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Letter

Macrofilaricidal Benzimidazole−Benzoxaborole Hybrids as an Approach to the Treatment of River Blindness: Part 1. Amide Linked Analogs

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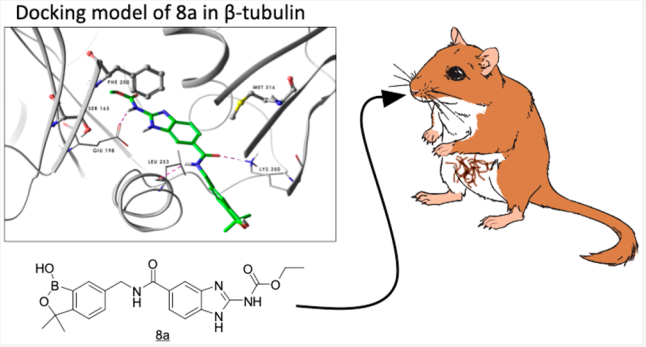
ABSTRACT: A series of benzimidazole−benzoxaborole hybrid molecules linked via an amide linker are described that exhibit good *in vitro* activity against *Onchocerca volvulus*, a filarial nematode responsible for the disease onchocerciasis, also known as river blindness. The lead identified in this series, **8a** (AN8799), was found to have acceptable pharmacokinetic properties to enable evaluation in animal models of human filariasis. Compound **8a** was effective in killing *Brugia malayi*, *B. pahangi*, and *Litomosoides sigmodontis* worms present in Mongolian gerbils when dosed subcutaneously as a suspension at 100 mg/kg/day for 14 days but not when dosed orally at 100 mg/kg/day for 28 days. The measurement of plasma levels of **8a** at the end of the dosing period and at the time of sacrifice revealed an interesting dependence of activity on the extended exposure for both **8a** and the positive control, flubendazole.



KEYWORDS: *onchocerciasis, lymphatic filariasis, flubendazole, tubulin, organoboron*

Diseases caused by infection of an individual with filarial worms are widespread and of particular concern in the endemic countries of the developing world. Two such diseases are onchocerciasis (river blindness), caused by the parasite *Onchocerca volvulus*, and elephantiasis (lymphatic filariasis, LF), caused by parasites *Wuchereria bancroftii*, *Brugia timori*, and *B. malayi*.[1] These diseases are endemic across Asia (LF) and sub-Saharan Africa (LF and onchocherciasis), with the parasites transmitted via black flies (onchocerciasis) or mosquitoes (lymphatic filariasis). Despite significant and long-term efforts to limit the impact of these parasitic infections on the population through mass drug administration (MDA) programs with microfilaricidal drugs (ivermectin for onchocerciasis; albendazole, ivermectin, and/or diethylcarbamazine for LF),[2−5] there remains an opportunity to discover, develop, and deliver new drugs that overcome limitations of existing therapies. For example, the current strategy for the treatment of onchocerciasis requires that an infected individual take ivermectin 1−3 times per year for 2−3 years over the lifetime of the adult worms (10−14 years for *O. volvulus* and 6−8 years for *Wuchereria* and *Brugia* spp.), which is logistically challenging in disease endemic areas.[2] Long-term treatment is required because the microfilaricidal drugs kill only the microfilariae of *O. volvulus* or LF; they have little effect on the adult macrofilariae.[4,4−7] In addition, coinfection of onchocerciasis or LF patients with the eye worm *Loa loa* can limit the utility of treatment with ivermectin due to significant side effects resulting from rapidly killing the *Loa loa* microfilariae.[8,9]

Flubendazole (**1**), an inhibitor of tubulin polymerization, has been shown to have the ability to kill adult filarial worms, providing promise that this molecule could have utility in the treatment of onchocerciasis and LF (Figure 1).[10−13] Despite this promise, flubendazole has several limitations that complicate its potential as a drug for these human infections. First, flubendazole has limited oral bioavailability, primarily a consequence of its poor aqueous solubility.[14,15] Second, though more selective than other members of the benzimida-

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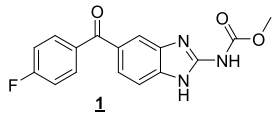


Figure 1. Structure of flubendazole.

