IJP: Drugs and Drug Resistance 8 (2018) 394–402

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

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

Validation of *Babesia* proteasome as a drug target

Marie Jalovecka[a,b], David Hartmann[a,b], Yukiko Miyamoto[c], Lars Eckmann[c], Ondrej Hajdusek[a], Anthony J. O'Donoghue[d,**], Daniel Sojka[a,*]

[a] Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, CZ-370 05, Ceske Budejovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, CZ-370 05, Ceske Budejovice, Czech Republic
[c] Department of Medicine, University of California, San Diego, La Jolla, USA
[d] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, USA

ARTICLE INFO

Keywords:
Proteasome
Babesia
Carfilzomib
Epoxyketone
Cytotoxicity

ABSTRACT

Babesiosis is a tick-transmitted zoonosis caused by apicomplexan parasites of the genus *Babesia*. Treatment of this emerging malaria-related disease has relied on antimalarial drugs and antibiotics. The proteasome of *Plasmodium*, the causative agent of malaria, has recently been validated as a target for anti-malarial drug development and therefore, in this study, we investigated the effect of epoxyketone (carfilzomib, ONX-0914 and epoxomicin) and boronic acid (bortezomib and ixazomib) proteasome inhibitors on the growth and survival of *Babesia*. Testing the compounds against *Babesia divergens* ex vivo revealed suppressive effects on parasite growth with activity that was higher than the cytotoxic effects on a non-transformed mouse macrophage cell line. Furthermore, we showed that the most-effective compound, carfilzomib, significantly reduces parasite multiplication in a *Babesia microti* infected mouse model without noticeable adverse effects. In addition, treatment with carfilzomib lead to an ex vivo and in vivo decrease in proteasome activity and accumulation of poly-ubiquitinated proteins compared to untreated control. Overall, our results demonstrate that the *Babesia* proteasome is a valid target for drug development and warrants the design of potent and selective *B. divergens* proteasome inhibitors for the treatment of babesiosis.

1. Introduction

Babesiosis is a malaria-related infection caused by the apicomplexan intracellular blood parasites of the genus *Babesia*, that are transmitted to their vertebrate hosts by the bite of ixodid ticks (Lantos and Krause, 2002). Despite routine epidemiologic surveillance babesiosis has long been recognized as an economically important disease of livestock (Zintl et al., 2003; Bock et al., 2004; Gohil et al., 2013), with growing incidence in both domesticated and wildlife animals (Schnittger et al., 2012; Gohil et al., 2013; Vannier et al., 2015). Only in the last 40 years has *Babesia* been recognized as an important human infection acquired naturally from interactions with established zoonotic cycles (zoonosis) (Yabsley and Shock, 2013; Vannier et al., 2015). Besides the natural infection by tick bites, humans are infected with *Babesia* also via blood transfusion with infected blood, or even congenitally during pregnancy (Ord and Lobo, 2015). The majority of human infections are reported in the United States (Vannier and Krause, 2012) where the principal agent of human babesiosis – *B. microti* – is one of the most common transfusion-transmitted pathogens (Leiby, 2011; Lobo et al., 2013; Yabsley and Shock, 2013; Vannier et al., 2015). In Europe, most reported medical cases of babesiosis have been attributed to *B. divergens* (Uhnoo et al., 1992; Haapasalo et al., 2010; Hildebrandt et al., 2013; Mørch et al., 2015).

A number of factors have contributed to the “emergence” of human babesiosis leading the US Centers for Disease Control and Prevention (CDC) to add babesiosis to the list of nationally notifiable conditions in 2011. The pathology in humans is a direct result of the parasite's ability to first recognize and then invade host red blood cells and ranges from clinically silent infections to intense malaria-like episodes resulting occasionally in death. Although many infections remain asymptomatic the burden of severe pathology resides within older or immunocompromised patients (Rosner et al., 1984; Benezra et al., 1987; Falagas and Klempner, 1996; Froberg et al., 2004; Häselbarth et al., 2007; Stowell et al., 2007; Krause et al., 2008) and is fatal in approximately 20% of cases where infection was acquired through blood transfusion (Vannier et al., 2015). This makes transfusion-transmitted babesiosis an emerging threat to public health as asymptomatic carriers donate blood, and there are as yet no licensed or regulated tests to

