1 Bumped kinase inhibitor prohibits egression in *Babesia bovis*

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34 Abstract (193 words)

35 Babesiosis is a global zoonotic disease acquired by the bite of a *Babesia*-infected
36 *Ixodes* tick or through blood transfusion with clinical relevance affecting humans and animals.
37 In this study, we evaluated a series of small molecule compounds that have previously been
38 shown to target specific apicomplexan enzymes in *Plasmodium*, *Toxoplasma* and
39 *Cryptosporidium*. The compounds, bumped kinase inhibitors (BKIs), have strong therapeutic
40 potential targeting apicomplexa-specific calcium dependent protein kinases (CDPKs). We
41 investigated if BKIs also show inhibitory activities against piroplasms such as *Babesia*. Using
42 a subset of BKIs that have promising inhibitory activities to *Plasmodium* and *Toxoplasma*, we
43 determined that their actions ranged from 100% and no inhibition against *B. bovis* blood
44 stages. One specific BKI, RM-1-152, showed complete inhibition against *B. bovis* within 48h
45 and was the only BKI that showed noticeable phenotypic changes to the parasites. Focusing
46 our study on this BKI, we further demonstrated that RM-1-152 has *Babesia*-static activity and
47 involves the prohibition of merozoite egress while replication and re-invasion of host cells are
48 unaffected. The distinct, abnormal phenotype induced by RM-1-152 suggests that this BKI can
49 be used to investigate less studied cellular processes such as egression in piroplasm.

50 1. Introduction

51 Emerging and re-emerging infectious diseases cause significant global impact not only
52 to human health (<http://www.cdc.gov/ncidod/eid/vol4no3/fauci.htm>) but also animal health with
53 consequent economic impact. These diseases frequently emerge or expand their range as a
54 result of changes in ecology, climate, and global trade (Jones et al., 2008). Babesiosis, a tick-
55 borne zoonotic disease, is an imminent threat to the U.S. livestock industry and economy.
56 Although eradicated from the continental U.S. (the Commonwealth of Puerto Rico remains
57 endemic) in the 1940s, re-emergence from Mexico, where the disease is endemic, is a major
58 risk especially for bovine babesiosis. This risk is increasing due to increased trade, expansion
59 of wildlife reservoirs for the tick vectors (*Rhipicephalus annulatus* and *R. microplus*), and the
60 development of multi-acaricide resistant ticks in Mexico. Current prophylaxis involves the
61 immunization of naïve cattle with live attenuated vaccine. Due to safety concerns, this
62 preventive care is not registered in the U.S. The administration of imidocarb dipropionate (3.5
63 mgkg⁻¹ intramuscularly) as a chemotherapy for infected animals is available but residual
64 metabolite in musculature and milk renders the treated animal unmarketable.

65 Similarly, human babesiosis is a growing public health concern in the United States
66 (Herwaldt et al., 2011). The incidence of this zoonosis, mostly due to *B. microti* infections has
67 increased significantly over the last decade with eighty percent (80%) of babesiosis cases
68 documented in the U.S. since 1969 were reported between 2000-2009 (Herwaldt et al., 2004).
69 Human babesiosis is especially problematic in the U.S. Northeast corridor (Rodgers and
70 Mather, 2007) although the rise of this zoonotic cases is a global trend (Herwaldt et al., 2004;
71 Kim et al., 2007; Senanayake et al., 2012) attributable to increased blood transfusions, climate
72 changes expanding tick habitats and human encroachment into tick-infested areas (Aguirre

73 and Tabor, 2008). Due to a lack of rapid and sensitive screening protocols for donated blood,
74 transfusion recipients such as children and immunocompromised patients are the primarily
75 affected cohort (Krause et al., 2008; Rodgers and Mather, 2007; Vannier and Krause, 2009).
76 Co-infection of babesiosis and Lyme disease is fairly common, given that both pathogens are
77 spread by the same tick vector, *Ixodes scapularis*. Some reports document 66% of Lyme
78 patients, may also be co-infected with *Babesia* (Krause et al., 1996). Although drug regimen,
79 mostly antibiotic combination therapies, inhibiting *B. microti* exist (Iseki et al., 2008; Salama et
80 al., 2012; Ueno et al., 2013), these chemotherapeutics either require high dosage to be
81 effective or have undesirable side effects (Galay et al., 2011; Munkhjargal et al., 2012; Salama
82 et al., 2012).

