 14.8 ± 0.62, 5.4 ± 0.20, and 0.60 ± 0.04 and the K_M values were determined to be 13.0 ± 1.3, 8.8 ± 0.97, 3.0 ± 0.89 for 19560 treatments of 0, 30, and 100 µmol/L. In panel (b), a Lineweaver-Burk plot of the substrate-velocity curve is shown. Values measured in triplicate, error bars represent SD for each glutamine concentration and are representative of three independent experiments.

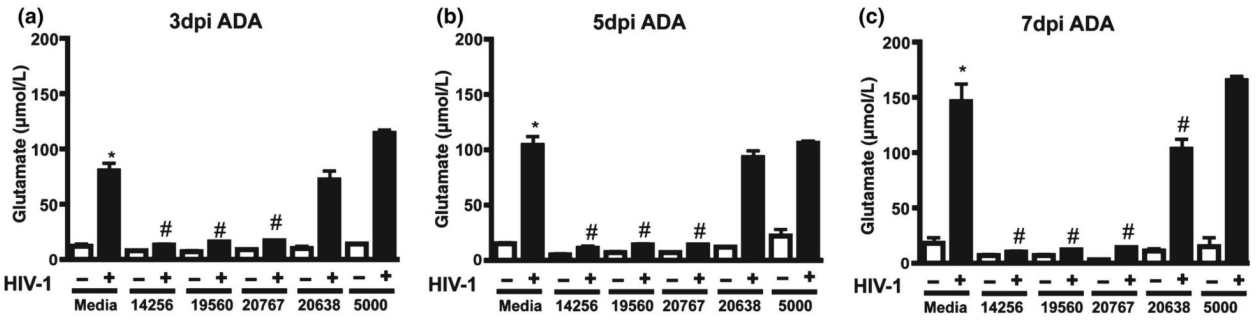

Fig 5.

Glutaminase inhibitor treatment at 3, 5, and 7 days post-infection of human macrophage. Human monocyte-derived macrophages were infected with HIV-1_{ADA} for either 3, 5, or 7 days and incubated in serum-free neurobasal media with or without glutaminase inhibitors. The glutaminase inhibitors were applied at a final concentration of 10 μmol/L. The concentration of glutamate in cell-free supernatants was determined by RP-HPLC. All data are expressed as absolute concentration of glutamate (μmol/L). Results are expressed as average ± SD of triplicate samples and are representative of three different donors. *Denotes $p < 0.01$ in comparison with control, #denotes $p < 0.01$ in comparison with HIV-1_{ADA}.

