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Efficacy of S-adenosylhomocysteine hydrolase inhibitors, D-eritadenine and (S)-DHPA, against the growth of *Cryptosporidium parvum* in vitro

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ABSTRACT

D-eritadenine and (S)-DHPA are aliphatic adenosine analogues known to target S-adenosylhomocysteine hydrolase (SAHH) and potent antiviral compounds. In the present study, we demonstrate that these two compounds also display efficacy against recombinant SAHH enzyme of the protozoan parasite *Cryptosporidium parvum*, as well as inhibition of parasite growth in vitro. Our data confirm that SAHH could serve as a rational drug target in cryptosporidial infection and antiviral adenosine analogues are potential candidates for drug development against cryptosporidiosis.

1. Introduction

More than three decades ago the compound (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA] was first identified as a broad spectrum antiviral adenosine analogue (De Clercq et al., 1978; Votruba and Holy, 1980). In the following years, (S)-DHPA (Fig. 1), D-eritadenine (Fig. 1) and several other adenosine analogues were found to target the S-adenosylhomocysteine hydrolase (SAHH) (De Clercq et al., 1984; De Clercq, 2008; Holy et al., 1985; Schanche et al., 1984; Votruba and Holy, 1982). SAHH (E.C. 3.3.1.1) is an essential enzyme in all cells that hydrolyzes S-adenosylhomocysteine (SAH) into adenosine and L-homocysteine. Therefore, this enzyme is also explored as potential drug target in many bacteria and parasites (Bitonti et al., 1990; Henderson et al., 1992; Parker et al., 2003; Singh et al., 2006; Tanaka et al., 2004). The inhibition of SAHH activity could result in the accumulation of SAH and reduce the S-adenosylmethionine (SAM):SAH ratio in the cell, in which SAH further acts as a potent feedback inhibitor, blocking the SAM-dependent methylation required for the metabolism of a wide variety of biological compounds such as nucleic acids, proteins, phospholipids and other small molecules (Chiang et al., 1996; Chiang, 1998; Nozaki et al., 2005).

We have previously cloned, expressed and functionally characterized SAHH from the parasite *Cryptosporidium parvum* (CpSAHH), and principally observed that (S)-DHPA, D-eritadenine and Ara-A could inhibit the activity of recombinant CpSAHH, implying that CpSAHH could be explored as a potential drug target in this parasite (Ctrnacta et al., 2007). Here, we extended that study by further testing detailed inhibitory kinetics of selected aliphatic adenosine analogues against recombinant CpSAHH, as well as their efficacies against the growth of *C. parvum* using an in vitro cryptosporidial infection model.

Cryptosporidium is a genus of unicellular parasites belonging to the Phylum Apicomplexa, of which *C. parvum* and *C. hominis* are the major species infecting humans. Their infection typically results in mild to severe, but self-limiting watery diarrhea in immunocompetent patients. However, their infection in immunocompromised individuals, such as AIDS patients, could be prolonged and life-threatening (Chen et al., 2002; Thompson et al., 2005; Tzipori and Widmer, 2008). Currently, no effective specific treatment is yet available to treat cryptosporidial infection in AIDS

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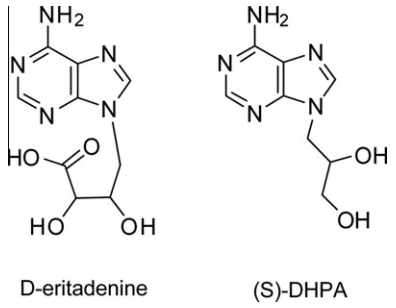

Fig. 1. Structures of the compounds D-eritadenine and (S)-DHPA.

patients. New, specific drugs against this parasite are still urgently needed. Our discovery that aliphatic nucleoside analogues could effectively block the growth of the parasite could be another step in long search for new anticryptosporidial drug candidates.

2. Materials and methods

2.1. Recombinant CpSAHH inhibition assays

The cloning and expression of maltose-binding protein (MBP)-fused CpSAHH protein has been previously reported by us (Ctrnacta et al., 2007). Briefly, the *CpSAHH* gene was engineered into a pMAL-c2x expression vector and the expression and purification with an amylose-resin-based chromatography followed the manufacturer's protocol (New England Biolabs). Purified MBP-CpSAHH fusion protein was digested with factor Xa to cleave the MBP-tag, and the tag was removed using a CHT 5-I hydroxyapatite column according to the manufacturer's protocol (Bio-Rad). The purity of recombinant CpSAHH without the MBP-tag was analyzed using SDS–PAGE, and concentrations were determined by a Bradford protein assay. Protein aliquots were stored at −20 °C until use.

