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Validation of *Babesia* proteasome as a drug target

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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

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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

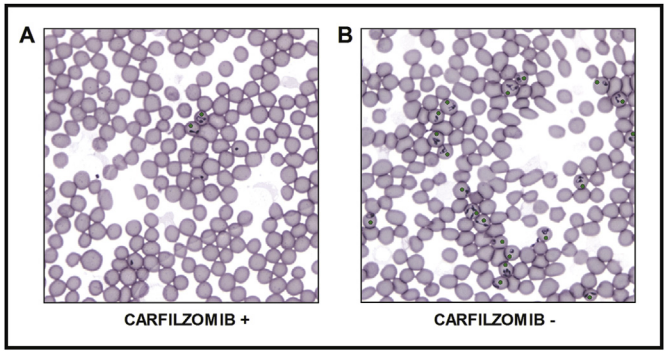

Fig. 1. Treatment of *B. divergens* ex vivo cultures: red blood cells infection. (A) Microscopy image of *B. divergens* ex vivo culture treated with 100 nM carfilzomib. (B) Microscopy image of *B. divergens* ex vivo culture treated with 0.008% DMSO. Smears were stained using DiffQuik staining set. Experimental conditions: starting parasitemia 2%, medium changed in 12 h intervals, total cultivation 48 h. Green dots indicate the parasitized red blood cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was used as a control. After 48 h cultivation (37 °C, 5% CO_2) cell viability was determined. Viability of HeLa cells was determined using AlamarBlue reagent (Invitrogen, USA) and replicate assays were performed on three separate days. PMJ2R cells (triplicated cultures) were stained with the Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (Biotium, USA) following the protocol provided by the manufacturer. Live cells stained by Calcein AM and dead cells stained with ethidium homodimer III were quantified by flow cytometry (BD FACSCanto II, BD Biosciences, USA) in BD FACSDiva software.

2.5. Lysate preparation

B. divergens infected RBCs (~15% parasitemia) were lysed with 0.15% saponin (Sigma-Aldrich, USA) and asynchronous parasites were isolated following extensive washing and centrifugation cycles with PBS. Pelleted parasites were supplemented with 20% glycerol and stored at −80 °C for further analysis. Parasites (~50 µl) were added to 500 µl hypotonic lysis buffer (20 mM HEPES pH 8.0, 1 µM E64, 0.03% SDS and 1 mM ATP; all Sigma-Aldrich, USA) and ruptured by ultrasonication with amplitude 0.5 for 3 × 15s (Ultrasonic processor UP200S, Hielscher, Germany). The resulting crude lysate, originating from the pool of 10 separate *B. divergens* cultures, was used for activity assays.