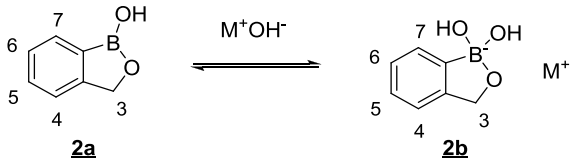


Figure 2. Equilibrium between three-coordinate, neutral benzoxaborole and four-coordinate, negatively charged benzoxaborole.

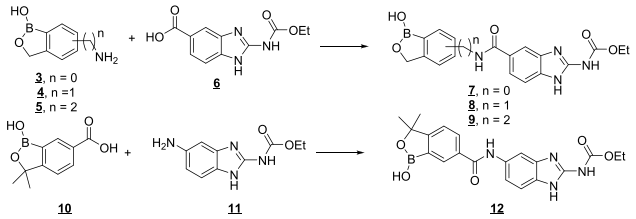


Figure 3. Strategy for the preparation of the initial benzoxaborole−benzimidazole hybrids.

zole class, flubendazole also exhibits affinity for the host's mammalian tubulin and, consequently, has been demonstrated to be potentially embryotoxic in both a rat whole embryo culture experiment and when dosed to pregnant female rats.[16,17] Third, flubendazole has been found to be an aneugen in both *in vitro* and *in vivo* micronucleus tests, although it has been argued that the lack of clastogenicity of flubendazole in these tests will limit the risk of carcinogenicity to patients.[18] However, the metabolism of flubendazole by the reduction of the ketone leads to short-lived clastogenic metabolites at low levels that may pose a minimal risk.[18]

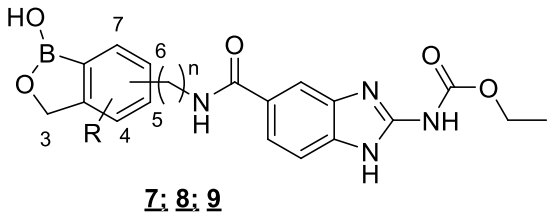
Our strategy was to prepare benzoxaborole−benzimidazole analogs of flubendazole that would overcome these limitations. We have observed in other discovery projects that the benzoxaborole core can improve aqueous solubility and oral bioavailability of otherwise poorly soluble molecular frameworks due to the ability of the boron atom to equilibrate between a three coordinate, neutral species (**2a**) and a four coordinate, negatively charged species (**2b**) under physiological conditions (Figure 2).[19−30]

A second potential advantage of the incorporation of the benzoxaborole moiety into a molecule was that the benzoxaborole could engage in unique interactions with the subunits of tubulin in a manner that could impart improved selectivity for inhibition of worm tubulin polymerization relative to mammalian host tubulin. Initial efforts to prepare benzoxaborole−benzimidazole hybrids related to flubendazole focused on simple amides. These compounds were easily prepared from an array of previously described amino-(**3**), aminoalkyl-(**4**,**5**), and carboxy-(**10**) substituted benzoxaboroles and the corresponding carboxy (**6**)[31−33] or amino (**11**)[31] benzimidazole as depicted in Figure 3.