* Corresponding author.
** Corresponding author. Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, CA, 92093-0657, La Jolla, USA.
E-mail addresses: ajodonoghue@ucsd.edu (A.J. O'Donoghue), sojkadan@gmail.com (D. Sojka).

https://doi.org/10.1016/j.ijpddr.2018.08.001
Received 8 December 2017; Received in revised form 3 August 2018; Accepted 6 August 2018
Available online 07 August 2018

screen blood products for this pathogen (Yabsley and Shock, 2013; Vannier et al., 2015). Reports of tick-borne cases within new geographical regions as well as identifications of new *Babesia* spp. as agents of severe human babesiosis suggest rapid changes in epidemiology of this disease making it a serious public health concern that requires novel intervention strategies (Leiby, 2011; Lobo et al., 2013; Yabsley and Shock, 2013; Vannier et al., 2015).

Babesiosis is generally treated using a combination of antimalarial drugs and antibiotics such as atovaquone and azithromycin (Vannier et al., 2015). However, the toxic effects of these treatments combined with an increase in parasite resistance (Wormser et al., 2010; Simon et al., 2017) and in numbers of relapsed immunocompromised and asplenic individuals (Lemieux et al., 2016), have made this widely used anti-babesial treatment regime less effective (Simon et al., 2017). Therefore, discovery of new drug targets and development of new and effective antibabesial drugs is urgently needed.

Proteasomes are large multi-component protein complexes that are constitutively expressed in all living cells and are involved in regulation of many cellular processes (Adams, 2004). The principal function of the constitutive proteasomes is to degrade poly-ubiquitinated proteins in the cytosol and nucleus via the ubiquitin-proteasome system (Voges et al., 1999; Bedford et al., 2010). A specialized form of the mammalian constitutive proteasome is the immunoproteasome with higher level of expression in antigen-presenting cells upon oxidative stress and cytokine stimulation (Ferrington and Gregerson, 2012). Proteasomes are composed of a barrel-shaped 20S core flanked by the 19S regulatory units on both ends (Voges et al., 1999; Bedford et al., 2010; Kish-Trier and Hill, 2013; Tomko and Hochstrasser, 2013). The function of the 19S subunits is substrate recognition, deubiquitinating, unfolding and translocation to the proteasome core for degradation (Voges et al., 1999; Tomko and Hochstrasser, 2013). The 20S core, the site of protein degradation, is formed by the two rings of α subunits surrounding the two stacked rings of seven β subunits. In the constitutive proteasome, three subunits on each of the β rings are proteolytically active with each subunit having a unique substrate cleavage preference. The β1 subunit preferentially cleaves on the C-terminal side of acidic residues. Fluorescent substrates that were originally developed for mammalian caspases are generally hydrolysed by this subunit. Therefore, the β1 subunit is often referred to as having “caspase-like” activity. In a similar manner, the β2 subunit cleaves on the C-terminal side of basic residues and has “trypsin-like” activity, while the β5 has “chymotrypsin-like” activity as it cleaves after non-polar residues (Verdoes et al., 2006; Kish-Trier and Hill, 2013). In the mammalian immunoproteasome, the chymotrypsin-like, trypsin-like and caspase-like proteolytic activities are performed by the β5, β2 and β1 subunit respective analogues LMP7, MECL1, and LMP2 (Ferrington and Gregerson, 2012).

Targeting the chymotrypsin-like activity of mammalian proteasome has been previously verified as a powerful strategy for anti-cancer therapy. Three proteasome inhibitors targeting the β5 subunit of mammalian proteasomes and have been approved for treatment of multiple myeloma (Kane et al., 2003, 2006; Groll et al., 2006; Kupperman et al., 2010; Kim and Crews, 2013; Bibo-Verdugo et al., 2017). These include the peptide epoxyketone carfilzomib and two peptide boronic acid derivatives, bortezomib and ixazomib. In addition, ONX-0914 selectively targets the chymotrypsin-like immunoproteasome subunit LMP7 and controls a pathogenic immune response in autoimmune disorders (Muchamuel et al., 2009). A recent revolution in the field of parasitology has been the development of proteasome inhibitors that selectively target parasitic organisms but with greatly reduced toxicity to the mammalian host (Bibo-Verdugo et al., 2017). Such selective inhibition may become a powerful strategy to combat infections such as malaria, leishmaniasis, sleeping sickness, and Chagas disease (Khare et al., 2016; Li et al., 2016b; LaMonte et al., 2017). In addition, the synergistic effect of proteasome inhibitors and artemisinin for the treatment of drug resistant *Plasmodium* has provided a potential new strategy for treating malaria (Dogovski et al., 2015; Li et al., 2016b; LaMonte et al., 2017).