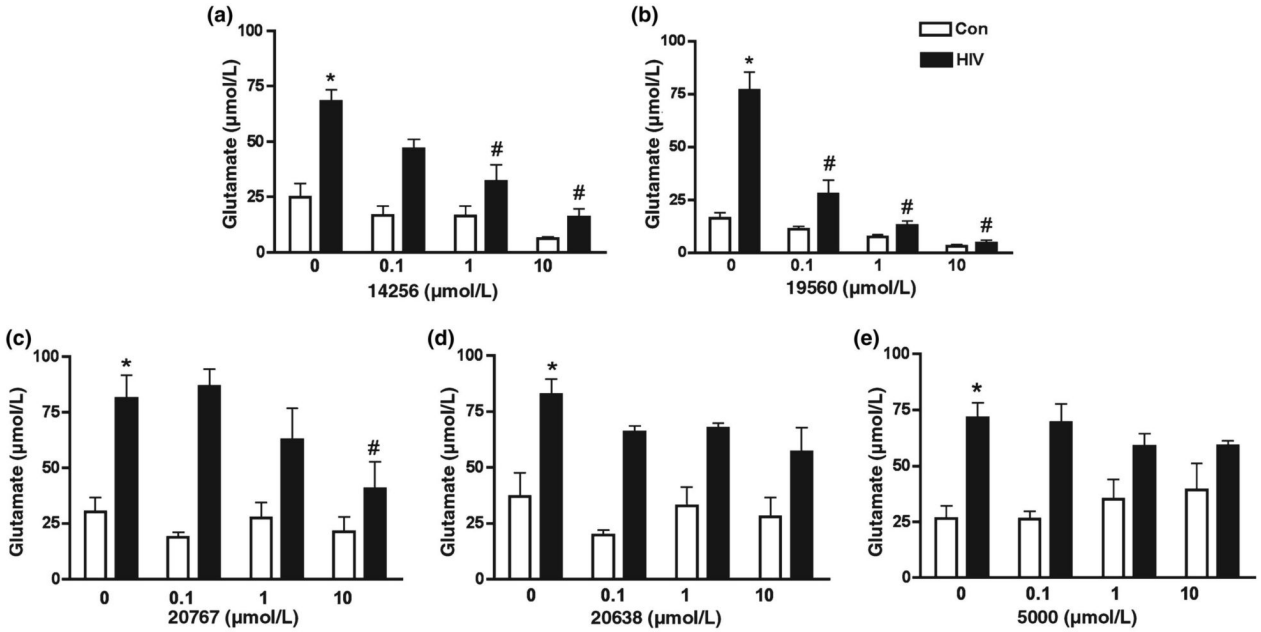

Fig 6.

Inhibitors reduced glutamate levels in infected macrophage cultures. Human monocyte-derived macrophages were infected with HIV-1_{ADA} for 7 days. Cells were washed and incubated in serum-free neurobasal media or in media containing glutaminase inhibitors at concentrations of 0, 0.1, 1, or 10 µmol/L. The concentration of glutamate in cell-free supernatants was determined by RP-HPLC. All data are expressed as absolute concentration of glutamate (µmol/L). Results are expressed as average ± SEM of data obtained from three different donors (triplicate from each donor). *Denotes $p < 0.01$ in comparison with control, #denotes $p < 0.05$ in comparison with HIV-1_{ADA}.

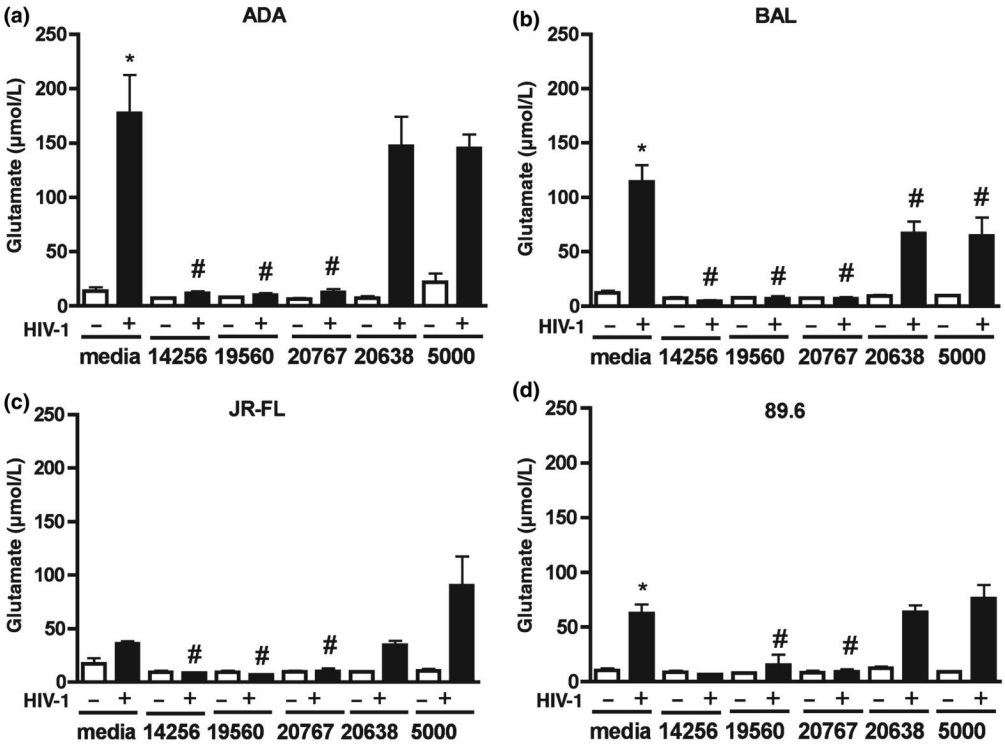

Fig 7.

The blocking of glutamate production induced by different viral strains by glutaminase inhibitors. Human monocyte-derived macrophages were infected with HIV-1_{ADA}, HIV-1_{JRFL}, HIV-1_{BAL} and HIV-$1_{89.6}$ for 8 days. Cells were incubated overnight in serum-free neurobasal media with or without glutaminase inhibitor. The concentration of glutamate in cell-free supernatants was determined by RP-HPLC. Data are expressed as absolute concentration of glutamate (µmol/L). Results are expressed as average ± SEM of data obtained from three different donors (triplicate from each donor). *Denotes $p < 0.01$ in comparison with control, #denotes $p < 0.05$ in comparison with HIV.

