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***In vitro* growth inhibition of *Theileria equi* by bumped kinase inhibitors**

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Abstract

Introduction—*Theileria equi*, an etiologic agent of equine piroplasmiasis, is a tick-transmitted hemoprotozoan of the phylum Apicomplexa. Recent outbreaks of piroplasmiasis in the United States have renewed interest in safe and effective treatment options. Although imidocarb dipropionate (IMD) is the drug of choice for clearance of *T. equi*, adverse reactions and recently documented resistance support the need for alternative therapeutic strategies. The recently described bumped kinase inhibitors (BKIs) are a new class of compounds that could potentially be used as safe and effective alternatives to IMD. In an initial effort to evaluate this potential, herein we determined the *T. equi* growth inhibitory activity of 11 BKIs relative to that of IMD and the previously tested BKI 1294. Because some BKIs have known human ether-à-go-go related gene (hERG) channel activity, we also assessed the hERG activity of each compound with the goal to identify those with the highest potency against *T. equi* coupled with the lowest potential for cardiotoxicity.

Results—Six BKIs inhibited *T. equi* growth *in vitro*, including the previously evaluated BKI 1294 which was used as a positive control. All six compounds were significantly less potent (higher 50% effective concentration (EC₅₀)) than IMD. Two of those compounds were more potent than BKI 1294 control but had similar hERG activity. Although the remaining three

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compounds had similar to lower potency than BKI 1294, hERG EC₅₀ was higher for three of them (BKI 1735, BKI 1369 and BKI 1318).

Conclusions—The BKI compounds evaluated in this study inhibited *T. equi* *in vitro* and had diverse hERG activity. Based on these considerations, three compounds would be suitable for further evaluation. While these results provide a foundation for future work, *in vivo* pharmacokinetic, pharmacodynamics, and safety studies are needed before BKI compounds can be recommended for clinical use in *T. equi* infected horses.

Keywords

bumped kinase inhibitor; apicomplexan; piroplasmosis; *Theileria equi*

Introduction

Theileria equi is a tick-transmitted hemoprotozoan of the phylum Apicomplexa and an etiologic agent of equine piroplasmosis (Mehlhorn and Schein, 1998). Horses infected with this parasite can have clinical or subclinical presentations and remain persistently infected (Holbrook, 1969; Ribeiro et al., 2013; Ueti et al., 2005). Although *T. equi* is not endemic in the United States, recent piroplasmosis outbreaks and the introduction of horses from endemic areas, have prompted a renewed interest in effective and safe treatment options (Short et al., 2012; Ueti et al., 2012).

The most commonly used drug to successfully treat equine piroplasmosis is imidocarb dipropionate (IMD)¹ (Grause et al., 2013; Ueti et al., 2012; Wise et al., 2013). However, adverse effects of IMD at therapeutic doses in horses include spasmodic colic and diarrhea due to the anticholinesterase activity of the drug (Tecles and Cerón, 2001). Other adverse effects include injection site reactions, hepatotoxicity, and renal toxicity (Adams, 1981; Lai et al., 2002; Meyer et al., 2005; Wang et al., 2009). In addition, variation in treatment response has been observed in natural *T. equi* infection, with treatment failure characterized by parasite persistence following treatment (Hines et al., 2015).

We recently reported that bumped kinase inhibitor (BKI)² 1294 inhibits *T. equi* growth *in vitro* (Hines et al., 2015). The BKIs are a group of compounds that are effective against some apicomplexan parasites both *in vitro* and *in vivo* (Castellanos-Gonzalez et al., 2013; Doerig et al., 2005; Johnson et al., 2012; Murphy et al., 2010; Ojo et al., 2014a; Ojo et al., 2010; Ojo et al., 2012; Ojo et al., 2014b). These compounds are an orthogonal set of ATP-competitive inhibitors that target calcium dependent protein kinases (CDPK)³ present in apicomplexans but absent in vertebrates (Larson et al., 2012). Selectivity is based on a small “gatekeeper” residue present in the CDPK of apicomplexan species (Doerig and Billker, 2010; Keyloun et al., 2014). Whereas most vertebrate protein kinases have a large gatekeeper that prevents inhibitors from accessing an hydrophobic pocket within the ATP binding site, the small gatekeeper of apicomplexan CDPK (threonine in *T. equi*) exposes the

¹IMD: Imidocarb dipropionate

²BKI: Bumped kinase inhibitor

³CDPK: Calcium dependent protein kinases

ATP-binding site and makes these parasites uniquely susceptible to BKI (Hines et al., 2015; Hui et al., 2015). Since apicomplexan parasites rely on calcium-mediated signaling for a variety of vital functions, and because calcium uptake is partially controlled by the function of CDPK, blocking these specialized systems results in parasite death (Nagamune et al., 2008). Therefore, BKIs offer selectivity for inhibition of apicomplexan CDPKs over mammalian kinases, and could represent effective alternatives for treating *T. equi*-infected horses.