Multi-gene analyses of apicomplexan parasites have positioned *Babesia* species as close relatives of *Plasmodium* species (Burki et al., 2009; Janouskovec et al., 2010; Arisue and Hashimoto, 2015; Schreeg et al., 2016) and therefore we predict that selective inhibition of the *Babesia* proteasome will represent a novel strategy for the treatment of babesiosis. In this study, we investigated the effect of proteasome inhibitors on *B. divergens* and *B. microti*, the causative agents of human babesiosis, with the goal of validating this target for future drug development efforts.

2. Materials and methods

2.1. Parasites

B. divergens 2210A G2 was cultivated in a bovine erythrocyte suspension obtained from a parasite-free cow (culture tested) by a previously described procedure (Malandrin et al., 2004). Parasites were cultivated in RPMI 1640 medium (Lonza, Switzerland; cat. no. BE12-115F) supplemented with 50 μg/ml gentamicin, 0.25 μg/ml amphotericin B and 20% heat-inactivated fetal calf serum (Lonza, Switzerland, inactivation at 56 °C for 30 min before use). *B. microti* (Franca) Reichenow (ATTC® PRA-99™, USA), was maintained by continuous passages in BALB/c female mice (Charles River Laboratories, Germany) and used for in vivo experiments. All animals were treated in accordance with the Animal Protection Law of the Czech Republic no. 246/1992 Sb., ethics approval No. 112/2016 and all in vivo experiments were approved by the institutional ethics committee.

2.2. Proteasome inhibitors

Carfilzomib (PR-171), bortezomib (PS-341), epoxomicin, ONX-0914 and ixazomib (MLN9708) were purchased from Selleckchem (USA) and dissolved to 5 mM in DMSO (dimethyl sulfoxide, Sigma-Aldrich, USA). For treatment of *B. divergens* cultures, inhibitors were diluted in culture medium. For treatment of *B. microti* infected mice, carfilzomib was diluted in sterile PBS (phosphate buffered saline).

2.3. Treatment of B. divergens ex vivo cultures

To evaluate the effect of proteasome inhibitors on *B. divergens* growth and determine their IC_{50} values, cultures containing 2% parasitemia were subsequently cultivated in media with proteasome inhibitor concentrations ranging from 6.25 nM to 400 nM. Assays were performed in triplicate wells in a 96-well plate format and media containing inhibitors were replaced at 12 h intervals. DMSO diluted in media served as a vehicle control. After 48 h of incubation, parasite replication was quantified as the number of infected red blood cells (RBCs) per 1000 RBCs on thin blood smears stained by Diff-Quik (Siemens, Germany) (Fig. 1); biological replicates are represented by three independent wells (RBC cultures) while technical replicates are represented by three different thin blood smears of each well (RBC culture) that the parasitemia was counted from. This whole inhibitor treatment assay was performed twice: firstly as a pilot experiment (Supplemetary Fig. 1) and than repeated with fresh 2% parasitemia *B. divergens* RBC cultures in order to determine IC_{50} values (Figs. 2 and 3).

2.4. Cell toxicity assay

PMJ2R mouse macrophages (ATCC® CRL2458™, USA) were cultivated in RPMI 1640 medium (Lonza, Switzerland; cat. no. BE12-115F) supplemented with 50 μg/ml gentamicin, 0.25 μg/ml amphotericin B and 10% heat-inactivated fetal calf serum. HeLa human epithelial cells (ATCC CCL-2) were grown in DMEM medium with 10% fetal calf serum. For cytotoxicity assays, compounds were serially diluted, and added to the mammalian cell cultures in 96-well plates. Vehicle alone