International Journal for Parasitology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Advances in bumped kinase inhibitors for human and animal therapy for cryptosporidiosis

Matthew A. Hulverson [a], Ryan Choi [a], Samuel L.M. Arnold [a], Deborah A. Schaefer [b], Andrew Hemphill [c], Molly C. McCloskey [a], Dana P. Betzer [b], Joachim Müller [c], Rama S.R. Vidadala [d], Grant R. Whitman [a], Kasey L. Rivas [a], Lynn K. Barrett [a], Robert C. Hackman [e,f,g], Melissa S. Love [h], Case W. McNamara [h], Thomas K. Shaughnessy [i], Alison Kondratiuk [i], Matthew Kurnick [i], Patricia N. Banfor [i], James J. Lynch [i], Gail M. Freiberg [i], Dale J. Kempf [i], Dustin J. Maly [d,j], Michael W. Riggs [b], Kayode K. Ojo [a,*], Wesley C. Van Voorhis [a,*]

[a] *Department of Medicine, Division of Allergy and Infectious Disease, Center for Emerging and Reemerging Infectious Disease (CERID), University of Washington, Seattle, WA 98109, USA*
[b] *School of Animal and Comparative Biomedical Sciences, College of Agriculture and Life Sciences, University of Arizona, Tucson, AZ 85721, USA*
[c] *Institute of Parasitology, Vetsuisse Faculty, University of Bern, Länggass-Strasse 122, CH-3012 Bern, Switzerland*
[d] *Department of Chemistry, University of Washington, Seattle, WA 98195, USA*
[e] *Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA*
[f] *Department of Pathology, University of Washington, Seattle, WA 98195, USA*
[g] *Department of Laboratory Medicine, University of Washington, Seattle, WA 98195, USA*
[h] *California Institute for Biomedical Research, La Jolla, CA, USA*
[i] *Research and Development, AbbVie, Inc, North Chicago, IL 60064, USA*
[j] *Department of Biochemistry, University of Washington, Seattle, WA 98195, USA*

ARTICLE INFO

Article history:
Received 10 May 2017
Received in revised form 26 August 2017
Accepted 29 August 2017
Available online xxxx

Keywords:
Cryptosporidiosis
Calcium-dependent protein kinase 1
Gatekeeper residue
Drug target
BKI 1369

ABSTRACT

Improvements have been made to the safety and efficacy of bumped kinase inhibitors, and they are advancing toward human and animal use for treatment of cryptosporidiosis. As the understanding of bumped kinase inhibitor pharmacodynamics for cryptosporidiosis therapy has increased, it has become clear that better compounds for efficacy do not necessarily require substantial systemic exposure. We now have a bumped kinase inhibitor with reduced systemic exposure, acceptable safety parameters, and efficacy in both the mouse and newborn calf models of cryptosporidiosis. Potential cardiotoxicity is the limiting safety parameter to monitor for this bumped kinase inhibitor. This compound is a promising pre-clinical lead for cryptosporidiosis therapy in animals and humans.

1. Introduction

Cryptosporidium infections have been associated with mortality and morbidity from diarrhoea in malnourished 6–18 month old children in resource limited environments (Kotloff et al., 2013; Platts-Mills et al., 2015). The only US Food and Drug Administration (FDA) approved therapeutic drug for cryptosporidiosis, nitazoxanide, is only approximately 30% effective in 1–5 year old malnourished children with cryptosporidiosis (Amadi et al., 2002). Nitazoxanide is not approved for use in children under 12 months of age (package insert, nitazoxanide). Furthermore, *Cryptosporidium* infections can be life threatening in immunocompromised HIV-positive individuals, in whom nitazoxanide has also been shown to lack efficacy (Amadi et al., 2009). Clearly, new effective therapeutic methods are needed for cryptosporidiosis.