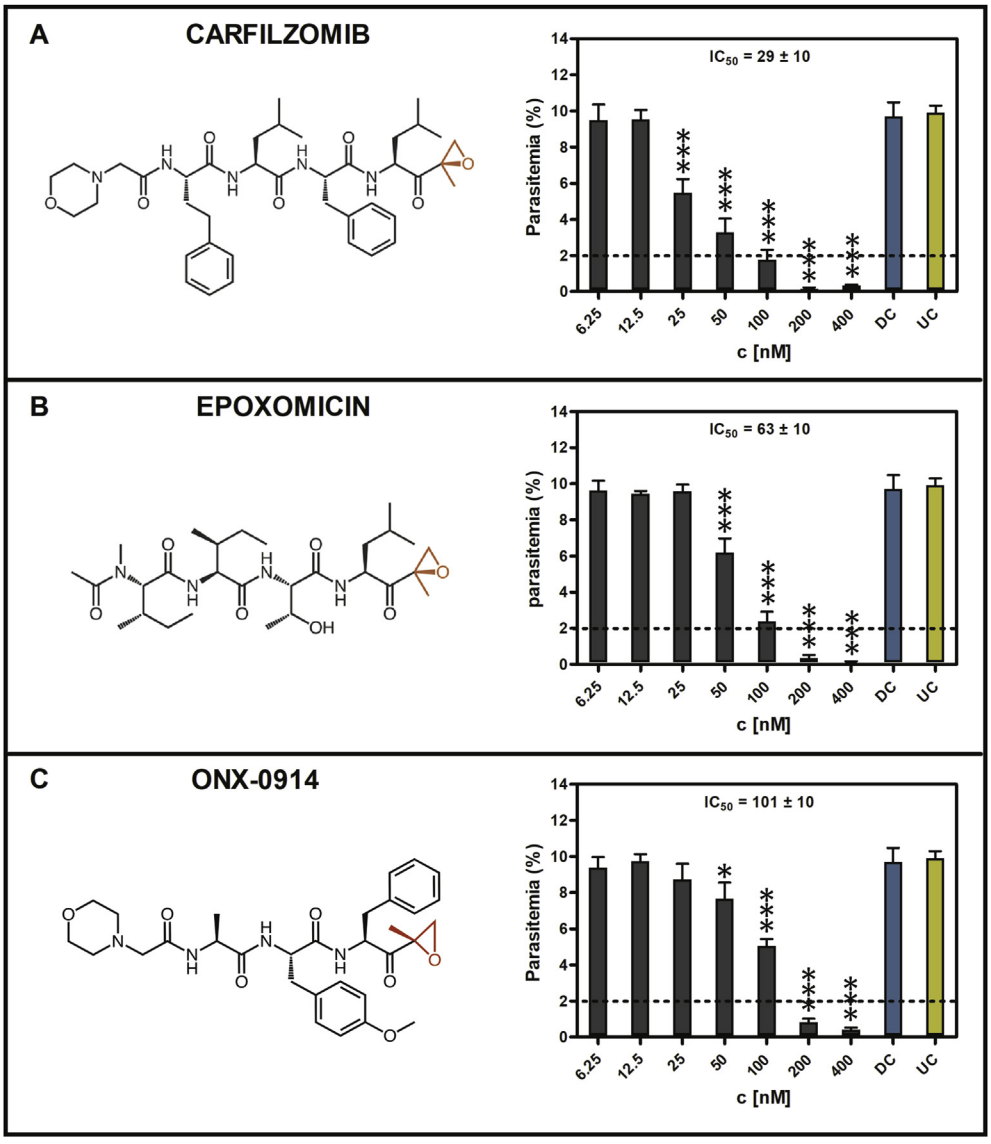

Fig. 2. Treatment of *B. divergens* ex vivo cultures with peptide epoxyketone inhibitors. Structure and IC_{50} determination of (A) carfilzomib, (B) epoxomicin, (C) ONX-0914. Data represent means of three independent biological replicates and the error bars indicate standard deviations. Smears were stained using DiffQuik staining set, parasitemia was counted at 1000 RBCs. Statistical analysis was performed in R using ANOVA (Kolmogorov-Smirnov test and the Bartlett test passed): * = $p < 0.05$, *** = $p < 0.001$ (compared to the DMSO treated culture). Experimental conditions: starting parasitemia 2% (dotted line), medium with inhibitory compounds exchanged in 12 h intervals, total cultivation duration 48 h. DC: DMSO treated culture (0.008%). UC: untreated culture. IC_{50}: half maximal inhibitory concentration. RBCs: red blood cells.