83 Advances in kinase biology have described a class of highly-conserved, apicomplexan-
84 specific serine/threonine enzymes known as calcium dependent protein kinases (CDPKs).
85 CDPKs are involved in multiple cellular processes such as host cell invasion, gliding motility
86 and secretion (Lovett et al., 2002; Lovett and Sibley, 2003). Homologues of apicomplexan
87 CDPKs have not been found in animals, hence, they have the potential to be exploited as
88 selective drug targets for apicomplexan parasites. This revelation led to the development of
89 bumped kinase inhibitors (BKIs) that specifically target ATP-binding domains with
90 characteristically small gatekeeper residues as found in *Plasmodium falciparum* CDPK4,
91 *Toxoplasma gondii* CDPK1 and *Cryptosporidium parvum* CDPK1 (Doerig et al., 2005; Johnson
92 et al., 2012; Murphy et al., 2010; Ojo et al., 2010; Ojo et al., 2012). BKIs have low inhibition of
93 host protein kinases, as ATP-binding domains of mammalian kinases typically have larger
94 gatekeeper residues (e.g. human SRC, ABL) that exclude the binding of BKIs (Doerig et al.,

95 2005; Johnson et al., 2012; Murphy et al., 2010; Ojo et al., 2010; Ojo et al., 2012). Therefore,
96 BKIs offer tremendous selectivity for inhibition of apicomplexan CDPKs over human kinases.

97 CDPK orthologues have not been characterized in piroplasms such as *Babesia* and
98 *Theileria* (Brayton et al., 2007; Cornillot et al., 2013; Cornillot et al., 2012; Kappmeyer et al.,
99 2012), although genome sequencing of these pathogens reveal putative CDPKs with
100 sufficiently small gatekeeper residues in the ATP-binding domains for selective BKI-targeting.
101 In fact, we recently identified such a CDPK, specifically a bona fide CDPK4 in *B. microti* and its
102 bovine relative, *B. bovis* (Keyloun et al., 2014). We also determined that additional protein
103 kinases may function as CDPKs or act as BKI-targets from our *in silico* structural analysis
104 using primary protein sequences of *Plasmodium* and *Toxoplasma* CDPKs and screening both
105 *B. microti* and *B. bovis* genomes (Table 1) although CDPK1 orthologues were not identified in
106 both genomes. In order to confirm that BKIs are effective in the inhibition of piroplasms such
107 as *Babesia*, this study (i) evaluates a subset of BKIs that were previously shown to be
108 inhibitory against related apicomplexans and (ii) reports those that are growth inhibitory against
109 *Babesia* asexual erythrocytic stages using *B. bovis* as a model system and (iii) suggests one
110 BKI target may be involved in egression and not in erythrocyte invasion.

111 2. Materials and Methods

112 2.1 Compound Library

113 Bumped kinase inhibitors were synthesized as previously described (Doerig et al.,
114 2005; Johnson et al., 2012; Murphy et al., 2010; Ojo et al., 2010; Ojo et al., 2012). Purity of
115 all compounds (>98%) was confirmed by reverse-phase HPLC and ¹H-NMR before
116 dissolution in 100% DMSO. At a final stock concentration of 20 mM, aliquots were stored at -
117 20°C.

118 2.2 *In vitro* cultivation of *B. bovis* T2Bo virulent strain

119 Parasites were grown and expanded in long-term microaerophilous stationery phase
120 conditions as previously described (Goff and Yunker, 1986; Levy and Ristic, 1980).

