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Development and evaluation of a host-targeted antiviral that abrogates herpes simplex virus replication through modulation of arginine-associated metabolic pathways

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ABSTRACT

Since their inception five decades ago, most antivirals have been engineered to disrupt a single viral protein or process that is essential for viral replication. This approach has limited the overall therapeutic effectiveness and applicability of current antivirals due to restricted viral specificity, a propensity for development of drug resistance, and an inability to control deleterious host-mediated inflammation. As obligate intracellular parasites, viruses are reliant on host metabolism and macromolecular synthesis pathways. Of these biosynthetic processes, many viruses, including Herpes simplex viruses (HSV), are absolutely dependent on the bioavailability of arginine, a non-essential amino acid that is critical for many physiological and pathophysiological processes associated with either facilitating viral replication or progression of disease. To assess if targeting host arginine-associated metabolic pathways would inhibit HSV replication, a pegylated recombinant human Arginase I (peg-ArgI) was generated and its *in vitro* anti-herpetic activity was evaluated. Cells continuously treated with peg-ArgI for over 48 h exhibited no signs of cytotoxicity or loss of cell viability. The antiviral activity of peg-ArgI displayed a classical dose-response curve with IC_{50}'s in the sub-nanomolar range. peg-ArgI potently inhibited HSV-1 and HSV-2 viral replication, infectious virus production, cell-to-cell spread/transmission and virus-mediated cytopathic effects. Not unexpectedly given its host-targeted mechanism of action, peg-ArgI showed similar effectiveness at controlling replication of single and multidrug resistant HSV-1 mutants. These findings illustrate that targeting host arginine-associated metabolic pathways is an effective means of controlling viral replicative processes. Further exploration into the breadth of viruses inhibited by peg-ArgI, as well as the ability of peg-ArgI to suppress arginine-associated virus-mediated pathophysiological disease processes is warranted.

1. Introduction

Herpes simplex virus (HSV) type-1 (HSV-1) and HSV type-2 (HSV-2) are closely related members of the human herpesvirus family. The prevalence of HSV-1 is estimated between 50 and 90% with 70% seropositive by adolescence and near 100% of adults 60 years or older positive for viral DNA (Hill et al., 2008; Xu et al., 2006). Although not as prevalent as HSV-1, HSV-2 is one of the most common sexually transmitted infections (Smith and Robinson, 2002). HSV is associated with genital herpes, herpes labialis, and herpetic keratitis; however, more severe disease manifestations can occur, including neonatal infections and encephalitis (Gnann and Whitley, 2002; Kimberlin, 2005; Whitley, 2002, 2006; Whitley and Gnann, 2002). Following an initial primary infection, HSV establishes a lifelong latent infection that sporadically reactivates leading to episodes of recurrent disease or asymptomatic viral shedding (Koutsky et al., 1990). Therefore, anti-herpetics are often utilized to treat recurrent disease or to

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prophylactically suppress viral transmission.

Synthetic nucleoside analogues, such as acyclovir, have proven a safe and effective means to inhibit HSV by targeting genomic replication (Dorsky and Crumpacker, 1987). However, the long-term use of these anti-herpetics has resulted in evolution of drug resistant viruses (Field, 1989; Field and Coen, 1986). Although acyclovir resistance was considered rare in immunocompetent individuals (Christophers et al., 1998; Fife et al., 1994; Nugier et al., 1992), some recent studies have shown a higher predilection for resistance within certain groups: children with oral herpetic lesions (≈4%) (Wang et al., 2011); patients with recurrent herpetic keratitis (≈6.5%) (Duan et al., 2009); patients with recurrent genital herpes (Cunningham et al., 2012; Kriesel et al., 2005). The prevalence of drug resistance in immunocompromised depends on the type of immunosuppression, varying between 3.5 and 7% in HIV patients (Englund et al., 1990; Levin et al., 2004; Reyes et al., 2003; Ziyaeyan et al., 2007) to 25% in allogeneic hematopoietic stem cell transplant recipients (Langston et al., 2002). These increasing trends have generated renewed interest in identifying novel targets that can suppress viral replication and minimize the likelihood of developing future resistance.

As obligate intracellular parasites, viruses are dependent on their host's metabolism and macromolecular synthesis pathways, often redirecting cellular processes to provide resources necessary for replication (Maynard et al., 2010). Of the numerous biosynthetic processes, many viruses are dependent on arginine bioavailability or *de novo* biosynthesis (Gonczol et al., 1975; Tankersley, 1964; Wigand and Kumel, 1978). Arginine is a versatile non-essential amino acid that is interconvertible with proline, ornithine, glutamate, and citrulline (Morris, 2006; Wu and Morris, 1998). Biochemically, arginine serves as a precursor for synthesis of protein, nitric oxide, polyamines and nucleotides (Morris, 2007). Physiologically, arginine and its metabolites have critical functions in innate and adaptive immunity, inflammation, wound healing, and vascularization (Morris, 2006, 2007; Wu et al., 2009; Wu and Morris, 1998). However, these normally physiological processes often contribute to severe virus-induced disease. Therefore, arginine bioavailability is a critical determinant of several physiological and pathophysiological processes that are involved both in facilitating viral replication and progression of disease.