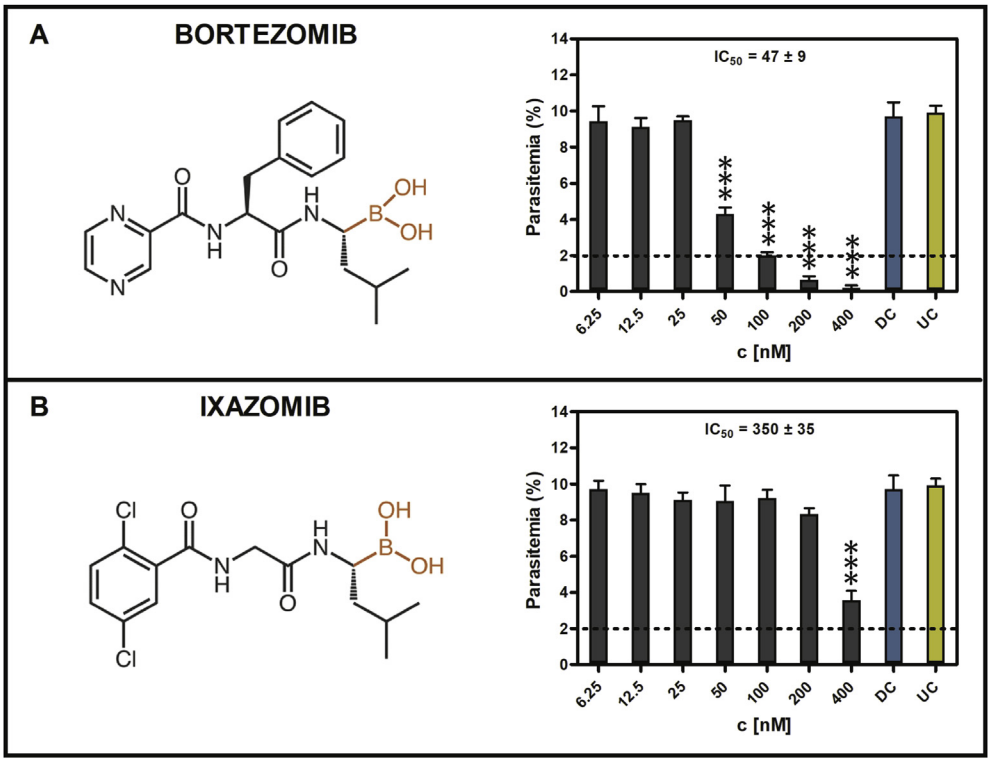

Fig. 3. Treatment of *B. divergens* ex vivo cultures with peptide boronic acid inhibitors. Structure and IC_{50} determination of (A) bortezomib and (B) ixazomib. Results represent means of three independent biological replicates, error bars indicate standard deviations. Smears were stained using DiffQuik staining set, parasitemia was counted at 1000 RBCs. Statistical analysis was performed in R using ANOVA (Kolmogorov Smirnov test and the Bartlett test passed): * = $p < 0.05$, *** = $p < 0.001$ (compared to the DMSO treated culture). Experimental conditions: starting parasitemia 2% (dotted line), medium with inhibitory compounds exchanged in 12 h intervals, total cultivation duration 48 h. DC: DMSO treated culture (0.008%). UC: untreated culture. IC_{50}: half maximal inhibitory concentration. RBCs: red blood cells.

2.6. Activity assays

Activity was measured in assays with the 20S proteasome chymotrypsin-specific substrate, Suc- LLVY-AMC (Bachem, Switzerland). Assays were performed in flat-bottom black 96-well plates (CoStar, USA) using 10 µl of *B. divergens* lysate per well in the reaction mixture of 20 mM HEPES (pH 8), 1 µM E64, 0.03% SDS, 1 mM ATP and 10 µM Suc-LLVY-AMC. Enzyme kinetics was monitored by Infinite M200 PRO reader (Tecan, Austria) as increasing fluorescence intensity at 360/465 nm excitation/emission wavelengths in 1 min intervals for 1 h at 37 °C. For specificity control, the parasite lysates were pre-incubated (30 min, 37 °C) with 1 µM concentration of all compounds in the assay buffer prior the addition of Suc-LLVY-AMC and kinetic assay measurements. To determine IC_{50} values of all tested inhibitors, the compounds were serially diluted and pre-incubated with parasite lysates prior the kinetic assay described above. All experiments were performed in triplicate with a single parasite lysate. Results were confirmed with repeated experiments using independently prepared *B. divergens* lysates.

2.7. Western blot analysis of poly-ubiquitinated proteins

Reducing SDS PAGE and Western blot analyses were performed as described previously (Perner et al., 2016). Briefly, *B. divergens* lysates were analysed by SDS-PAGE using the NuPAGE 4–12% Bis-Tris Protein Gels and visualization by Coomassie Brilliant Blue staining (Thermofisher). For Western blot the unstained SDS PAGE gel loads were electro blotted to a PVDF (polyvinylidene difluoride) membrane using the Trans-Blot Turbo system (BioRad, USA). The membrane was blocked in 3% (w/v) non-fat skimmed milk in 1 × PBS with 0.05% Tween 20 (PBS-T; Sigma-Aldrich, USA). For immunostaining the membrane was first exposed to Anti-Ubiquitin K48 Linkage antibodies (Boston Biochem, USA) diluted by 1:2000 in PBS-T, washed (3 × 5 min) in PBS-T, and subsequently exposed to the goat anti-rabbit IgG-peroxidase secondary antibody (Sigma) diluted by 1: 2000 in PBS-T. After the final wash (4 × 5 min in PBS-T) the membrane was developed using the ClarityWestern ECL substrate (BioRad, USA), visualized in the ChemiDoc MP imager and analysed using Image Lab Software (BioRad, USA).

2.8. Treatment of B. microti infected mice

Six female BALB/c mice (~20 g each) were intra-peritoneally injected with 150 µl of *B. microti* infected blood (50% parasitemia, ~800 × 10^6 of infected red blood cells) obtained from a previously infected mouse. Starting on the first day post infection (DPI), mice were treated daily with either 0.75 mg/kg of carfilzomib in PBS (three mice) or a vehicle control (three mice) by intra-peritoneal injection of 150 µl target or control treatment volume per mouse. Treatment continued for 7 DPI and the health status of the mice was assessed by observation of activities and physical appearances. Parasite load was monitored daily for 15 DPI on thin blood smears as described above; blood for examination was obtained by tail snip.

2.9. Pre-treatment of B. microti infected red blood cells

Three mice with *B. microti* infection (50% parasitemia) were bled out and pooled infected blood was treated either with Carfilzomib (final concentration 400 nM) or with DMSO in appropriate concentration for 1 h at 37 °C and 5% CO_2. Treated blood samples were then intra-peritoneally injected into three experimental and three control BALB/c mice (dose volume 150 µl, 800 × 10^6 of infected red blood cells). Parasitemia was monitored daily for 8 DPI on thin blood smears as described above.

2.10. Activity assay and western blot analysis of poly-ubiquitinated proteins in B. microti infected mice

To evaluate the in vivo on target effect of carfilzomib, eight female BALB/c were intra-peritoneally infected with *B. microti* as described above. Mice were then treated with either 0.75 mg/kg of carfilzomib in PBS (four mice) or a vehicle control (four mice) on 5 and 6 DPI. On 7 DPI, the blood was collected, and the proteasome activity was analysed on crude cell lysates prepared from pooled blood taken from treated/

untreated mice groups by the above described protocol. In addition, Western blot analysis of poly-ubiquitinated proteins was performed with the parasite lysates as described above.