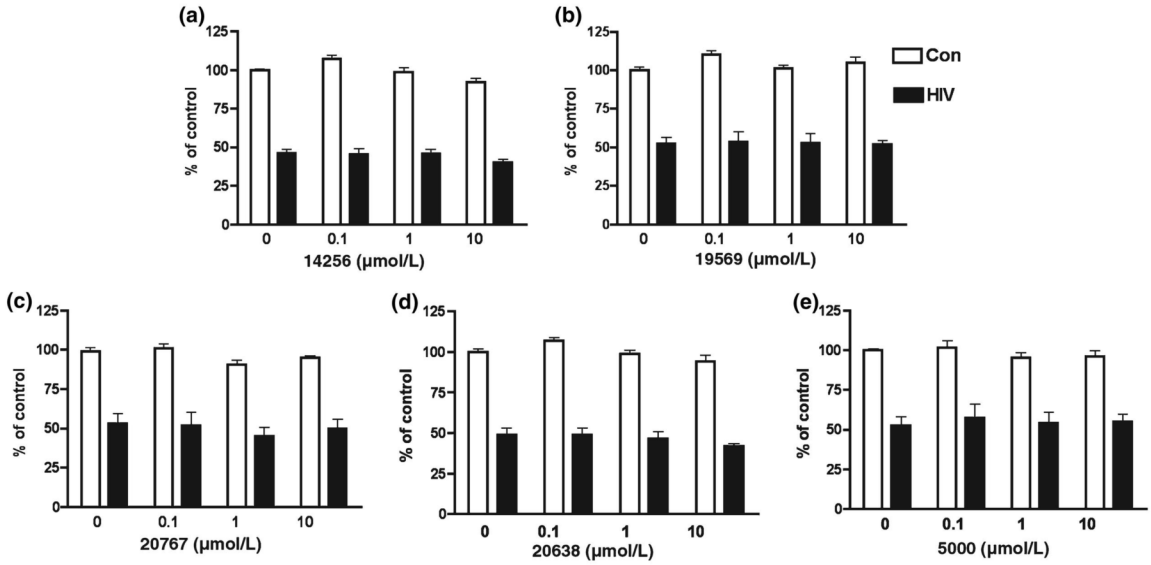

Fig 8.

The effect of glutaminase inhibitor treatment on cell viability. Human monocyte-derived macrophages were infected with HIV-1_{ADA} for 7 days. Cells were washed and incubated in serum-free neurobasal media or in media containing glutaminase inhibitors at concentrations of 0, 0.10, 1.0, or 10 μmol/L. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were incubated with 200 μL of 10% MTT solution after macrophage conditioned media collection. Cell culture plates were incubated for 20-30 min at 37°C. After incubation, MTT solution was removed and 200 μL dimethylsulfoxide was added to each well. Cell viability was determined by measuring optical density at 490 nm in a microplate reader. Results are expressed as average ± SD (n = 3) and are representative of three different donors.