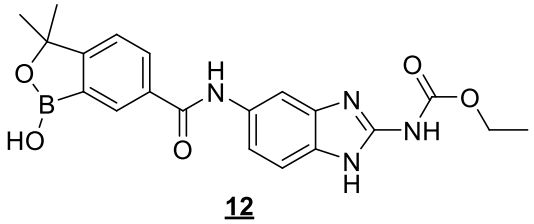
The first three analogs from the benzimidazole 5-carboxylic acid, namely **7a** (from 6-aminobenzoxaborole), **8a** (from 6-aminomethyl-3,3-dimethylbenzoxaborole), and **8b** (from 3-aminomethylbenzoxaborole), were prepared and tested in an *Onchocerca volvulus* L3 larval molting assay[34,35] and a mammalian G2/M arrest assay[36] to evaluate antifilarial activity and selectivity, respectively (Table 1). *O. volvulus* is the causative agent of river blindness, and the L3 molting assay is the only widely available and reproducible assay of this filariid, the target of novel macrofilaricidal drugs.[34,35] Other animal models (e.g., *Brugia* in gerbils) recapitulate aspects of lymphatic filariasis and are also used as surrogate screens for *O. volvulus* infections. Of the three compounds tested, **8a** emerged as the most attractive compound on the basis of potency and selectivity.

Addition of the 3,3-dimethyl substituents to the 6-aminobenzoxaborole core (**7b**) resulted in a loss of *O. volvulus* activity, as did removal of the 3,3-dimethyl substituents from the 6-aminomethyl benzoxaborole (**8c**). As previous work in the benzoxaborole class had demonstrated that pharmacokinetic properties of 3,3-dimethyl analogs were generally superior to the 3,3-unsubstituted analogs, we focused our

Table 1. Initial Benzoxaborole−Benzimidazole Amide Leads[a]



7; **8**; **9**



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ID	link atom	*n*	R	*O. volvulus* IC_{50} (μM)[31,32]	G2/M arrest IC_{50} (μM)[33]	MDCK-MDR1 P_{app} (A-B, $\times 10^6$ cm/s)[34−36]
1	NA	NA	NA	0.004	0.67	15.6
7a	6	0		4.55	>100	NT
7b	6	0	3,3-Me_2	>10	NT	NT
8a	6	1	3,3-Me_2	0.300	13	0.8
8b	3	1		0.426	10	NT
8c	6	1		0.421	>100	NT
8d	5	1	3,3-Me_2	0.042	2.54	0.5
9a	6	2	3,3-Me_2	0.004	0.555	0.7
12a	6	0	3,3-Me_2	>10	>100	NT

[a]NT = not tested.

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Table 2. *In Vivo* Efficacy and Terminal Plasma Concentrations of 8a and Flubendazole[a]

in vivo model	drug treatment	worm count median ± SEM (range)	adult worm reduction/animals with no worms	$P \leq$	drug concentration (μM)		
					24 h after last dose	interim	necropsy
B. malayi; necropsy on day 42	vehicle (SC study), $n = 5$	12 ± 0.73 (11−15)			N/A	N/A	N/A
	flubendazole, 10 mg/kg × 5 days, QD, SC, $n = 10$	0 ± 0 (0−0)	100%/100%	0.0001	0.173	NM	0.043
	8a, 150 mg/kg × 14 days, QD, SC (solution), $n = 6$	2 ± 2.14 (0−12)	83.3%/50%	0.0622	2.47	0.07 (day 28)	0.01
	8a, 100 mg/kg × 14 days, QD, SC (suspension), $n = 16$	0 ± 0.19 (0−3)	100%/87.5%	0.0001	4.10	5.36 (day 28)	3.38
	vehicle (PO study), $n = 5$	8 ± 0.81 (5−10)			N/A	N/A	N/A
	8a, 100 mg/kg × 28 days, QD, PO (suspension), $n = 10$	7 ± 1.38 (4−16)	12.5%/0%	0.9999	0.128	NM	<LOQ
B. pahangi; necropsy on day 63; $n = 5$ per group	vehicle	89 ± 12.79 (64−146)			N/A	N/A	N/A
	flubendazole, 10 mg/kg × 5 days, QD, SC	0 ± 0 (0−0)	100%/100%	0.0009	0.178	NM	0.018
	8a, 100 mg/kg × 14 days, QD, SC (suspension)	0 ± 0.333 (0−2)	100%/83%	0.0024	5.87	NM	1.15
	8a, 100 mg/kg × 28 days, QD, PO (suspension)	67.5 ± 14.86 (2−114)	24%/0%	0.9999	0.13	NM	<LOQ
L. sigmodontis; necropsy on day 63; $n = 4$ vehicle; $n = 6$ other groups	vehicle	8.5 ± 3.28 (5−20)			N/A		N/A
	flubendazole, 10 mg/kg × 5 days, QD, SC	0 ± 0 (0−0)	100%/100%	0.0208	0.283	NM	0.040
	8a, 100 mg/kg × 14 days, QD, SC (suspension)	0 ± 0.34 (0−2)	100%/33.3%	0.1207	9.30	NM	0.069
	8a, 300 mg/kg × 7 days, QD, SC (suspension)	0 ± 0 (0−0)	100%/100%	0.0208	7.85	10.6 (day 21) 4.64 (day 42)	0.48
	8a, 100 mg/kg × 28 days, QD, PO (suspension)	20 ± 6.92 (5−50)	−135.3%/0%	0.999	0.210	0.006 (day 42)	0.003