2.11. Statistical analysis and graph design

Statistical analysis of *B. divergens* ex vivo culture treatment was performed in R (version 3.2.2) using ANOVA (normal distribution confirmed by Kolmogorov-Smirnov test, Bartlett test of homogeneity of variances passed). IC_{50} was calculated and all graphs were designed in GraphPad Prism software (version 5). The in vivo application of carfilzomib was analysed in R using AUC (Area Under Curve), C_{max} and T_{max} calculation. AUC was calculated using series of Reiman sums. The AUC, T_{max} and parasitemia values were compared using the two-sample (independent) *t*-test (Kolmogorov-Smirnov test and the Bartlett test passed). Data from cell toxicity and proteasome activity assays were compared using the two-sample (independent) *t*-test (Kolmogorov-Smirnov test and the Bartlett test passed). Mean values (± SD, standard deviation) were counted from the biological triplicates (independent experiments) and used for the graphical representations of the results and their statistical analyses.

3. Results

3.1. Clinically approved proteasome inhibitors reduce B. divergens burden in ex vivo cultures

Carfilzomib is a covalent and irreversible peptide epoxyketone inhibitor of the β5 subunit of the human proteasome and has been approved for clinical treatment of multiple myeloma. *B. divergens* infected RBCs were treated with 6.25–400 nM of carfilzomib for 48 h and parasitemia was quantified on thin blood smears (Fig. 1). Growth of *B. divergens* was significantly inhibited by carfilzomib at 25 nM and above, with an IC_{50} of 29 nM (Fig. 2A). In addition, *B. divergens* ex vivo cultures were treated with two additional peptide epoxyketone inhibitors: epoxomicin, a natural product isolated from actinomycetes, and ONX-0914, a selective immunoproteasome inhibitor. Both inhibitors were effective, but showed slightly lower potency than carfilzomib, with epoxomicin and ONX-0914 having IC_{50} values 63 nM (Figs. 2B) and 100 nM (Fig. 2C), respectively. We next evaluated two covalent reversible peptide boronic acid inhibitors, bortezomib and ixazomib, in *B. divergens* ex vivo cultures. Bortezomib significantly blocked parasite replication at 50 nM concentration with an IC_{50} = 47 nM (Fig. 3A) while ixazomib treatment had no effect on parasite replication at 200 nM and reduced parasitemia by 64% at 400 nM (Fig. 3B).

3.2. Epoxyketone and boronic acid inhibitors reduce the chymotrypsin-like activity of babesial proteasome

We anticipated that the proteasome inhibitors were targeting the β5 catalytic subunits of the *B. divergens* proteasome as they are known to target the β5 subunit of mammalian proteasomes. To confirm this, we prepared crude cellular lysates from *B. divergens* and used a standard β5 proteasome substrate, Suc-LLVY-AMC, to detect proteasome activity. The lysate was pre-treated with 1 μM of E−64 to inhibit cysteine proteases that can hydrolyse the Suc-LLVY-AMC substrate (Giguere and Schnellmann, 2008). Pilot experimets consisting of the addition of 1 μM of epoxyketone proteasome inhibitors carfilzomib, epoxomicin and ONX-0914 to *B. divergens* cultures reduced Suc-LLVY-AMC measured activity by > 90% with carfilzomib treatment completely inhibiting this activity. Similarly, 1 μM of the boronic acid inhibitors – bortezomib and ixazomib – reduced the activity by 96.0% and 93.3%, respectively (data not shown). The IC_{50} values of all five tested proteasome inhibitors obtained from the Suc-LLVY-AMC activity assays with *B. divergens* crude cell lysates (Fig. 4A) correlate with the IC_{50} values of the antiparasitic effects obtained from ex vivo *B. divergens* cultures

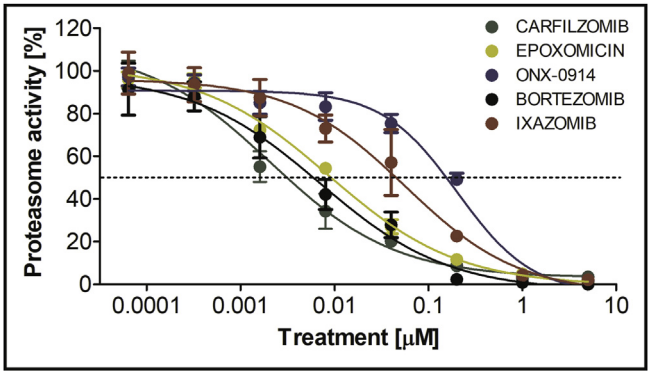

Fig. 4. Detection and inhibition of *B. divergens* proteasome β5 subunit activity. IC_{50} values of all tested proteasome inhibitors on *B. divergens* crude cell lysates. Parasite lysates were obtained from ex vivo cultures and analysed using the fluorogenic peptidyl substrate, Suc-LLVY-AMC. Results represent the means of three independent replicates, error bars indicate standard deviations. IC_{50} values were analysed using GraphPad Prism software (version 5).