121 2.3 Evaluation of *Babesia* growth inhibition of BKIs

122 Using the *B. bovis* asexual erythrocytic stage expansion culture, parasites were
123 seeded in flat bottom 96-well tissue culture plates. Each well contained 150 µl of culture at
124 10% hematocrit with a starting percent parasitized erythrocytes (PPE) of 0.5 as determined by
125 microscopic counting of smears stained with Diff-Quick® (Dade Behring). Drug compound
126 stock solutions were provided at 20 mM in 100% DMSO and aliquots were diluted daily to 30
127 µM with HL-1 culture media (BioWhittaker®). Controls included no treatment (NT) and
128 imidocarb dipropionate (ID) (Imizol® Schering-Plough Animal Health) which is a well
129 established *Babesia*-cidal compound. Final DMSO concentration was 0.15% for all
130 conditions. Eighty percent of drug containing media was replaced at 24h intervals. PPE was
131 enumerated over a 72h period by manual counting of 2 µl culture smears stained with Diff-
132 Quick® using a Zeiss Axioskop microscope fitted with a 100x oil-immersion phase contrast

133 objective. Initial drug screening to determine which BKIs were inhibitory against *B. bovis* was
134 performed in a single assay. Percent parasitemia was measured to evaluate the proportion of
135 morphological abnormal parasites to normal ones in both 24h and 48h treated cultures.
136 Compounds that exhibited significant parasite growth retardation when assayed at 30 μ M
137 were further tested at 0.3 μ M-12.0 μ M in the same manner as described above in triplicates.

138 *2.4 Determination of parasite-static verses -cidal effect of RM-1-152*

139 RM-1-152 at 30 μ M was prepared as described above. *B. bovis* asexual erythrocytic
140 stage culture at 2.5 PPE and 10% hematocrit was used for all conditions. RM-1-152
141 evaluations were conducted in quadruplicates. ID, clindamycin hydrochloride (CL) (Sigma,
142 St. Louis, MO), both at 20 μ M were used as *Babesia*-cidal and -static controls, respectively
143 (Arai et al., 1998; Brasseur et al., 1998; Rashid et al., 2008) while NT was included as the
144 negative control. Culture media were changed daily and 50 μ l was collected from each
145 condition for real time PCR analysis at designated time points described below. Cultures in
146 the presence of drugs were exposed $\leq$ 48h followed by 3x washing with HL-1 medium to
147 remove all drug traces. The cultures were then incubated in normal medium for the remaining
148 of the study. The experiment was terminated after 144h upon which the cultures were treated
149 with Trizol for RNA (Invitrogen, Grand Island, NY) and cDNA preparation made with the
150 RETRO script Reverse Transcription kit (Ambion, Grand Island, NY). Fifty μ l of culture was
151 collected at time 0h and the same volume was collected at the termination of the experiment
152 (i.e. 144h). cDNA templates were amplified in quadruplicate using iSYBR Green Supermix
153 (Bio-Rad, Hercules, CA) according to manufacturer protocol on a Bio-Rad CFX96 instrument.
154 The cycling conditions were as follows, 95°C for 3 min, followed by 40 cycles of 95°C for 10 s,
155 55°C for 10 s, and 72°C for 30 s. Melt curve analysis was done using 95°C for 10 s, 65°C to

156 95°C with 0.5°C increment of 5s/step. Bio-Rad CFX Manager 3.1 software was used for data
157 analysis. Two genes were selected to determine if RM-1-152 effect was *Babesia*-cidal or -
158 static. These were topoisomerase II (BBOV_II004820) and 40S ribosomal protein S3
159 encoding gene (BBOV_II001650). Both genes are single copy genes within the genome and
160 are highly expressed during the asexual erythrocytic stages as determined previously from
161 our microarray analysis (Pedroni et al., 2013). Gene specific primers were designed
162 (MacVector v.12.0) to amplify product sizes of 100-200 bp. Because topoisomerase II gene
163 has no intron, primers designed for 40S ribosomal protein S3 encoding gene span the intronic
164 region to rule out gDNA contamination (Table S1).