Bumped kinase inhibitors (BKIs) have been developed to selectively target *Cryptosporidium parvum* and *Toxoplasma gondii* calcium-dependent protein kinase 1 (CDPK1) (Murphy et al., 2010). These BKIs have excellent activity against *C. parvum* and *T. gondii* parasites in vitro and in vivo. A recently reported series of pyrazolo[2,3-*d*]pyrmidine (PP) BKIs are effective in experimental therapeutic treatments for cryptosporidiosis (Hulverson et al., 2017). Since that publication, additional data make it clear that

* Corresponding authors. Fax: +1 206 616 4898.
E-mail addresses: ojo67kk@u.washington.edu (K.K. Ojo), wesley@uw.edu (W.C. Van Voorhis).

http://dx.doi.org/10.1016/j.ijpara.2017.08.006
0020-7519/

one compound, BKI 1369, is emerging as a pre-clinical lead for cryptosporidiosis therapy.

The study presented here describes the pharmacokinetic, safety and efficacy parameters of BKI 1369. BKI 1369 is very active at low doses in the *C. parvum*-infected interferon-γ knockout (IFN-γ KO) mouse model. It is also efficacious in the *C. parvum*-infected newborn calf clinical model, abrogating diarrhoea and reducing parasite excretion. In addition, this compound is safe in that it lacks mutagenic activity, is not toxic to a variety of human cell lines, is safely administered orally to mice at up to 150 mg/kg of body weight for 7 days, and is safe for both mothers and fetuses in a mouse pregnancy model. One limitation of BKI 1369 is potential cardiotoxicity, both from ether-à-go-go (hERG) inhibition and negative inotropic activity. However, there is a sufficient safety window regarding cardiotoxicity to suggest BKI 1369 will be safe in humans and animals at therapeutic doses. BKI 1369 has low plasma exposure after oral administration, and has two major identified metabolites, each of which appears to be non-toxic to human cells. A pharmacodynamic model is presented here to further explain the therapeutic efficacy of BKI 1369.

2. Materials and methods

2.1. Previously described methods

Methods for the propagation of *C. parvum* (IOWA-II strain) oocysts in calves (Riggs and Perryman, 1987; Riggs et al., 1989), propagation of transgenic Nanoluciferase (Nluc) expressing *C. parvum* (UGA1 strain) oocysts in mice (Vinayak et al., 2015; Hulverson et al., 2017), in vitro microsomal stability (Tatipaka et al., 2014), pharmacokinetic analysis of mouse plasma, faecal, and urine BKI concentrations by LC-MS/MS analysis (Ojo et al., 2012; Schaefer et al., 2016; Hulverson et al., 2017), in vitro protein binding using dialysis membranes (Tatipaka et al., 2014), Nluc expressing *C. parvum* in vitro growth inhibition in infected HCT-8 cells (Hulverson et al., 2017), in vitro cytotoxicity in CRL-8155 and HepG2 cells (Tatipaka et al., 2014), and in vivo mouse gastrointestinal (GI) tract tissue exposure by LC-MS/MS analysis (Arnold et al., 2017) have all been previously described. Detailed descriptions of these methods are included in Supplementary Data S1. Methods for high-content imaging of *Cryptosporidium* proliferation in HCT-8 cells (Love et al., 2017), QPatch hERG (Danker and Moller, 2014), rat cardiovascular screening (Banfor et al., 2016), in vitro micronucleus assay (Nicolette et al., 2011), the 24-well Ames screening assay, kinome profiling (Goedken et al., 2015), Nluc *C. parvum* in infected adult IFN-γ KO mouse efficacy (Hulverson et al., 2017), Gastroplus (Simulations Plus, Inc., Lancaster, CA, USA) modelling of efficacy and GI tissue and lumen exposures (Arnold et al., 2017), and IOWA-II strain *C. parvum* in infected neonatal calf efficacy (Schaefer et al., 2016) have all been previously described. All LC-MS/MS analytes were measured with an Acquity ultra performance liquid chromatography (UPLC) system in tandem with a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). For all statistical analyses, $P < 0.05$ was considered significant. Methods for toxicity profiling in mice to determine the maximum tolerated single dose and toxicity related to multiple dosing were previously described (Vidadala et al., 2016). In toxicity profiling experiments, mice were monitored twice daily and dosing was halted after the first observable signs of toxicity. Necropsy, obtaining blood for complete blood counts (CBCs) and serum chemistries, and histology of organs (brain, heart, lungs, spleen, liver, stomach, small intestine, large intestine and kidney) were performed within 48 h of the final dose. Examination of organ histology included routine H&E and Oil-Red-O (lipid)-stained organs from the treated mice and a set of untreated matched controls. Pathological examination was performed by a board certified pathologist who was blinded to the treatment and control group identities.