Although BKIs have demonstrated safety in rodents and other species, off-target effects may be a concern with some BKI compounds in some species. In particular, the human ether-à-go-go related gene (hERG)⁴-encoded ion channel is inhibited by some BKIs (Castellanos-Gonzalez et al., 2013; Ojo et al., 2014a). The hERG channel conducts K⁺ out of cardiomyocytes, which is critical for membrane repolarization during the production of a cardiac action potential (Gutman et al., 2005). In humans, the pharmacological inhibition of hERG results in long QT syndrome (LQTS)⁵, which can lead to cardiac arrhythmia and predisposition to sudden death (Curran et al., 1995; Sanguinetti and Tristani-Firouzi, 2006). Although LQTS has not been specifically described in horses, sudden death is a well-recognized problem in horses and in some cases may be due to cardiac arrhythmias (Kiryu et al., 1999; Lyle et al., 2011). Interestingly, the hERG nucleotide and encoded amino acid sequences are 93% and 99% homologous to the equine ERG orthologue and inhibition of the equine ERG channel results in a significant increase in the cardiac refractory period in equine ventricular muscle (Pedersen et al., 2015). Thus, hERG activity (and by extension equine ERG activity) should be considered a potential cardiac liability for drugs intended for use in horses.

The purpose of this study was to determine and compare the *in vitro* effectiveness and potency of 12 BKI compounds (including BKI 1294) against *T. equi*. In addition, given the genetic and functional homology between human and equine ERG, we report the hERG activity for all BKIs tested.

Methods

A 72 hr *in vitro* parasite growth inhibition assay was performed as previously described (Hines et al., 2015) using the USDA Florida strain of *T. equi* (FL) (Kappmeyer et al., 2012). In brief, individual wells of 96 well plates contained 120 µl of equine erythrocytes in culture medium, with a starting percent parasitized erythrocytes (PPE)⁶ of approximately 0.3%. Growth inhibition was evaluated for IMD (Imizol® Merck Animal Health), and BKIs 1294, RM-1-132, RM-1-152, 1369, 1318 (Johnson et al., 2012), 1547 (Vidadala et al., 2016) 1649 (unpublished data, Hulverson et al., 2017), 1735 (pyrazolo [2,3-*d*] pyrimidine (PP) compounds), 1643, 1571, 1597, and 1586 (Huang et al, 2015) (Table 1). Each BKI was synthesized as described as was over 95% pure by HPLC and NMR determination (Huang et al., 2015; Vidadala et al., 2016) (unpublished data, Hulverson et al, 2017). Synthesis of 1735: ¹H NMR (500 MHz, CD₃OD) δ 8.30 (s, 1H), 8.12 (s, 1H), 7.98 (d, *J*=9.5 Hz, 1H),

⁴hERG: human ether-à-go-go related gene

⁵LQTS: Long QT syndrome

⁶PPE: Percent parasitized erythrocytes

7.90 (d, $J=9.5$ Hz, 1H), 7.88 (d, $J=9.1$ Hz, 1H), 7.34 (s, 1H), 7.24 (d, $J=9.1$ Hz, 1H), 4.70 (s, 2H), 4.60-4.40 (m, 2H), 4.20 (q, $J=6.0$ Hz, 2H), 4.00-3.98 (m, 2H), 3.0 (s, 3H), 1.51 (t, $J=6.6$ Hz, 3H); MS (ESI) 404.4 m/z [MH⁺], C₂₂H₂₄N₆O₂ requires 404.2. HPLC >98%; synthesis methods for 1735 are provided in supporting information.

