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Kinetic characterization of ebselen, chelerythrine and apomorphine as glutaminase inhibitors

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ABSTRACT

Glutaminase catalyzes the hydrolysis of glutamine to glutamate and plays a central role in the proliferation of neoplastic cells via glutaminolysis, as well as in the generation of excitotoxic glutamate in central nervous system disorders such as HIV-associated dementia (HAD) and multiple sclerosis. Both glutaminase siRNA and glutaminase inhibition have been shown to be effective in *in vitro* models of cancer and HAD, suggesting a potential role for small molecule glutaminase inhibitors. However, there are no potent, selective inhibitors of glutaminase currently available. The two prototypical glutaminase inhibitors, BPTES and DON, are either insoluble or non-specific. In a search for more drug-like glutaminase inhibitors, we conducted a screen of 1280 *in vivo* active drugs (Library of Pharmacologically Active Compounds (LOPAC^{1280})) and identified ebselen, chelerythrine and (*R*)-apomorphine. The newly identified inhibitors exhibited 10 to 1500-fold greater affinities than DON and BPTES and over 100-fold increased efficiency of inhibition. Although non-selective, it is noteworthy that the affinity of ebselen for glutaminase is more potent than any other activity yet described. It is possible that the previously reported biological activity seen with these compounds is due, in part, to glutaminase inhibition. Ebselen, chelerythrine and apomorphine complement the armamentarium of compounds to explore the role of glutaminase in disease.

1. Introduction

Glutamine is a major source of energy for highly proliferative and neoplastic cells via glutaminolysis [1,2]. Glutamine is transported into cells where glutaminases (EC 3.5.1.2) hydrolyze glutamine to glutamate [3]. Glutamate is further catabolized through the tricarboxylic acid cycle to ATP. Glutaminase has been shown to play a critical role in glutaminolysis via a c-Myc regulated process [4]. Glutaminase siRNA, a prototype glutaminase inhibitor and glutamine deprivation have all been shown to cause significant decreases in cell proliferation in various cancer lines [4–9] suggesting a role for small molecule glutaminase inhibitors for the treatment of cancer.

Glutaminase is also thought to play a critical role in the generation of glutamate, a key excitatory neurotransmitter in the CNS [10,11]. HIV-infected macrophages were shown to express increased glutaminase levels and to produce significantly more glutamate that was glutamine dependent [12]. Prototype glutaminase small molecule inhibitors and glutaminase specific siRNA were able to abrogate the increases in glutamate caused by HIV-infected macrophages [13]. These results suggest a fundamental role of glutaminase in HIV-induced neurotoxicity. Glutaminase-mediated glutamate release from microglia was also shown to occur in models of multiple sclerosis [14] and ischemia [15], suggesting glutaminase inhibition could be of broad therapeutic interest for neuroinflammatory and neurodegenerative disorders.

Even though glutaminase inhibition could have therapeutic utility, to date, there are no known potent and selective glutaminase

Abbreviations: Apomorphine, 5,6,6a,7-tetrahydro-6-methyl-4*H*-dibenzo[*de*,*g*]quinoline-10,11-diol; Berberine, 5,6-dihydro-9,10-dimethoxy-benzo[*g*]-[1,3]benzodioxolo[5,6-a]quinolizinium; BPTES, bis-2-(5-phenylacetimido-1,2,4-thiadiazol-2-yl)ethyl sulfide; CNS, central nervous system; Chelerythrine, 1,2-dimethoxy-*N*-methyl[1,3]benzodioxolo[5,6-*c*]phenanthridinium; DON, 6-diazo-5-oxo-L-norleucine; Ebselen, 2-phenyl-1,2-benzisoselenazol-3[2*H*]-one; GAC, c-type glutaminase; GLS, glutaminase; HIV, human immunodeficiency virus; HRP, horse radish peroxidase; KGA, kidney-type glutaminase; LGA, liver-type glutaminase; Nitidine, 2,3-dimethoxy-*N*-methyl[1,3]benzodioxolo[5,6-*c*]phenanthridinium; Norsanguinarine, [1,3]-benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*]phenanthridine; Sanguinarine, 13-methyl-[1,3]-benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*]phenanthridinium.

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inhibitors available. DON, the earliest known inhibitor of glutaminase, is an active site directed inhibitor [16,17] that has been used as a tool compound to help elucidate potential involvement of glutaminase in HIV-associated dementia (HAD) pathogenesis and multiple sclerosis [12,14]. However, DON is a non-selective toxic reagent that inhibits several glutamine utilizing enzymes [18]. DON also has weak millimolar inhibitory potency in *in vitro* models of disease [12,14] and is not well tolerated *in vivo* [19–21]. Elan Pharmaceuticals described a glutaminase inhibitor (Newcomb, US Patent, 2002) termed BPTES with low micromolar potency (K_i = 0.2 μM) and an uncompetitive mode of inhibition [22]. Although more potent than DON, BPTES is not a drug-like compound as it has high molecular weight (534), poor solubility and low bioavailability [23]. Structure Activity Relationship (SAR) studies around BPTES has not yielded significantly better analogs [8,23]. Consequently, in order to identify more drug-like inhibitors, we conducted a screen of a Library of Pharmacologically Active Compounds (LOPAC^{1280}, Sigma) comprising small molecule modulators and approved drugs from all major drug classes. Here, we report on the kinetic characterization of three new inhibitors identified from our screening endeavor and on their direct comparison to DON and BPTES.