Importantly, arginine is essential for replication of many viruses, including HSV (Gonczol et al., 1975; Tankersley, 1964; Wigand and Kumel, 1978). To evaluate the potential of targeting host arginine-associated metabolic pathways for control of HSV, we generated a pegylated human recombinant Arginase I (peg-ArgI). peg-ArgI exhibited no demonstrable toxicity and effectively inhibited HSV replication, infectious virus production, cell-to-cell transmission, and virus-induced cytopathic effects. These findings illustrate that targeting host arginine-associated metabolic pathways is as effective as current nucleoside analog anti-herpetics in its ability to control HSV replication.

2. Materials and methods

2.1. Viruses and cells

Primary human corneal epithelial cells (HCEC) were from Invitrogen. HSV-1(McKrae) was the parental wildtype HSV-1 strain utilized. The G strain of HSV-2 was obtained from ATCC. The parental HSV-1 (KOS) and its drug resistant derivatives that specified mutations in either the polymerase (PAAr5) or thymidine kinase (tkLTRZ1; tkG7dG.2) genes were a kind gift from Dr. Donald Cohen (Coen and Schaffer, 1980; Fleming and Coen, 1984; Griffiths and Coen, 2003; Horsburgh et al., 1998). All HSV viral stocks were propagated in Vero cells and stored as infectious cell preparations at −80 °C.

2.2. Production, pegylation and protein analysis of recombinant human ArginaseI

Recombinant human ArginaseI (ArgI) was expressed in *E. coli* and purified by AbboMax (San Jose, CA). Protein concentrations were determined by the Pierce BCA Protein Assay kit. To increase enzymatic activity and protein stability, ArgI was covalently conjugated to O-[2-(N-Succinimidyloxycarbonyl)-ethyl]-O′-methyl-polyethylene-glycol (PEG) 5000mw (Sigma-Aldrich) (Cheng et al., 2007; Tsui et al., 2009) at a protein:PEG molar ratio of 1:18. Samples were maintained at a constant pH = 7.5 using 1N NaOH and stirred at RT for 3 h. After PEGylation the peg-ArgI solution was brought to a pH = 7.0. The relative purity of ArgI and the extent of protein conjugated to PEG were assessed by SDS-PAGE with Novex SimplyBlue Safe Stain. The specificity of the protein expressed and purified as human Arginase I was assessed by western analysis using an antibody to human Arginase I (Cell Signaling). The extent of PEG covalently incorporated onto ArgI or BSA was assessed by western with an antibody against covalently attached or free PEG molecules (abcam; ab133471).

2.3. Arginase activity assays and determination of arginine and glutamine levels by HPLC

The enzymatic activity of peg-ArgI was determined across a 2-fold dilution series (from 5000 to 19.5 ng) by measuring the production of L-ornithine from L-Arginine, relative to a standard curve of L-ornithine as previously reported (Rodriguez et al., 2004). For determination time-dependent depletion of arginine for each dosage of drug, pre-warmed DMEM, or medium containing 2-fold serial dilutions of peg-ArgI (from 10,000 to 78 ng/ml) was incubated at 37 °C. At 5, 10, 20, 40, 60, 180, or 360 min post treatment, media was deproteinized with methanol, centrifuged and supernatants were stored at −80 °C until analysis. Deprotonated media was derivatized with 0.2 mol/L ophthalaldehyde/β-mercaptoethanol and fifty microliters per sample was analyzed by high-performance liquid chromatography (HPLC)-electrochemical detector on an ESA CoulArray Model 540, with an 80 × 3.2 column with 120A pore size. Standards of L-arginine and L-glutamine in methanol were run with each experiment and the levels of each amino acid in a test sample were determined relative to this standard curve. Experiments for each time point and dosage were repeated at least three times.

2.4. Cytotoxicity and cell viability assays

The cytotoxic effects of peg-ArgI and other anti-herpetics was assessed on Vero cells (according to Clinical Laboratory Standards Institute (CLSI) guidelines) (Sweirkosz et al., 2004), as well as primary HCEC. 8,000 cells/well were seeded to 96-well plates. The next morning, media was aspirated and replenished with media containing either: 1) 10 μg/ml of peg-ArgI; 2) 10 μg/ml of peg-BSA; 3) Mock treatment; 4) 1% triflouorothymidine (TFT); 5) 50 μM Acyclovir (ACV; Sigma Chemical); or 6) 300 μM ACV. The relative cytotoxic effect of each compound was determined for 5 replicates at 4 h, 24 h, and 48 h later using the Promega CellTox Green Cytotoxicity Assay according to the manufacturer's directions on a Tecan Infinite M200 scanning microplate spectrofluorometer. Trypan blue exclusion was utilized to visualize drug toxicity effects over the course of 15 days of continuous drug treatment. Subconfluent Vero cell monolayers were mock treated or treated with: peg-ArgI at 1 μg/ml or 10 μg/ml; 1% trifluorthymidine (TFT); or ACV at 1 μg/ml or 67 μg/ml. At 12 h, 24 h, 48 h, 8 days, and 12days post