The enzymatic activity of the recombinant protein CpSAHH was spectrophotometrically assayed in the hydrolytic direction (Lozada-Ramirez et al., 2006) using 50 μM SAH as a substrate. The drugs used in this study were neutral (S)-DHPA [9-(S)-(2,3-dihydroxypropyl)adenine] and acidic derivate of (S)-DHPA, D-eritadenine [(2R,3R)-4-(6-aminopurin-9-yl)-2,3-dihydroxy-butanoic acid] (Fig. 1). Both inhibitors are adenosine analogues with sugar moieties replaced by aliphatic chains. Inhibitors were provided by Professor Antonin Holy at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Inhibition of CpSAHH was evaluated using various concentrations of D-eritadenine (0.01–1 μM) or (S)-DHPA (1–300 μM). The assay was carried out by pre-incubating 5 μL of 1 mg/ml CpSAHH with different concentrations of inhibitors for 10 min at 37 °C. The reaction started with the addition of the CpSAHH–inhibitor mixture into an enzyme reaction buffer (50 μM S-adenosylhomocysteine, 4 U Ado deaminase, 250 mM DNTB in 50 mM potassium phosphate buffer with 1 mM EDTA, pH 7.2) in a final volume of 1 ml. Enzyme activity was spectrophotometrically detected at 412 nm at 37 °C using a Shimadzu UV 1601 spectrophotometer. Controls included reactions without inhibitors, and those containing inhibitors, but no enzyme. Reactions were performed in at least four replicates.

2.2. Cultivation of parasite in vitro and drug treatments

All experiments used *C. parvum* oocysts (Iowa-1 strain) that were less than 3 months old, purified by Percoll gradient centrifugation and bleached as previously described (Nesterenko and Upton, 1996). HCT-8 (ATCC # CCL-244) cells (1.0×10^5 per well) were seeded into 48-well plates and allowed to grow until reaching ~80% confluence at 37 °C with 5% CO_2 in RPMI 1640 medium containing 10% fetal bovine serum, 15 mM HEPES, and other supplements as previously described (Cai et al., 2005; Upton et al., 1995). For the generation of parasite standard curves, host cells were infected with 10-fold serial dilutions of oocysts (50–50,000). For all drug testing experiments, host cells were infected with 5000 oocysts per well. Parasites were allowed to incubate with host cells at 37 °C for 4 h to allow for excystation and invasion into host cells. At this time, an exchange of culture medium was performed to remove parasites that failed to invade the host cells. The compounds D-eritadenine and (S)-DHPA were dissolved in water and added to the infected cell cultures at the specified final concentrations (0.01–1000 μM) during the medium exchange. Parasite-infected cultures were then incubated for 44 h at 37 °C in the presence of 5% CO_2 (Cai et al., 2005). Each experimental condition was assayed in at least duplicates, and all experiments were repeated at least three times. Negative controls included cultures that received no parasites, and parasite-infected cultures that received no drug. Positive controls used various concentrations of paromomycin that is a commonly used standard inhibitor of *Cryptosporidium* growth in vitro (Cai et al., 2005).

Cytotoxicity of both inhibitors at 1 mM concentration on uninfected HCT-8 host cells was also examined using a standard fluorescent LIVE/DEAD viability/cytotoxicity assay for mammalian cells (Invitrogen/Molecular Probes). Cytotoxicity values for each inhibitor were also compared to cultures with and without the presence of paromomycin.

2.3. Efficacy of aliphatic adenosine analogs assayed by RT-qPCR

Total RNA was isolated from infected cultures 48 h post-infection (i.e., 44 h after drug addition) using an RNeasy isolation kit (Qiagen). The concentration and quality of RNA from each sample was determined by measuring their absorbance at 260 nm and 280 nm, and adjusted to a final concentration of 20 ng/μl. A SYBR green-based one-step real-time RT-qPCR method was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen). Each PCR reaction contained 20 ng of total RNA and proper amounts of reaction components as recommended by the manufacturer. The detection of parasite and human host cell 18S rRNA were detected using following primers: Cp18S-1011F (5′ TTG TTC CTT ACT CCT TCA GCA C 3′) and Cp18S-1185R (5′ TCC TTC CTA TGT CTG GAC CTG 3′) for *C. parvum*, and Hs18S-1373F (5′ CCG ATA ACG AAC GAG ACT CTG G 3′) and Hs18S-1561R (5′ TAG GGT AGG CAC ACG CTG AGC C 3′) for human host cells. Quantitative real-time RT-PCR was carried out in an iCycler iQ real-time PCR detection system (Bio-Rad). Reagents and primers were incubated at 48 °C for 30 min to synthesize cDNA, heated to 95 °C for 15 min to inactivate the reverse transcriptase. Then 40 thermal cycles were performed to amplify cDNA (95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s). At least two replicate reactions were performed for each sample in all PCR amplifications. The melting analysis showed a clear single peak without shoulder for each amplicon from each organism. Data was generated and analyzed following previously described methods (Cai et al., 2005) using GraphPad Prism v.5 software.

3. Results and discussion

The two potent acyclic adenosine analogues, (S)-DHPA and D-eritadenine, displayed a dose-dependent inhibitory effect against the recombinant CpSAHH. The 50% inhibitory concentrations (IC_{50} values) calculated by nonlinear regression were at 30 nM (8.3 ng/ml) for D-eritadenine and 24 μM (5 μg/ml) for (S)-DHPA, respectively (Fig. 2). It was described previously that D-eritadenine