165 2.5 Determination of RM-1-152 effect influence on re-invasion

166 *B. bovis* culture was also used to investigate if RM-1-152 inhibits *Babesia* reinvasion.
167 Two groups of culture were set up where group one consisted of 30 µM RM-1-152 treatment
168 for ≤ 48h while group two was the NT control. This time point was chosen as the culture
169 begins to die off approximately 48h under continuous RM-1-152 treatment (Figure 1).
170 Infected erythrocytes were lysed, washed 3x with HL-1 media and centrifuged at 1,800 rpm
171 for 5 min at room temperature to pellet the freed merozoites. These merozoites were divided
172 into two aliquots and used to re-invade fresh erythrocytes in the presence or absence of 30
173 µM RM-1-152 for a total of 72h. NT-free merozoites in the presence and absence of RM-1-
174 152 were included as controls. The experiment was conducted in triplicates in the same
175 culture condition as described in section 2.4. Blood smears were stained with Diff-Quick®
176 and using a Zeiss Axioskop microscope fitted with a 100x oil-immersion phase contrast
177 objective, PPE was enumerated and morphological changes were recorded.

178 2.6 Statistical analysis

179 Mean and standard error means (SEM) of PPE under the influence of the BKIs based
180 on replicates and non-linear regression curve were calculated using PRISM v. 5.0a.

3. Results and Discussion

To determine whether BKIs inhibit asexual erythrocytic *Babesia* growth, 18 BKIs were chosen based either on successful inhibitory effect against related apicomplexans (Larson et al., 2012) or because they were closely related to the active compounds (Figure S1, Table 2). Our results showed that constant exposure of the parasites to these BKIs at 30 μ M demonstrated a diversity of action. Figure 1A illustrates six BKIs that had intermediate or no inhibitory activities on *B. bovis* growth while the remaining 12 had complete inhibitory action as early as 24h post-incubation (Figure 1B). A plausible explanation as to why only 66% (n=12) of the tested BKIs were effective against *Babesia* sp. is because these inhibitors were originally designed to bind *T. gondii* CDPK1, which contains a hydrophobic pocket with a glycine gatekeeper residue. *B. bovis* does not appear to contain a bona fide CDPK1 orthologue and that all *Babesia* CDPKs that we identified do not have such an atypically small gatekeeper (Table 1). Presumably, the “bumped” chemical group of BKIs exhibits steric clash with the larger gatekeeper residues, disfavoring binding. The seven putative kinases identified in *B. microti* and *B. bovis* genomes with conserved ATP-binding domains do contain “relatively” small gatekeeper residues which suggest that the inhibitory action of the 12 BKIs that was observed may be target specific after all. Furthermore, we cannot rule out that additional regions on the *Babesia* CDPKs may also influence the effectiveness of the BKIs’ overall action and that these regions differ from those in *Plasmodium*, *Toxoplasma* and *Cryptosporidium*. Studies have shown that some BKIs affect host cell invasion by binding to specific CDPKs such as CDPK1 (Johnson et al., 2012). With that said, although these BKIs may have different *B. bovis* targets as in other apicomplexans, the final outcome/readout is the same.

204 Effective concentration (EC)₅₀ values of all 20 BKIs were evaluated as shown in Table 2
205 where compound 1291 and 1354 showed the highest and lowest EC₅₀ values at 26 μM and 3
206 μM, respectively. The remaining 10 BKIs have similar EC₅₀ values that range from 5.0 μM
207 such as RM-1-132 to 11.2 μM as in RM-1-152. Many of the tested BKIs were previously
208 evaluated with favorable results for specificity against human kinases such as SRC whose
209 minimum inhibitory concentrations are also represented in Table 2. Certain BKIs, namely, RM-
210 1-132, 1318, and 1294 are designed to be exquisitely selective which means they are likely to
211 interact with a single kinase in *B. bovis*.