BKI stock solutions were provided at 20 mM in 100% DMSO and diluted in culture media HL2A-FBS to 30 μ M, 12 μ M, 9 μ M, 6 μ M, 3 μ M, and 0.3 μ M (Hines et al., 2015). For IMD, two-fold serial dilutions ranged from 2.7 nM to 344 nM. Each concentration was evaluated in triplicate two times. Controls included infected red blood cells treated with DMSO (iRBC DMSO⁷), non-infected red blood cells treated with plain media (nRBC⁸), and infected red blood cells treated with IMD or BKI 1294. After 72 hrs, all samples were stained with hydroethidine and parasite growth was evaluated using flow cytometry as previously described (Hines et al., 2015). The PPE of each well was calculated based on the percentage of RBC exhibiting FL-2 fluorescence. Fluorescent profiles were recorded for analysis with FCS Express software (De Novo software, Los Angeles, CA). Quadrant gates of generated dot plots (FL-2 vs. side scatter) were set based on stained nRBC controls. The 50% effective concentrations (EC₅₀)⁹ for each compound were determined by fitting the curves with nonlinear regression. One-way ANOVA with Tukey's test for multiple comparisons were used to compare EC₅₀ values using a two-tailed alpha level of 0.05. Linear regression analysis was used to determine the correlation between *T. equi* potency and hERG activity for all tested BKI compounds (for BKIs lacking *T. equi* inhibition, an EC₅₀ value of 30 μ M was used). All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The hERG activity was determined using a thallium assay to measure the inhibition of ion transport of hERG as previously reported (Bridal et al., 2010; Ojo et al., 2014a). The erythrocytes used for this experiment were obtained from healthy non-infected horses under a protocol approved by the Washington State University Institutional Animal Care and Use Committee.

Results and discussion

As expected, *T. equi* growth was not inhibited in the negative control iRBC DMSO while *T. equi* growth inhibition occurred in the IMD and BKI 1294 treated iRBC DMSO positive controls (Figure 1), confirming the results of previous studies (Grause et al., 2013; Hines et al., 2015; Ueti et al., 2012; Wise et al., 2013). Of the 11 additional BKIs evaluated, RM-1-132, RM-1-152, 1735, 1369 and 1318 effectively inhibited *T. equi* growth (Figure 1). The inhibition by RM-132 and RM-1-152 was consistent with previously described inhibition of other apicomplexan species by these compounds (Ojo et al., 2014b; Pedroni et al., 2016). Based on a previous report, it was possible that the effective BKIs were interfering with RBC invasion and egression by the parasite (Doerig et al., 2005). However, high resolution microscopic analysis revealed that none of the RBC cultures treated with the effective BKIs demonstrated abnormal morphology or merozoite entrapment compared to the RBC cultures control (data not-shown). Moreover, hydroethidine would have stained

⁷iRBC DMSO: Infected red blood cells treated with DMSO

⁸nRBC: Non-infected red blood cells

⁹EC₅₀: 50% effective concentrations

entrapped merozoites resulting in an increased PPE, which was not observed. In fact, after three day-treatment with the effective BKIs, cultures demonstrated very low PPE (0.2 to 0.87%). The mechanism by which these BKIs inhibited *T. equi* growth remains to be determined.

The growth inhibition curves and EC₅₀ values indicated that IMD was much more potent than the six BKIs that inhibited *T. equi* (Table 2). The remaining six BKIs did not achieve 50% growth reduction (Table 3). Although significantly less potent than IMD, the effective BKIs had variable potency against *T. equi*, with RM-1-152 and 1735 being the most and the least potent drugs, respectively (Figure 2).

Previous reports indicate that certain BKIs inhibit hERG, giving them potential cardiac liability (Curran et al., 1995; Ojo et al., 2014a; Sanguinetti and Tristani-Firouzi, 2006). Given the sequence and functional homology between equine ERG and hERG (Pedersen et al., 2015), we determined the hERG activity for the evaluated BKIs. Interestingly, the BKIs with the lowest hERG activity (EC₅₀ >11 µM) did not inhibit *T. equi*, while those with greater hERG activity (EC₅₀ ≤ 11 µM) demonstrated *T. equi* inhibition (Table 4). The lack of *T. equi* inhibition in the former compounds might have been related to modifications in the piperidine ring designed to reduce hERG activity (Vidadala et al, 2016). Compounds RM-1-132 and RM-1-152 were more potent against *T. equi* than the positive control BKI 1294 and had higher hERG EC₅₀, suggesting lower potential cardiac liability (Table 4). Although the remaining effective three compounds BKI 1735, 1369 and 1318 had similar to lower potency than BKI 1294, the higher hERG EC₅₀ activity, suggested even less risk of cardiotoxicity than RM-1-132 and RM-1-152 (Table 4).