[a]NM = not measured. N/A = not applicable. Statistical significance was tested by Kruskal−Wallis followed by Dunn's multiple comparisons test.

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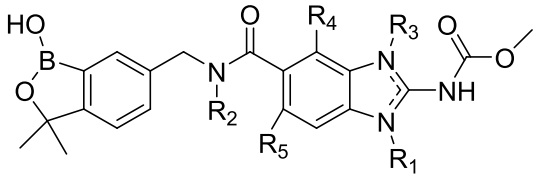
Table 3. Analogs Designed To Overcome the Permeability Challenge



ID	R1	R2	R3	R4	R5	*O. volvulus* IC_{50} (μM) or % inhibition of molting at 1 μM	G2/M IC_{50} (μM)	MDCK-MDR1 P_{app} (A-B, $\times 10^6$ cm/s)
8a	H	H	H	H	H	0.300	13	0.8
8e	CH_3	H	H	H	H	24%[a]	>100	1.2
8f	H	H	CH_3	H	H	29%[a]	>100	1.8
8g	H	CH_3	H	H	H	NT	4.1	0.6
8h	H	$CH_2CH_2NMe_2$	H	H	H	NT	35	0.14
8i	H	H	H	F	H	0%[a]	16	2.35
8j	H	H	H	H	F	0.13	1.26	2.83

[a]% of *O. volvulus* larvae that completed molting from L3 to L4 compared to control worms.

attention on this substitution pattern.[25] Increasing the length of the linker between the benzoxaborole and benzimidazole cores as in **9a** resulted in a significant increase in *O. volvulus* potency, but also in the G2/M arrest assay we were using as a functional indicator of the interaction with mammalian tubulin. Similarly, changing the point of attachment on the benzoxaborole core from 6- to 5- to afford **8d** was also accompanied by increased potency in both the *O. volvulus* and G2/M arrest assays. Lastly, preparation of a reverse amide from the 5-amino benzimidazole and 6-carboxybenzoxaborole (**12a**) resulted in loss of activity.