2. Materials and methods

2.1. Materials

DON, ebselen, LOPAC^{1280}, sanguinarine chloride, *R* and *S*-apomorphine and *R*(−)-apocodeine hydrochloride were purchased from Sigma (St. Louis, MO, USA). The chloride salts of chelerythrine, nitidine, berberine and norsanguinarine were obtained from LC Laboratories (Woburn, MA, USA), Ontario Chemicals Inc (Guelph, Ontario, Canada), MP Biomedicals (Solon, OH, USA) and Quality Phytochemicals LLC (Brunswick, NJ, USA), respectively and *R*(−)-propylnorapomorphine hydrochloride was bought from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). BPTES was synthesized in-house [23]. The plasmid encoding the cDNA for hKGA was graciously provided by Dr. Norman P. Curthoys (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA). Finally, mouse liver glutaminase (mLGA) and mouse c-type glutaminase (mGAC) were generous gifts from Dr. Andre Ambrosio (Laboratório Nacional de Biociências, Centro Nacional de Pesquisa em Energia e Materiais, Campinas-SP 13083–970, Campinas, Brazil).

2.2. Methods

Unless otherwise noted, glutaminase activity in the presence and absence of compounds was determined using the Amplex UltraRed fluorescent assay [24]. A counter-screen was carried to weed out autofluorescent compounds and quenchers and a second counter-screen was performed to eliminate inhibitors of glutamate oxidase and horse radish peroxidase and/or compounds that reacted with Amplex UltraRed. Competition studies (K_i determinations) were carried out with enzyme (*E*) exposed to substrate (*S*) and inhibitor (*I*) at the same time. K_m and V_{max} for all compounds were determined from glutamine saturation profiles at the various inhibitor concentrations using GraphPad Prism, employing a least-squares fit of the Michaelis–Menten equation: $v = V_{max}[S]/(K_m + [S])$. During inactivation studies (k_{inact} determinations) enzyme was incubated with inhibitor for varying times and the substrate used as a tool to determine the percent of remaining enzyme activity [25]. Due to BPTES' limited solubility [23], a factor that interferes with fluorescence measurement, the radiolabel assay [26] was used to determine the kinetic parameters of BPTES. [^{3}H]-Glutamine was used as the substrate in this assay. The radiolabel assay was also employed for DON in the inactivation experiment as DON hydrolyzes to glutamate [16], a product of the glutaminase reaction, which results in spurious fluorescence output. Data were analyzed using GraphPad Prism's non-linear regression analysis, with variable slope, of log [inhibitor] *vs.* normalized values.

3. Results

3.1. Primary glutaminase screen

The LOPAC^{1280} were screened for glutaminase activity. Of the 1280 compounds screened, 123 were active (percent inhibition $\geqslant$50 and $IC_{50} \leqslant 10$ μM). After counter screening, 23 were confirmed as bona fide glutaminase inhibitors. From these, ebselen, chelerythrine chloride and *R*-apomorphine hydrochloride were identified as most potent. Ebselen and chelerythrine chloride inhibited both isoforms of GLS1 (hKGA$_{\Delta 1}$ and its splice variant, mGAC) with similar potency and exhibited 5 to 10-fold less activity against GLS2 (mLGA). In contrast, *R*-apomorphine hydrochloride had similar activity against both GLS1 isoforms and GLS2 (Table 1).

3.2. Kinetic characterization of ebselen, chelerythrine, apomorphine, DON and BPTES

To determine the kinetics of inhibition of ebselen, glutamine saturation experiments were performed in the presence of different concentrations of ebselen (Supplementary: Fig. 1a). As the concentration of inhibitor was increased, the apparent Michaelis constant (K_{mapp}) increased with a concomitant 6-fold decrease in V_{max}. A double reciprocal plot of the data yielded lines with varying slopes that intersected in the second quadrant (Supplementary: Fig. 1b), indicative of mixed non-competitive inhibition. A secondary plot of the slopes for each line of the double reciprocal plot (K_{mapp}/V_{max}) versus inhibitor concentration gave a K_i of approximately 15 nM (Table 2; Supplementary: Fig. 1c).

The mode of inhibition of glutaminase by ebselen was further investigated by performing a time-dependent inhibition experiment [25]. The semilogarithmic plot of the remaining activity of glutaminase versus time showed pseudo-first order kinetics. Additionally, inactivation of glutaminase by ebselen was dependent on both concentration and time of incubation (Supplementary: Fig. 1d). The calculated K_i of ebselen was approximately 14 nM and the k_{inact} of glutaminase by ebselen was 1.32 /h (Table 3; Supplementary: Fig. 1e).

Similar experiments were carried out with chelerythrine, apomorphine and prototype inhibitors, BPTES and DON. Results are shown in tables 2 and 3 (and in Supplementary: Figs. 2, 3, 4 & 5). Competition studies with BPTES showed that K_{mapp} decreased with a concomitant decrease in V_{max} (Supplementary: Fig. 4a). A double reciprocal plot of the data yielded parallel lines (Supplementary: Fig. 4b), indicative of uncompetitive inhibition. A secondary plot of the reciprocal of K_{mapp} versus inhibitor concentration gave a K_i of 125 nM (Table 2; Supplementary: Fig. 4c). Competition studies could not be carried out with DON since it acts as both a substrate

Table 1
Specificity of ebselen, chelerythrine chloride and apomorphine hydrochloride for GLS1 (hKGA$_{\Delta 1}$, mGAC) and GLS2 (mLGA).

Compound ID	Average IC_{50} (μM) Against		
	hKGA	mGAC	mLGA
Ebselen	0.008	0.02	0.1
Chelerythrine chloride	0.03	0.07	0.3
Apomorphine hydrochloride	0.4	1.0	0.3