Table 1
Summary of IC_{50} values for *B. divergens* and HeLa cells.

Proteasome Inhibitor	IC_{50} values (nM)		
	B. divergens ex vivo cultures	*B. divergens* activity assay	HeLa cells
Carfilzomib	29 ± 10	2 ± 13	59 ± 36
Epoxomicin	63 ± 10	9 ± 5	4 ± 1
ONX-0914	101 ± 10	203 ± 7	350 ± 143
Bortezomib	47 ± 9	7 ± 14	7 ± 1
Ixazomib	350 ± 35	57 ± 14	810 ± 10

(Table 1) for all tested compounds except for ONX-0914. Taken together these results confirmed that the Suc-LLVY-AMC substrate can be used to specifically monitor *B. divergens* proteasome proteolytic activity from crude cell lysates and that the peptide-epoxyketone and peptide-boronic acid inhibitors target the parasite proteasome. In order to further confirm the on-target effect we immunoblotted parasite lysates with antibodies that recognize the K48-linked ubiquitin to demonstrate accumulation of poly-ubiquitinated proteins in *B. divergens* lysates following 12 h treatment of ex vivo cultures with 200 nM carfilzomib (Supplementary Fig. 2). These data indicated that the cultured *B. divergens* were unable to degrade poly-ubiquitinated proteins following treatment with carfilzomib, thereby confirming that the parasite proteasome was indeed inhibited by the drug. In mammalian cells, accumulation of ubiquitinated proteins induces apoptosis and ultimately cellular death (Demo et al., 2007; Khare et al., 2016); therefore exposure of *B. divergens* to carfilzomib may also directly lead to cellular death.

3.3. Carfilzomib is less toxic to immune host cells than to the parasite

With the exception of epoxomicin the proteasome inhibitors evaluated in this study were developed to target the human proteasome and therefore we predicted that they would be more cytotoxic to human cells than to *B. divergens*. To evaluate this, we assessed cytotoxicity of these compounds with the proteasome inhibitor sensitive human cervical cancer cell line, HeLa. Bortezomib and epoxomicin displayed IC_{50} values 7 nM and 4 nM making them significantly more cytotoxic to mammalian cells compared to *B. divergens*, therefore ruling them out for use in our rodent infection studies. Carfilzomib, ONX-0914 and ixazomib were 2–3.5-fold more potent against *B. divergens* compared to HeLa. Out of these three compounds, carfilzomib had the highest potency with IC_{50} of 59 nM and therefore we further investigated

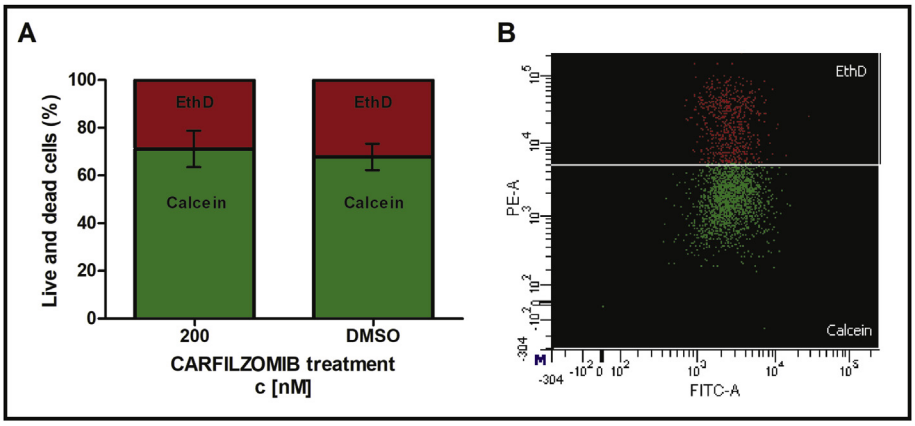

Fig. 5. Cell viability assay: carfilzomib treatment of macrophage cell line. (A) Results represent means of three biological replicates for (i) carfilzomib (200 nM) and (ii) control (medium + 0.004% DMSO) experiments; PMJ2R cells (medium changed every 12 h, total cultivation 48 h). Error bars indicate standard deviation. Statistical analysis was performed in R using unpaired *t*-test (Kolmogorov-Smirnov test and the Bartlett test passed). (B) Dot plot results of flow cytometry analysis of carfilzomib treated cells: dead cells (red color) stained by EthD (measured in PE channel, emission 620 nm) and live cells (green color) stained by Calcein (FITC channel, emission 517 nm). DC: 0.004% DMSO treatment control. EthD: ethidium homodimer. PE: phycoerythrin. FITC: fluorescein isothiocyanate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

carfilzomib cytotoxicity in a non-transformed mouse macrophage cell line PMJ2R. Macrophages treated with 200 nM of carfilzomib, corresponding to 2.5-times the *B. divergens* IC_{50} value, did not show any toxicity. The survival rate of PMJ2R cells treated with carfilzomib (71.1% ± 7.4) did not significantly differ from untreated cells (67.8% ± 5.5) (Fig. 5). These data showed that *B. divergens* is more sensitive to carfilzomib than non-transformed cells allowing us to evaluate this drug in a rodent infection model for *Babesia* parasites.