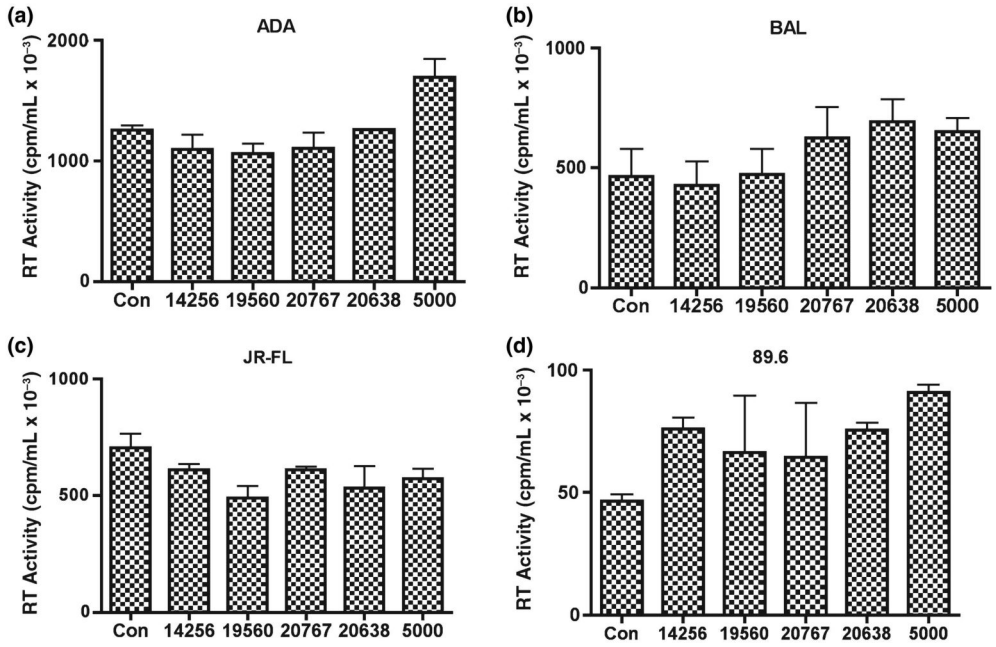

Fig 9.

The effect of glutaminase inhibitor treatment on HIV infection. Human monocyte-derived macrophages were infected with HIV-1_{ADA} (a), HIV-1_{BAL} (b), HIV-1_{JRFL} (c), and HIV-$1_{89.6}$ (d) for 8 days. Cells were then incubated with glutaminase inhibitors 14256, 19560, 20767, 20638, and 5000 at concentrations of 0 and 10 μmol/L. Quantitation was verified through measurement of reverse-transcriptase (RT) activity in cell-free supernatants from monocyte-derived macrophages infected by different HIV strains. Results are expressed as average ± SD of three samples and are representative of three different donors.

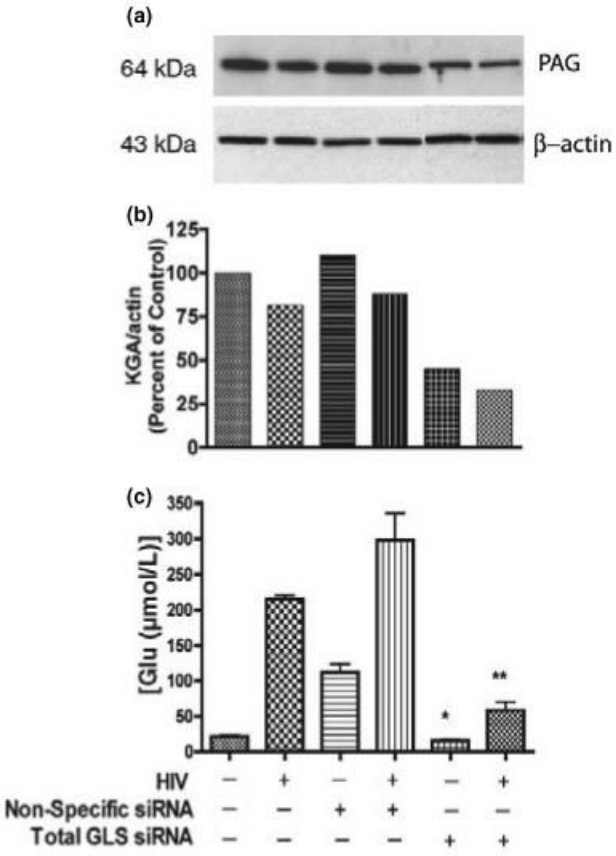

Fig 10.

siRNA treatment targeting glutaminase in infected human macrophage. Human monocyte-derived macrophages were grown in culture and then infected with HIV-1_{ADA}. Infected monocyte-derived macrophages were then transfected with siRNA targeting glutaminase or non-specific control. (a) Whole cell lysates were collected and analyzed by western blot for phosphate activated glutaminase. (b) Bands were quantitated and values expressed are as compared with β-actin. (c) Supernatants were analyzed for glutamate concentration. Results are expressed as average ± SD of three samples and are representative of three different donors.

Table 1

Inhibitor constants derived from enzyme activity assay

Inhibitor	IC_{50} (μmol/L)	Inhibitor concentration (μmol/L)	V_{MAX}	K_M	K_I (μmol/L)
14256	38	0	15.0 ± 0.47	12.3 ± .99	9.84 ± 1.21
		30	5.0 ± 0.25	7.7 ± 1.27	
		100	0.75 ± 0.05	2.5 ± 1.03	
19560	48	0	14.8 ± .62	13.0 ± 1.3	13.5 ± 0.79
		30	5.4 ± 0.20	8.8 ± 0.97	
		100	0.60 ± 0.04	3.0 ± 0.89	