We characterized the *in vitro* ADME properties of **8a** in preparation for evaluation of this compound in our primary *in vivo* model in gerbils. Metabolic stability of **8a** in gerbil microsomes was good (Cl_{int} < 4 μL/min/mg), but it was found to be poorly permeable in an MDR1-MDCK monolayer assay (P_{app} (A-B) = 0.8 × 10^{-6} cm/s).[37−39] The P_{app} in this assay when the P-glycoprotein (Pgp) efflux inhibitor GF-120918 was added increased to 2.7 × 10^{-6} cm/s, suggesting that the compound was potentially a substrate for this efflux mechanism.[40,41] Taken together, these data prompted us to explore the activity of **8a** following subcutaneous administration to gerbils infected by implantation of adult *Brugia malayi* or *Brugia pahangi* parasites in the peritoneal cavity.[42] We were pleased to find that **8a** was able to kill 100% of both male and female worms in the gerbil peritoneum when dosed subcutaneously at 100 mg/kg/day for 14 days as a suspension in a nonsolubilizing HEC/Tween vehicle. Interestingly, when **8a** was dosed subcutaneously at 150 mg/kg/day as a solution in a DMSO/water vehicle, *in vivo* efficacy was substantially reduced. The subsequent assessment of the pharmacokinetics of **8a** from these two dosing paradigms provided an interesting observation that we believe to be important in understanding the PK−PD requirements for achieving efficacy in this animal model. In the suspension dose group, plasma levels of **8a** were maintained above the *in vitro* IC_{50} in the *O. volvulus* assay (300 nM) for over 42 days after the last dose, whereas in the solution dose group, plasma levels fell below this IC_{50} within a few days after the last dose. We had made the same observation in a positive control group using flubendazole at a subcutaneous dose of 10 mg/kg/day for 5 days in the nonsolubilizing HEC/Tween vehicle, namely, that plasma levels of flubendazole were maintained above the *in vitro* IC_{50} (4 nM) for over 42 days, consistent with data reported in the literature.[43] As anticipated from our *in vitro* ADME data, **8a** was not efficacious when dosed by the oral route (at 100 mg/kg/day for 28 days), and plasma levels of the drug were found to be well below the *in vitro* IC_{50} at all time points. These observations suggest that the efficacy observed in the *in vivo* model was dependent upon long-term exposure of worms to the drug, perhaps a consequence of the "depot-like" properties of the subcutaneous suspension.[44] We next examined **8a** in two additional *in vivo* models, the first where L3 *B. pahangi* larvae were injected into the peritoneum of gerbils and allowed to develop into adult worms[45] and a second where gerbils were naturally infected by the filarial nematode *Litomosoides sigmodontis*.[46,47] In both of these models, **8a** was administered subcutaneously at 100 mg/kg for 14 days or orally at 100 mg/kg for 28 days as a suspension. As with the adult worm infection model, **8a** was quite effective via the subcutaneous route in these additional models but essentially inactive when dosed orally as summarized in Table 2. Additionally, when plasma obtained from treated animals at necropsy was analyzed for **8a**, we observed an outcome similar to that observed in the adult implantation model, e.g., that measurable levels were present at this time point in the subcutaneous-dosed groups but not in the orally dosed groups, weeks after administration of the drug. In a final *L. sigmodontis* experiment with **8a**, we dosed the compound subcutaneously at 300 mg/kg for 7 days as a suspension. As anticipated, this study demonstrated good activity of **8a**, as plasma concentrations of the drug were in excess of the *in vitro* IC_{50} for at least 42 days.

These observations of the dependence of activity on the extended exposure of *B. malayi*, *B. pahangi*, and *L. sigmodontis* worms to the drug (either flubendazole or **8a**) were consistent with observations made in an *ex vivo B. malayi/pahangi* assay,[48] namely, that a short (<7 days) exposure of worms to these drugs was not effective in killing the worms. Taken together, these results suggest that the mechanism of action of these benzimidazole drugs (inhibition of tubulin polymerization) requires a long (>28 day) exposure to the drug to be effective.

While we were encouraged by the proof of concept demonstrated by **8a** in these *in vivo* models, it was clear that this molecule would not meet our target candidate profile that required an orally active drug candidate.