3.4. Treatment of B. microti infected mice with carfilzomib displays significant effects on the parasite

BALB/c mice intra-peritoneally infected with *B. microti* (Franca) Reichenow (strain Peabody mjr) were treated daily from 1 DPI up to 7 DPI with 0.75 mg/kg of carfilzomib. Growth curves counted as percent parasitemia were generated from thin blood smears obtained by tail snips. Treatment of infected mice with carfilzomib significantly reduced peak parasitemia levels and the area under curve (AUC) compared to PBS alone (Fig. 6A). Carfilzomib treatment had no adverse effects on the spontaneous activity and physical appearance of mice. In addition, treatment did not change the microscopic appearance of non-parasited RBCs (data not shown). *B. microti* parasite cultures pre-treated with 400 nM carfilzomib for 1 h prior to mouse infection displayed a delayed onset of parasitemia in mice (Fig. 6B). In addition, the C_{max} value was reduced by 31.2% on T_{max} (= 7 DPI) when compared to the control group. Longer exposure of *B. microti* infected erythrocytes to carfilzomib prior to mice infection was not possible as the untreated ex vivo parasites were unable to efficiently infect the host when incubated for longer than 1 h.

3.5. Carfilzomib reduces the chymotrypsin-like activity of B. microti proteasome in vivo and results in accumulation of poly-ubiquitinated proteins

Treatment of *B. microti* infected mice with 0.75 mg/kg of carfilzomib only at 5 and 6 DPI (high parasitemia) resulted in decreased parasite load in experimental mice from 47.4 ± 6.3% at 5 DPI to 41.5 ± 2.9% at 7 DPI as compared to control mice where parasitemia gradually increased from 47.1 ± 4.6% to 58.8 ± 4.4%. Therefore, on 7 DPI there was a significant reduction in parasitemia ($p = 0.0006$) upon treatment with carfilzomib. The *B. microti* crude cellular lysate was prepared from mice blood collected at 7 DPI. The Suc-LVVY-AMC activity assays showed a reduction by 33% ($p = 0.0057$) in carfilzomib treated babesial lysates compared to the untreated control (Fig. 6C). Immunoblot comparison of lysates confirmed accumulation of poly-ubiquitinated proteins in *B. microti* originating from carfilzomib treated mice using primary antibodies that recognize the K48-linked ubiquitin (Fig. 6D). In both assays (Fig. 6C and D), equal amounts of parasite proteins were used (visualized by coomassie stained SDS PAGE, Fig. 6D). These results indicate that the in vivo exposure of *B. microti* to carfilzomib results in significant decrease of parasite proteasome activity followed by increased inability to degrade poly-ubiquitinated proteins and, thus, confirms that *B. microti* proteasome is inhibited by carfilzomib in the in vivo infected mouse model.

4. Discussion

In this study, we demonstrated that the proteasome of *Babesia* parasites represents a novel therapeutic target. While genes encoding proteasome subunits have been documented in the EST database of *Babesia bovis* intra-erythrocytic stages (de Vries et al., 2006; Aboulaila et al., 2010), the functional role of this enzyme complex in *Babesia* species has yet not been characterised. Using a selection of peptide boronic acid and peptide epoxyketone inhibitors we have confirmed that the proteasome is enzymatically active in babesial protein extracts and that this activity is important for ex vivo parasite growth. Although babesiosis in humans is mostly asymptomatic the findings reported here are of importance to both veterinary and human medicine especially when antimalarial drugs and antibiotics have been reported to fail due to suspected parasite drug resistance (Vial and Gorenflot, 2006; Vannier et al., 2015).

Our cell-based assays and proteasome activity assays performed with parasite lysates determined that all compounds reduce the chymotrypsin-like activity of babesial proteasome. Comparison of IC_{50} values identified carfilzomib, epoxomicin and bortezomib as the most potent inhibitors however, the latter molecules were significantly more cytotoxic towards the human cell line, HeLa (Supplementary Fig. 3). These cells have been used extensively for evaluating drug toxicity and have been shown to be sensitive to proteasome treatment (Gu et al., 2014). Therefore, we evaluated potency of carfilzomib in a non-transformed macrophage cell line and found that treatment with 200 nM did not affect cell viability. These findings support previous studies with non-transformed human fibroblast cells (HFF) that carfilzomib kills with an IC_{50} of 492 nM (Li et al., 2012). Using this value, carfilzomib is 8.4-fold more selective for *B. divergens* cultures over HFF cells.