Conclusions

For the first time we have shown that in addition to BKI 1294, five BKI compounds effectively inhibit *T. equi* growth *in vitro*. Taking hERG activity and potential cardiac liability into consideration, BKI compounds BKI 1735, 1369 and 1318 are the most likely candidates for further evaluation. However, *in vivo* studies evaluating pharmacodynamics, pharmacokinetics, and safety are needed to identify the best drug candidate and dosage regimen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Theileria equi* is the etiologic agent of equine piroplasmosis
- Imidocarb dipropionate is the drug of choice for clearance of *T. equi*, however it induces undesirable side effects
- Bumped kinase inhibitors could potentially be used as safe and effective alternatives to Imidocarb dipropionate
- Six Bumped kinase inhibitors inhibited *T. equi* growth *in vitro*
- Considering some probable off target effects of Bumped kinase inhibitors, three of them would be suitable for further evaluation

Figure 1. In vitro evaluation of bumped kinase inhibitors inhibiting *T. equi* growth

Means of percent parasitized erythrocytes (PPE) for positive and negative controls, and for BKIs tested at 30 μ M against *T. equi*. Results are representative of two independent experiments. Error bars are SE.

	IMD	1294	RM-1-132	RM-1-152	1735	1369
1294	****					
RM-1-132	****	*				
RM-1-152	***	***	*			
1735	****	*	****	****		
1369	****	ns	***	****	ns	
1318	****	ns	ns	*	***	**

Figure 2. Inhibitory concentrations (EC₅₀) of bumped kinase inhibitors and statistical comparisons of potency

Mean EC₅₀ levels for BKIs with inhibitory effects. Results are representative of 2 independent experiments. Error bars are SE. *P* 0.001(***), *P* 0.01(**), *P* 0.05(*). ns, not significant.

Table 1

Chemical structures of bumped kinase inhibitors and mean hERG EC50 activity of BKI.

Name	Structure
Imidocarb	
BKI 1294	
BKI RM-1-132	
BKI RM-1-152	
BKI 1597	
BKI 1571	
BKI 1643	
BKI 1586	
BKI 1547	
BKI 1735	

Name	Structure
BKI 1369
BKI 1318
BKI 1649

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Table 2
Dose response values of BKIs that inhibited *T. equi* growth at 72 hrs post-incubation.

Drug concentration (µM)	% maximum PPE						
	BKI RM-1-152	BKI RM-1-132	BKI 1294	Imidocarb	BKI 1735	BKI 1369	BKI 1318
0.000001				100			
0.002688				96.18736			
0.005375				42.37473			
0.01075				18.76362			
0.0215				16.8573			
0.043				13.48039			
0.086				12.5			
0.172				10.10349			
0.344				9.586057			
0.3	93.48066	101.0076	93.88814		99.78663	103.2062	98.72056
3	47.66115	88.0719	73.34693		96.37959	90.57835	85.90233
6	16.27118	25.54466	36.53111		75.68211	48.07812	26.36193
9	11.07373	8.687364	17.32657		52.31479	24.60825	13.07516
12	9.977422	8.714597	10.19955		28.1807	16.63537	9.42342
30	7.436282	5.283224	6.94913		7.720447	10.24971	7.771934

Table 3
Dose response values of BKIs that did not inhibit *T. equi* growth at 72 hrs post-incubation.

Drug concentration (µM)	% maximum PPE									
	BKI 1571	BKI 1643	BKI 1547	BKI 1597	BKI 1586	BKI 1649	BKI 1753	BKI 1767		
0.3	83.65385	89.42308	98.07692	100	100	94.51004	95.60915	105.3791		
3	93.75	95.67308	100.2404	99.03846	88.46154	96.07438	93.59926	99.8899		
6	96.63462	97.11538	102.4038	100	97.59615	93.90496	90.90909	99.32369		
9	93.75	100.9615	103.8462	106.7308	98.55769	94.70189	87.80148	99.93709		
12	95.67308	100.9615	103.8462	101.9231	99.03846	95.63164	88.72913	97.90815		
30	98.07692	100.9615	100.4808	97.35577	100.9615	87.79516	69.75881	95.78484		

Table 4

Potential therapeutic indices of bumped kinase inhibitors.

Name	Mean T. equi EC ₅₀ (μM)	hERG EC ₅₀ (μM)	Potential therapeutic index
Imidocarb	0.005	NA	
BKI 1294	5.717	0.3	19.057
BKI RM-1-132	4.111	1.66	2.477
BKI RM-1-152	2.439	1.9	1.284
BKI 1597		>30	
BKI 1571		27.5	
BKI 1643		>30	
BKI 1586		22.75	
BKI 1547		>30	
BKI 1735	7.81	11	0.710
BKI 1369	6.931	10	0.693
BKI 1318	4.171	10	0.417
BKI 1649		>30	