2.2. Mouse pregnancy safety

Female BALB/c mice aged 8–10 weeks were mated for 3 days, after which the males were removed. Weights were monitored daily to confirm pregnancy. Starting at day 9 after mating, treated groups ($n = 6$ or 7) were dosed by oral gavage (PO) with 0.1 mL of BKI suspended in 100% corn oil and control groups were dosed PO with corn oil only for 5 days. On day 18 after mating, mice that were positively identified as pregnant were moved to individual housing and monitored 2–3 times daily. After birth, pups were observed for an additional 3 days, counted and dead pups were removed.

2.3. Metabolite identification, formation and excretion

Adult female BALB/c mice were given a single 0.2 mL oral dose of 60 mg/kg of body weight in 3% ethanol/7% Tween80/90% saline (ETS). Blood was taken by cardiac puncture from mice ($n = 3$) at time points of 1, 4, 8 and 24 h post dose. Control mice given ETS only were also sampled at 8 h post dose. A list of expected metabolites was generated by analysing the structure of BKI 1369. Plasma was analysed on LC MS/MS to confirm the presence of these metabolites in vivo. Pure samples of two of the probable metabolites were synthesised for use as standards to quantify sample concentration by LC MS/MS.

Additional adult female BALB/c mice ($n = 3$) were given a single oral BKI dose of 60 mg/kg of body weight in 0.2 mL of ETS and housed in metabolic cages to sample urine and faeces at 8 h intervals for a total of 48 h. Additional mice ($n = 3$) were given the same 60 mg/kg dose and blood was collected at 1, 4, 8 and 24 h post dose. Mice dosed with ETS only were used as controls. Plasma, urine and faeces were extracted with acetonitrile and analysed by LC MS/MS to determine the concentrations of 1369 and its metabolites identified from previously obtained plasma samples.

2.4. Cytochrome P450 (CYP) inhibition

To determine the inhibition kinetics for the reversible inhibition of CYP3A4 and CYP2D6 by BKI 1369, experiments were performed in triplicate with either midazolam (CYP3A4) or dextromethorphan (CYP2D6) concentrations of 1 μM, which is fivefold below the reported Michaelis constant (K_m) values for each of these substrates. The incubations were performed in 100 mM potassium phosphate buffer (pH 7.4), and final incubation volumes were 100 μL. BKI 1369 at concentrations of 0.01–100 μM, human liver microsomes (0.1 mg/mL) and substrate were pre-incubated at 37 °C before reactions were initiated by adding NADPH (1 mM, final concentration). The reactions were terminated after 4 min (midazolam) or 20 min (dextromethorphan) by addition of an equal volume of ice-cold acetonitrile containing internal standard. The samples were vortexed followed by centrifugation at 4,000*g* for 15 min. The supernatants were removed and analysed by LC MS/MS. The samples were normalised by the no inhibitor controls to determine the percentage of activity remaining. Prism (GraphPad, La Jolla, CA, USA) was used to fit the normalised data to a log(dose) –response curve and calculate a half maximal inhibitory concentration IC_{50}.

2.5. Animal ethics

All animal experiments conducted at the University of Washington, USA, and University of Arizona, USA, were approved by the Institutional Animal Care and Use Committees. Animal experi-