Bortezomib is known to inhibit several serine proteases, however, the epoxyketone reactive group of carfilzomib exclusively targets the active site threonine residue of proteasomes (Arastu-Kapur et al., 2011; Kisselev et al., 2012). Carfilzomib, the FDA approved drug for treatment of multiple myeloma in patients not responding to bortezomib (Kuhn et al., 2007) was developed from the scaffold of epoxomicin, a natural product of actinomycetes (Kim and Crews, 2013). Epoxomicin has been previously shown to exhibit anti-babesial activity on intra-erythrocytic multiplication of Babesia species *B. bovis*, *B. bigemina*, *B.*

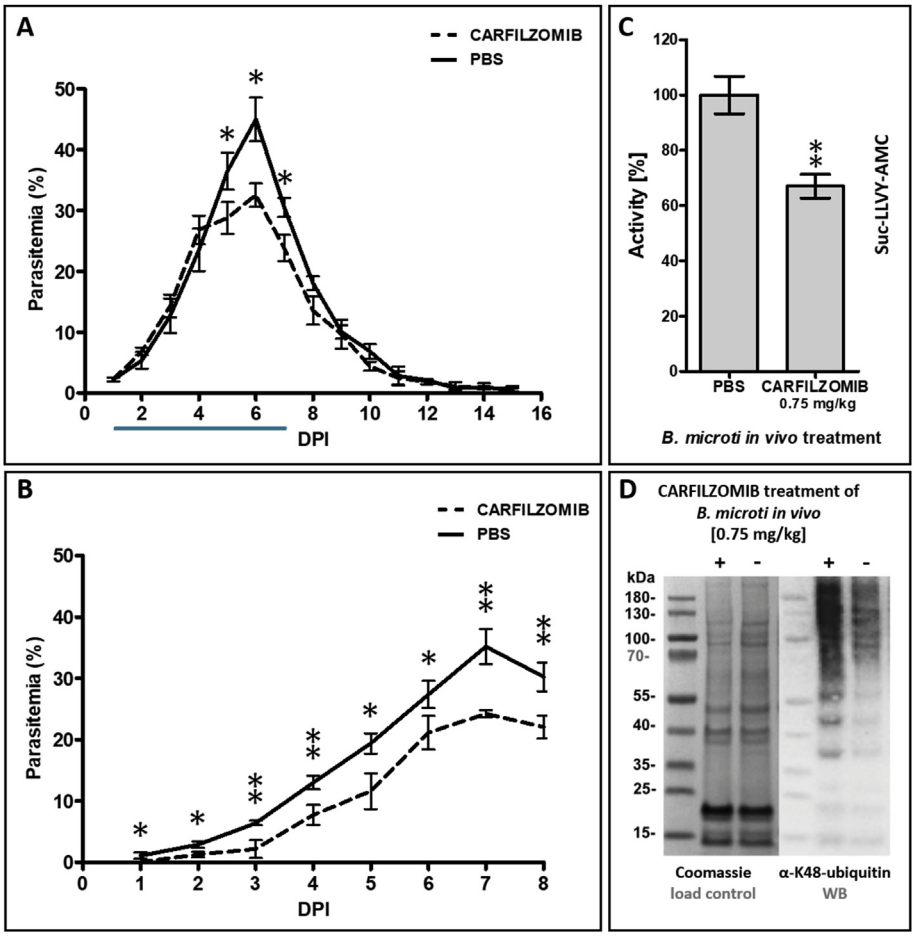

Fig. 6. Treatment of *B. microti* infected mice. (A) The growth curve of *B. microti* in mice treated with either carfilzomib (0.75 mg/kg) or PBS (control). Treatment (intraperitoneal application) was performed daily from 1 DPI up to 7 DPI (blue line in the graph). (B) Growth curve of *B. microti* pre-treated with either 400 nM carfilzomib for 1 h or vehicle control prior to injection into mice. Respective values of AUC (Area Under Curve) and parasitemia values were compared using the two-sample (independent) *t*-test; AUC and parasitemia values differed significantly: * = $p < 0.05$, ** = $p < 0.01$. DPI: days post infection. (C) Inhibition of proteasome chymotrypsin-like activity of *B. microti* isolated from mice on 7 DPI. The infected animals were treated with carfilzomib at 5 and 6 DPI. Activity was detected in crude cell lysates using Suc-LLVY-AMC fluorescent peptidyl substrate using equal amounts of parasite proteins (visualized at coomassie gel, Fig. 6D). Results represent the means of technical triplicates, error bars indicate standard deviations. Respective values were compared using the two sample *t*-test: ** = $p < 0.01$. (D) Detection of poly-ubiquitinated proteins in in vivo inhibited *B. microti* proteasome on 7 DPI in *B. microti* crude cell lysate obtained from mice previously treated on 5 and 6 DPI. Coomassie stained SDS PAGE visualizes separated protein loads of treated and untreated parasite lysates prior electro-blotting to PVDF membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ovata, B. caballi and *B. microti* with 3 day IC_{50} values ranging from 4 nM to 40 nM (Aboulaila et al., 2010). Our studies with *B. divergens* identified carfilzomib as a more potent compound than epoxomicin while also having lower mammalian cytotoxicity. Therefore carfilzomib was selected for in vivo studies with *B. microti* infected mice.