212 Bumped kinase inhibitors have previously been associated with phenotypic inhibition of
213 invasion and egression of parasites from mammalian host cells (Doerig et al., 2005; Johnson
214 et al., 2012; Murphy et al., 2010; Ojo et al., 2010; Ojo et al., 2012). High resolution
215 microscopic analysis of all drug-treated cultures revealed only one anti-babesial BKI, RM-1-
216 152, that showed phenotypic effects (merozoite entrapment and abnormal morphology) that
217 suggest interference with parasite egression. Under normal conditions, *B. bovis* merozoites
218 invade erythrocytes, each developing into a single trophozoite per cell. The trophozoite
219 replicates by binary fission and results in two, tear-drop shaped merozoites (Figure 2A) that
220 subsequently egress independently to re-invade uninfected erythrocytes. However, under the
221 constant influence of 30 μM RM-1-152, *Babesia* replicated within host cells but failed to
222 egress. These trapped merozoites remained within the infected cell and cumulated in
223 numbers over 24h (Figure 2C-F). Trapped merozoites within infected erythrocytes also
224 appeared round/oval instead of the typical tear-dropped shape. The maximum and minimum
225 numbers of RM-1-152 treated-round merozoites within a single host cell were 16 and three,
226 respectively. Percent parasitemia of morphologically abnormal *B. bovis* under RM-1-152

227 treatment for 24h and 48h were approximately 56% and 30%, respectively. The decrease of
228 percent abnormal parasites over time was unexpected. One possible explanation is that there
229 was an overall parasitemia decline over 24h due to RM-1-152's inhibitory effect and the
230 parasites that were already affected by the treatment simply died while those that escaped
231 from the drug treatment continued to replicate asexually. Nonetheless, presence of RM-1-152
232 remains lethal by 72h.

233 Due to the fact that RM-1-152 was the only BKI where we captured a phenotypic
234 change in the parasite's appearance, we focused our study by determining the inhibitory action
235 of this BKI. In order to determine if RM-1-152 causes irreversible consequences to *B. bovis*
236 growth, two assays were carried out. In the first assay, *B. bovis* cultures were either left
237 untreated (NT control) or exposed to RM-1-152 $\leq$ 48 h followed by merozoite isolation for re-
238 invasion ability assessment. Using equal quantity of free merozoites in both groups confirmed
239 via light microscopy, merozoites that were previously exposed to RM-1-152 reinvaded
240 uninfected erythrocytes regardless of the presence (abnormal numbers of merozoites) (Figure
241 3A) or absence (normal number of merozoites) (Figure 3B) of RM-1-152 in the media.
242 However, once these merozoites became intracellular, those that were re-exposed to RM-1-
243 152 in the media developed abnormal morphology (Figure 3C) but the previously drug-
244 exposed merozoites recovered in the absence of RM-1-152 (Figure 3D). Similarly, free
245 merozoites from the NT group (Figure 3B) reinvaded fresh erythrocytes in the presence and
246 absence of RM-1-152. However, these intracellular merozoites when re-exposed to RM-1-
247 152, developed abnormal morphology (Figure 3E) while those that were allowed to propagate
248 free of RM-1-152, showed normal morphology (Figure 3F). These results demonstrate that
249 RM-1-152 does not prohibit host cell invasion by the merozoites that were previously exposed

250 to the compound but that it interferes with egression alone. One plausible explanation for this
251 observation is that different enzymes, such as CDPKs, are responsible for the two independent
252 processes where RM-1-152 is target-specific to the kinase involved in egress. The fact that
253 after NT-merozoites exposure to RM-1-152 became intracellular but failed to egress, further
254 strengthens the suspicion that RM-1-152 targets egress-specific CDPKs.

255 In the second assay, qRT-PCR was employed to confirm that RM-1-152 treated
256 *Babesia* remains viable because RM-1-152 is a *Babesia*-static inhibitor. Clindamycin
257 hydrochloride was used as a *Babesia*-static inhibitor (Arai et al., 1998) where ID, was used as
258 the *Babesia*-cidal inhibitor controls. The detection of topoisomerase II (Figure 3G) and 40s
259 ribosomal (Figure 3H) protein transcripts confirmed that the parasites were viable upon
260 removal of the compound. Furthermore, *B. bovis* in the NT, CL and BKI groups were
261 successful in invasion, replication and egression of the erythrocytes, which were in contrast to
262 the ID group. Among the NT, CL and BKI groups, slight statistically significant higher
263 parasitemia was observed in the NT group with lowest cycle titration (CT) values than CL and
264 BKI groups while BKI and CL groups had the same level of parasitemia. It is possible that CL
265 and BKI treatment groups resulted in permanent damage to the parasites or that the parasites
266 required more than 144h to recover from the static effect of both inhibitors. As expected,
267 parasites previously treated with ID failed to recover (Figure 3G, H).