It has been suggested in the literature that the propensity for Pgp efflux is much greater in compounds containing more that 2−3 hydrogen bond donors (HBDs).[49,50] Our lead compound

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(**8a**) has four potential donors (B−OH, amide NH, benzimidazole NH, and carbamate NH). In order to ameliorate this potential Pgp liability, we prepared and evaluated compounds with fewer HBDs (Table 3). As anticipated on the basis of the flubendazole literature,[31] alkylation of the benzimidazole NH (**8e**, **8f**) resulted in loss of activity and also did not improve permeability. Alkylation of the amide nitrogen (**8g**, **8h**) also did not improve permeability. The incorporation of a fluorine substituent on the benzimidazole ring adjacent to the amide (**8i**, **8j**), a strategy that has been shown to "mask" an amide hydrogen bond donor,[50,51] did improve the permeability but also affected the potency in both the *O. volvulus* and G2/M arrest assays. Interestingly, 4-F analog **8i** lost activity in the *O. volvulus* assay, whereas the 7-F analog **8j** exhibited greater potency (and hence, poorer selectivity) in both assays.

On the basis of these results, it was clear that more substantial changes needed to be made to the benzoxaborole−benzimidazole hybrids to achieve our objective. The exploration of an additional series of hybrid molecules, most specifically those containing a ketone linker analogous to that found in flubendazole, will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00396.

Synthesis methods for benzimidazole−benzoxiborole hybrids and methods for testing compounds in larval molt assays and *in vivo* studies (PDF)

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R.T.J., D.S.C., Y.R.F., and JAS wrote the manuscript and contributed equally to the design and execution of the project. T.A., R.T.J., D.S.C., and J.J.P. designed and coordinated the synthesis of the compounds. R.T.J., K.J., Y.R.F., C.S.L., E.E.E., F.R., J.J.P., R.S., J.M., A.H., J.A.S., and S.L. provided scientific leadership and management of the project. P.W.B. designed, coordinated, and interpreted the *in vitro* and *in vivo* pharmacokinetics studies. C.F., C.A.B., K.C.L., B.M.S., N.T., A.M., U.D., S.M., B.C., and S.S. conducted the *in vitro* and *in vivo* biological assays, which were designed and coordinated by Y.R.F., C.S.L., F.R., K.J., M.P.H., S.S., A.H., S.L., J.A.S., and J.W.M. The manuscript was edited by R.T.J., D.S.C., Y.R.F., P.W.B., A.H., M.P.H., SL, C.A.B., and J.A.S.

Notes

The authors declare no competing financial interest.

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(32) Synthesis of compound **8a**. Step 1. A mixture of 3-amino-4-(methylamino)benzoic acid (750 mg, 4.51 mmol) and bis(methoxycarbonyl)-2-methylisothiourea (1.86 g, 9.00 mmol) in AcOH (10 mL) was stirred at 80 °C for 20 min and filtered, and the filter cake washed with methanol (20 mL) and ethyl acetate (20 mL), dried *in vacuo* to give 2-((methoxycarbonyl)amino)-1-methyl-1*H*-benzo[d] imidazole-5-carboxylic acid **6** (850 mg, 76%) as a white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.98 (s, 1H), 7.80 (dd, J = 8.6, 1.4, 1H), 7.43 (d, J = 8.8 Hz, 1H), 3.62 (s, 3H), 3.50 (s, 3H). Step 2. A mixture of **6** (80 mg, 0.32 mmol), 6-(aminomethyl)-3,3-dimethylbenzo[c]-[1,2]oxaborol-1(3*H*)-ol (61 mg, 0.32 mmol), HATU (182 mg, 0.48 mmol), and DIPEA (124 mg, 0.96 mmol) in DMF (10 mL) was stirred at room temperature for 16 h under N_2. The mixture was concentrated, and the residue was purified by Prep-HPLC to give **8a** (40 mg, 30%) as a white solid; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.06 (t, J = 5.6 Hz, 1H), 8.00 (s, 1H), 7.84 (d, J = 8.4 Hz, 1H), 7.60 (s, 1H), 7.56 (d, J = 8.8 Hz, 1H), 7.41 (d, J = 7.6 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 4.49 (d, J = 4.8 Hz, 2H), 3.70 (s, 3H), 3.60 (s, 3H), 1.40 (s, 6H).
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