Carfilzomib treatment of *B. divergens* in culture and *B. microti* in mice results in the accumulation of endogenous poly-ubiquitinated proteins within the parasite cell cytoplasm. These results further confirm that carfilzomib targets the parasite cell proteasome and indicate that carfilzomib kills babesial infections by a mechanism relating to proteasome inhibition known to cause cell death in mammals (Kuhn et al., 2007).

B. microti was selected for in vivo studies because the rodent infection model for *B. divergens* has not yet been described. Daily intraperitoneal treatment of *Babesia*-infected mice with carfilzomib resulted in lower parasitemia on 5, 6 and 7 DPI compared to untreated control. Under these conditions we did not observe any differences in animal physical activity and red blood cell microscopic appearance between the treated and untreated animal groups. Activity assays and Western blots with *B. microti* lysates were performed to demonstrate that the babesial proteasome is the target of carfilzomib in infected mice. Animals were twice treated with carfilzomib at the peak of parasitemia on 5 and 6 DPI only and blood was collected at 7 DPI to prepare *B. microti* lysates originating from carfilzomib treated and untreated mice. While in all the four untreated (control) infected mice the individually counted parasitemia persisted or increased during 5, 6 and 7 DPI, in the carfilzomib treated (experimental) mo use group it decreased in three out of four individuals, when the one mouse without the decrease had a significantly reduced (by 22%) initial parasitemia at 5 DPI (Supplementary Table 1). The 33% decrease in Suc-LLVY-AMC activity in *B. microti* lysates from pooled samples of carfilzomib treated mice and the Western blot analysis demonstrating the accumulation of poly-ubiquitinated proteins provide clear in vivo evidence of effective parasite proteasome inhibition by carfilzomib in *B. microti* infected mice.

To evaluate whether carfilzomib treatment completely eliminates the parasite or if it only temporarily blocks its replication, we have performed pre-treatment of *B. microti* infected erythrocytes with carfilzomib prior peritoneal mice infection. Surprisingly, pre-treated parasites were able to infect the host but with an obvious delay and lowered parasitemia. We propose that the delay may be due to the synthesis of new proteasome by the parasite as well as metabolic degradation of carfilzomib in mice as this compound displayed high plasma clearance (195–319 ml/min/kg), a short-terminal half-life (5–20 min), and rapid and wide tissue distribution in rats (Yang et al., 2011). It was previously shown that *Babesia* spp. parasites can grow once the epoxomicin treatment is complete (Aboulaila et al., 2010). This would only happen when parasites are treated with less than 50 nM of drug. Similarly, the low doses of carfilzomib temporarily arrested the growth of *Plasmodium* spp. but higher dosing resulted in parasite death (Li et al., 2012). Our ex vivo data with *B. divergens* erythrocyte cultures display similar dose dependent effects when carfilzomib concentrations ≥100 nM (Fig. 2A) result in parasitemia reduced below the starting point of 2% indicating parasite killing by higher compound concentrations.

While we have validated the *Babesia* proteasome as a target for drug development, the toxicity associated with carfilzomib precludes it for being used for treatment of babesiosis. Carfilzomib was also used to validate the *Plasmodium* proteasome as a drug target (Li et al., 2012) and subsequent studies have shown that compounds with 379-fold

selectivity for the parasite can be developed (LaMonte et al., 2017). Future studies will involve screening of compounds that selectively inhibit the malarial proteasome for their ability to target *Babesia* species. Substrate specificity characterization of the 20S catalytic subunits of *Babesia* proteasome(s) will allow for rational design of peptide inhibitors that are selective for the parasite proteasome over the mammalian host constitutive proteasome and immunoproteasome. Also detailed structure studies using cryoEM (Li et al., 2016a) may uncover differences between the catalytic subunits of the babesial 20S proteasome and the mammalian proteasomes to that can be used to design novel selective inhibitors of the *Babesia* proteasome on the way to determine highly selective therapeutic compounds.

Competing interests

The authors declare that they have no competing interests. The funding agencies played no role in the design or implementation of the study, analysis or interpretation of the data, or the preparation and submission of the manuscript.

Authors' contributions

Conceived and designed the experiments: DS, MJ, AJO, DH. Performed the experiments: MJ, YM, DH. Analysed the data: DS, MJ, LE, DH. Contributed reagents/materials/analysis tools: DS, AJO, LE, OH. Wrote the paper: DS, MJ, LE, AJO. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijpddr.2018.08.001.

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