268 In summary, this study reports anti-*Babesia* activity of BKIs. Among the 12 BKIs tested
269 to be inhibitory, RM-1-152's action is correlated with the inhibition of *B. bovis* egress process
270 but not invasion of the host cell. Under continuous exposure of the BKIs, *B. bovis* replicated
271 but the culture eventually died. This is most likely attributed to factors such as diminishing
272 supply of host nutrients, as trapped parasites remained replicative prior to 48h. Earlier

273 observations suggest that each CDPK has defined regulatory functions at distinct stages of the
274 apicomplexan life cycle. Expression levels and specific functions of CDPKs in each organism
275 may vary based on the specific life cycle requirements (Keyloun, et al., 2014), and that RM-1-
276 152 inhibits a different BKI than the other BKIs. It is also concluded that the inhibitory action of
277 RM-1-152 is *Babesia*-static, consistent with previously described effects of BKIs (Doerig et al.,
278 2005; Johnson et al., 2012; Murphy et al., 2010; Ojo et al., 2010; Ojo et al., 2012). With that
279 said, our recent study evaluating the action of these BKIs in *Theileria equi* suggests that these
280 compounds are broadly inhibitory against members of the apicomplexan phylum which is
281 potentially good news as these may be possible treatment option for human babesiosis caused
282 by *B./T. microti* (Cornillot et al., 2012; Hines et al., 2015). Future efforts will be directed
283 towards the identification of BKI targets, refinement of BKI structural designs for optimizing
284 target specificity and host toxicity, and the investigation of potential resistance to BKI treatment
285 using *B. bovis* as the *in vitro* model system. Future experiments also include *in vivo* evaluation
286 of these BKIs' action on *B. microti* as *in vitro* culture system is not feasible for this
287 *Babesia/Theileria* (Cornillot et al., 2012). Last but not least, host cell egression mechanism is
288 a knowledge gap in the piroplasm field. With the help of RM-1-152, characterization of such
289 an essential and potential drug-target pathway can finally be initiated.

290

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425 Figure legends

426

427 Figure 1. Effect of bumped kinase inhibitors (BKIs) at 30 μ M on *Babesia* asexual erythrocytic
428 stages over 72h. (A) BKIs with partial to no inhibition and (B) BKIs with inhibitory effect as
429 early as 24h post-incubation. NT, no treatment control; ID, imidocarb dipropionate positive
430 control; PE, parasitized erythrocytes.

431 Figure 2. Visualization of *Babesia bovis* under the influence of 30 μ M RM-1-152 after 24h. (A)
432 No treatment control, showing twin tear-drop shaped merozoites occupying a single
433 erythrocyte. (B-C) Five circular merozoite-like parasites within a single erythrocyte and (D-E)
434 infected erythrocytes harboring >5 circular merozoites per cell under continuous presence of
435 RM-1-152.

436 Figure 3. *Babesia bovis* successfully re-invaded uninfected erythrocytes upon the removal of
437 bumped kinase inhibitor's effect. Merozoites from the RM-1-152-treated (A) and no treatment
438 (NT) (B) groups were freed by erythrocyte lysis. (C) and (D) correspond to merozoites-infected
439 erythrocytes previously exposed to RM-1-152 in the presence (C) and absence (D) of 30 μ M
440 RM-1-152 at 72h, respectively while (E) and (F) correspond to NT-merozoites reinvaded
441 erythrocytes in the presence and absence of 30 μ M RM-1-152 at 72h, respectively.
442 Comparison of *B. bovis*-infected erythrocytes using quantitative RT-PCR amplifying (G)
443 BBOV_I1004820 and (H) BBOV_I1001650 transcripts in four treatment groups at 144h. No
444 treatment, NT control; BKI, RM-1-152 at 30 μ M; CL, 20 μ M clindamycin hydrochloride and ID,
445 20 μ M imidocarb dipropionate. *, statistically significant with $p < .05$.

446 Supplementary materials

447 Figure S1. Structures of the six bumped kinase inhibitors that had no effect on *Babesia bovis*
448 asexual erythrocytic stage growth.

449 Table S1. Specifications of the two pairs of gene specific primers used for quantitative real
450 time RT-PCR.

1 Table 1. Putative bumped kinase inhibitor targets in *Babesia microti* and orthologous proteins
2 in *B. bovis* based on conserved ATP-binding domain. Gatekeeper residue and putative
3 function for each protein is also shown.

<i>B. microti</i>	gatekeeper	annotation	<i>B. bovis</i>	gatekeeper	annotation
CCF74481	Thr131	CDPK-like	BBOV_IV003210	Thr141	CDPK4
CCF74596	Val183	CDPK-like	BBOV_II007640	Met219	CDPK, putative
CCF75526	Thr87	AMP-dependent PK	BBOV_III009120	Met119	PK containing domain
CCF75730	Thr133	cAMP-dependent PK	BBOV_III005470	Met374	PK containing domain
CCF75355	Thr623	cGMP-dependent PK	BBOV_I004690	Thr654	cGMP-dependent PK
CCF75428	Ala163	Ser/Thr kinase	BBOV_III007550	Thr155	PK containing domain
CCF75969	Met131	Ser/Thr kinase	BBOV_II000760	Ser460	PK containing domain

4 PK, protein kinase; Thr, threonine; Ser, serine; Val, valine; Met, methionine; Ala, alanine

5

Table 2. Effective concentration (EC)₅₀ values, structures and Src cytotoxicity values of bumped kinase inhibitors that were inhibitory to *Babesia bovis* blood stage grown over 72h.

BKIs	1244	1263	1264	1282	1291	1292	1294	1308	1318	1354	RM-1-132	RM-1-152
Structures
EC ₅₀ (μM)	8.0	3.0	9.0	5.9	26.0	9.9	7.4	5.5	11.0	2.2	5.0	11.2
Src Cytotoxicity (μM)	NA	NA	>10	>5.02	>10	>5.58	>10	0.73	>10	0.08	>20	>10

NA, not available

Figure 1. Effect of bumped kinase inhibitors (BKIs) at 30 μ M on *Babesia* asexual erythrocytic stages over 72h. (A) BKIs with complete, partial or no inhibition and (B) BKIs with complete inhibitory effect as early as 24h post-incubation. NT, no treatment control; ID, imidocarb propionate positive control; PE, parasitized erythrocytes.

Figure 2. Visualization of *Babesia bovis* under the influence of 30 μ M RM-1-152 after 24h. (A) No treatment control, showing twin tear-drop shaped merozoites occupying a single erythrocyte. (B-C) Five circular merozoite-like parasites within a single erythrocyte and (D-E) infected erythrocytes harboring >5 circular merozoites per cell, under continuous presence of RM-1-152.

Figure 3. *Babesia bovis* successfully re-invaded uninfected erythrocytes upon the removal of bumped kinase inhibitor's effect. Merozoites from the RM-1-152-treated (A) and no treatment (NT) (B) groups were freed by erythrocyte lysis. (C) and (D) correspond to merozoites-infected erythrocytes previously exposed to RM-1-152 in the presence (C) and absence (D) of 30 μ M RM-1-152 at 72h, respectively while (E) and (F) correspond to NT-merozoites reinvaded erythrocytes in the presence and absence of 30 μ M RM-1-152 at 72 h, respectively. Comparison of *B. bovis*-infected erythrocytes using quantitative RT-PCR amplifying (G) BBOV_II004820 and (H) BBOV_II001650 transcripts in four treatment groups at 144h. No treatment, NT control; BKI, RM-1-152 at 30 μ M; CL, 20 μ M clindamycin hydrochloride and ID, 20 μ M imidocarb dipropionate. *, statistically significant with $p < .05$.

Graphical Abstract (for review)

No treatment

BKI treated